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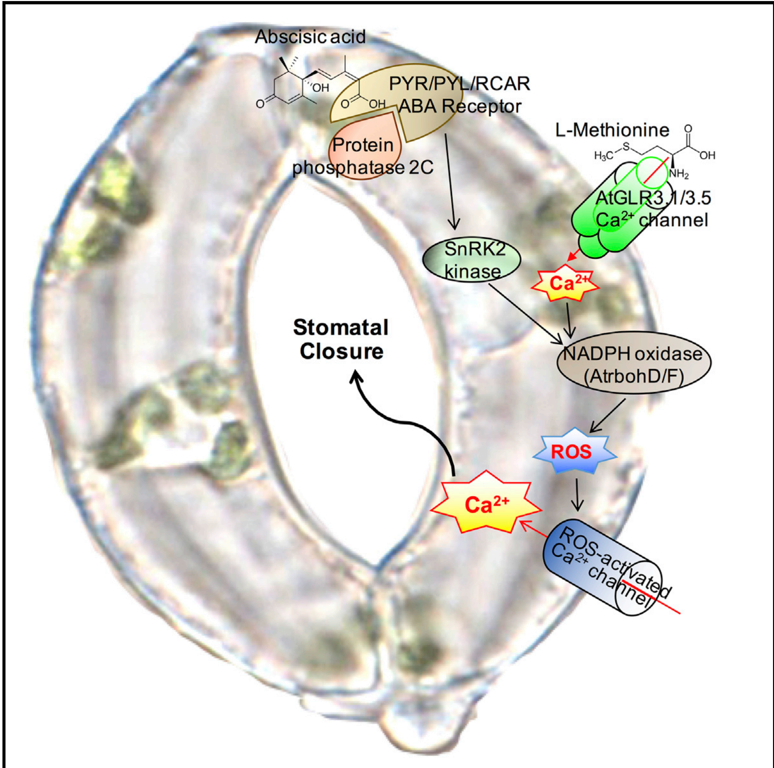
L-Met Activates *Arabidopsis* GLR Ca^{2+} Channels Upstream of ROS Production and Regulates Stomatal Movement

Graphical Abstract



Highlights

- GLR3.1 and GLR3.5 form guard cell Ca^{2+} channels specifically activated by L-Met
- GLR3.1/3.5 Ca^{2+} channels contribute to maintenance of basal cytosolic Ca^{2+} levels
- GLR3.1/3.5 Ca^{2+} channels act upstream of reactive oxygen species
- GLR3.1/3.5 Ca^{2+} channels play a role in plant growth and stomatal movement

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In Brief

Kong et al. show that *Arabidopsis* glutamate receptor homologs GLR3.1 and GLR3.5 form L-methionine-activated Ca^{2+} channels. The GLR3.1/3.5 Ca^{2+} channels are distinct from ROS-activated Ca^{2+} channels and play a role in maintaining basal cytosolic Ca^{2+} levels, thereby regulating stomatal movements and plant growth.

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L-Met Activates *Arabidopsis* GLR Ca^{2+} Channels Upstream of ROS Production and Regulates Stomatal Movement

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SUMMARY

Plant glutamate receptor homologs (GLRs) have long been proposed to function as ligand-gated Ca^{2+} channels, but no in planta evidence has been provided. Here, we present genetic evidence that *Arabidopsis* GLR3.1 and GLR3.5 form Ca^{2+} channels activated by L-methionine (L-Met) at physiological concentrations and regulate stomatal apertures and plant growth. The *glr3.1/3.5* mutations resulted in a lower cytosolic Ca^{2+} level, defective Ca^{2+}-induced stomatal closure, and Ca^{2+}-deficient growth disorder, all of which involved L-Met. Patch-clamp analyses of guard cells showed that GLR3.1/3.5 Ca^{2+} channels are activated specifically by L-Met, with the activation abolished in *glr3.1/3.5*. Moreover, GLR3.1/3.5 Ca^{2+} channels are distinct from previously characterized ROS-activated Ca^{2+} channels and act upstream of ROS, providing Ca^{2+} transients necessary for the activation of NADPH oxidases. Our data indicate that GLR3.1/3.5 constitute L-Met-activated Ca^{2+} channels responsible for maintaining basal $[Ca^{2+}]_{cyt}$, play a pivotal role in plant growth, and act upstream of ROS, thereby regulating stomatal aperture.

INTRODUCTION

In response to abiotic and biotic signals, plant cells employ a network of signal transduction pathways that elicit intracellular responses. Cytosolic Ca^{2+} plays a key role in these signal transduction networks, serving as a universal second messenger in a variety of cellular activities (Dodd et al., 2010; Ranty et al., 2016). Plasma membrane Ca^{2+} channels contribute to dynamic changes in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) levels, which in turn generate a variety of cellular responses, including cell proliferation, differentiation, circadian rhythm, pollen tube growth, stomatal movement, and microorganism interactions (Sanders et al., 2002).

Guard cells have been used to investigate Ca^{2+}-mediated physiological changes in response to different environmental and endogenous signals (McAinsh et al., 2000). Endogenous and environmental cues, including plant hormones and nutrient ions, regulate stomatal movement by modulating cytosolic Ca^{2+} signals. For example, the plant hormone abscisic acid (ABA) causes $[Ca^{2+}]_{cyt}$ elevation in guard cells via Ca^{2+} influx through plasma membrane Ca^{2+}-permeable channels and Ca^{2+} release from internal stores, which leads to stomatal closure by activating S-type anion channels or inhibiting inward-rectifying K^+ channels and H+ ATPases (Hedrich, 2012; Hedrich et al., 1990; Kwak et al., 2003, 2008; MacRobbie, 2000; Pei et al., 2000). Although many studies have shown that Ca^{2+}-permeable channels play a central role in regulating stimulus-specific $[Ca^{2+}]_{cyt}$ increase and stomatal movements (Cosgrove and Hedrich, 1991), the molecular identity and electrophysiological properties of plasma membrane Ca^{2+} channels in plant cells remain elusive.

Glutamate receptor homologs (GLRs) that share sequence similarity with animal ionotropic glutamate receptors (iGluRs) are present in various plant genomes (Lam et al., 1998). iGluRs are ligand-gated Ca^{2+}-permeable channels and function as neurotransmitter receptors (Pinheiro and Mulle, 2008). Like their counterparts, plant GLRs have been shown to be involved in various Ca^{2+}-mediated developmental regulations and physiological responses, including stomatal closure (Cho et al., 2009),

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Ca^{2+} utilization in response to Na^+/K^+ ionic stress (Kim et al., 2001), mechanical stress responses (Meyerhoff et al., 2005), pollen tube growth (Michard et al., 2011), lateral root development (Vincill et al., 2013), and wound signaling (Mousavi et al., 2013). In addition, it has been suggested that Ala, Asn, Cys, Gly, Glu, Ser, and glutathione act as agonists for plant GLRs (Demidchik et al., 2004; Dennison and Spalding, 2000; Michard et al., 2011; Qi et al., 2006). However, because of the genetic redundancy in this gene family and the potential combinations among different GLR family members, direct electrophysiological evidence for the physiological function of GLR channels remained to be collected.

In this study, we show that L-methionine (L-Met) acts as an agonist at physiological concentrations and activates the GLR3.1/3.5 Ca^{2+} channels in guard cells, leading to cytosolic Ca^{2+} elevation, production of reactive oxygen species (ROS) by NADPH oxidase, and stomatal closure. We also show that defects in GLR3.1/3.5 Ca^{2+} channels result in reduction in the basal cytosolic Ca^{2+} level and Ca^{2+}-deficient growth, all of which can be rescued in part by L-Met. The identification of stereospecific L-Met, in addition to the previously suggested agonists for plant GLR channels, as an activator of the *Arabidopsis* GLR3.1/3.5 Ca^{2+} channel acting upstream of ROS provides an opportunity to identify the physiological function of the entire family in plant signaling and growth.

RESULTS

L-Methionine Enhances Ca^{2+}-Induced Stomatal Closure

Studies have suggested that *Arabidopsis* GLR proteins are ligand-gated Ca^{2+} channels (Kong et al., 2015; Michard et al., 2011; Vincill et al., 2013), but ligand activation of GLR channels has not been directly demonstrated in plants. To obtain in vivo evidence that GLR proteins are Ca^{2+} channels activated by agonists, we chose guard cells as a model system, because they are composed of Ca^{2+} channels that play a crucial role in stomatal movements but have yet to be identified at the molecular level (Hamilton et al., 2000; McAinsh et al., 2000; Pei et al., 2000). Considering that a few amino acids have been suggested to act as agonists of GLR channels (Dennison and Spalding, 2000; Michard et al., 2011; Tapken et al., 2013), we tested the effects of amino acids on Ca^{2+}-induced stomatal closure and found that stomatal closure in wild-type (WT) plants was greatly enhanced by a cocktail of 20 standard amino acids (Figure S1A). Of the five groups into which these amino acids were randomly divided, only one enhanced Ca^{2+}-triggered stomatal closure (Figure S1A). Subsequent stomatal aperture analyses of the roles of four individual amino acids in this group led to the identification of methionine (Met) as a factor significantly enhancing Ca^{2+}-induced stomatal closure in a dose-dependent manner (Figure 1A), whereas the other three amino acids in the group showed no clear effect (Figure S1B).

L-Met Elevates Ca^{2+} Influx via GLR3.1/3.5 Channels in Guard Cells

To investigate the potential role of GLRs in Met-enhanced Ca^{2+}-induced stomatal closure, we evaluated the expression levels of GLR genes in guard cells (Cho et al., 2009; Leonhardt et al., 2004) and selected *GLR3.1* and *GLR3.5*, which are highly expressed in guard cells, as candidates (Figures S1C and S1D). Analyses of GFP-fused GLR3.1 and GLR3.5 proteins showed that the proteins are localized in the plasma membrane (Figure S1E). A study reported that GLR3.5 is localized in various cellular compartments, including mitochondria and chloroplast (Teardo et al., 2015). To further verify the localization of GLR3.5, we generated a GLR3.5-GFP fusion construct and found that GLR3.5-GFP was localized in the plasma membrane, as well as chloroplasts (Figure S1F).

Assuming functional redundancy in these genes, we generated *glr3.1/3.5* double mutants (Figure S1G). Stomatal movement analyses of the *glr3.1/3.5* mutants showed not only that Ca^{2+}-induced stomatal closure was drastically attenuated but also that Met promotion of Ca^{2+}-induced stomatal closure was significantly impaired (Figures 1A, S1H, and S1I; $p < 0.001$). Stomatal guard cells from both *glr3.1* and *glr3.5* plants did not respond to extracellular Ca^{2+}, which is similar to the results for *glr3.1/3.5* double mutants (Figure S1H). We further analyzed changes in stomatal aperture over a period in response to experimentally imposed Ca^{2+} transients (Allen et al., 2001). The *glr3.1/3.5* mutants showed a pronounced defect in Ca^{2+}-reactive stomatal closure but not in the maintenance of stomatal closure (Figure 1B; $p < 0.001$ at both 20 and 30 min). These results suggest that Met requires the GLR3.1 and GLR3.5 proteins for rapid stomatal closure induced by external Ca^{2+}.

Analyses of plants expressing the Ca^{2+} indicator aequorin revealed that resting $[Ca^{2+}]_{cyt}$ in *glr3.1/3.5* mutants was ~25% lower than that in the WT (Figures 1C and 1D). Our previous study showed a similarly low resting $[Ca^{2+}]_{cyt}$ level in the *GLR3.5* RNAi lines (Kong et al., 2015). We accordingly investigated whether the low basal $[Ca^{2+}]_{cyt}$ in *glr3.1/3.5* mutants affected stimulus-induced $[Ca^{2+}]_{cyt}$ increases by measuring $[Ca^{2+}]_{cyt}$ in seedlings grown in media containing 0–40 mM Ca^{2+} (Ca^{2+} concentrations found in soils) (Rorison and Robinson, 1984). In the WT, $[Ca^{2+}]_{cyt}$ was elevated as Ca^{2+} in the medium increased, whereas this response was dramatically reduced in *glr3.1/3.5* mutants (Figure 1E; $p < 0.001$). Cytosolic Ca^{2+} levels in *glr3.1/3.5* mutants were significantly reduced at all tested concentrations of Ca^{2+} in media (Figure 1E), probably due to reduction in Ca^{2+} transport into the cytoplasm. Furthermore, Met greatly enhanced $[Ca^{2+}]_{cyt}$ elevation in WT but not in *glr3.1/3.5* mutants (Figure 1F), indicating that GLR3.1/3.5 and Met play a role in maintaining $[Ca^{2+}]_{cyt}$, which could explain the impaired stomatal movement of *glr3.1/3.5* mutants (Figure S1I).

Calcium is required for plant growth and development (Niu and Liao, 2016; Schulz et al., 2013). Considering the reduced basal $[Ca^{2+}]_{cyt}$ in *glr3.1/3.5* mutants, we investigated the growth phenotype of the plants under limited Ca^{2+} conditions. As shown in Figure 1G, most *glr3.1/3.5* plants displayed yellow necrotic lesions at the tips of their inflorescences. This symptom resembled blossom-end rot, a Ca^{2+}-deficiency disorder associated with insufficient Ca^{2+} supply that is commonly found in fruits and vegetables and can be controlled with direct Ca^{2+} treatment (White and Broadley, 2003). Ca^{2+} is an immobile nutrient that is not transported from old tissues to rapidly growing young tissues, where substantial Ca^{2+} is required (White and Broadley, 2003). The necrosis regions indicated by arrows are the rapidly

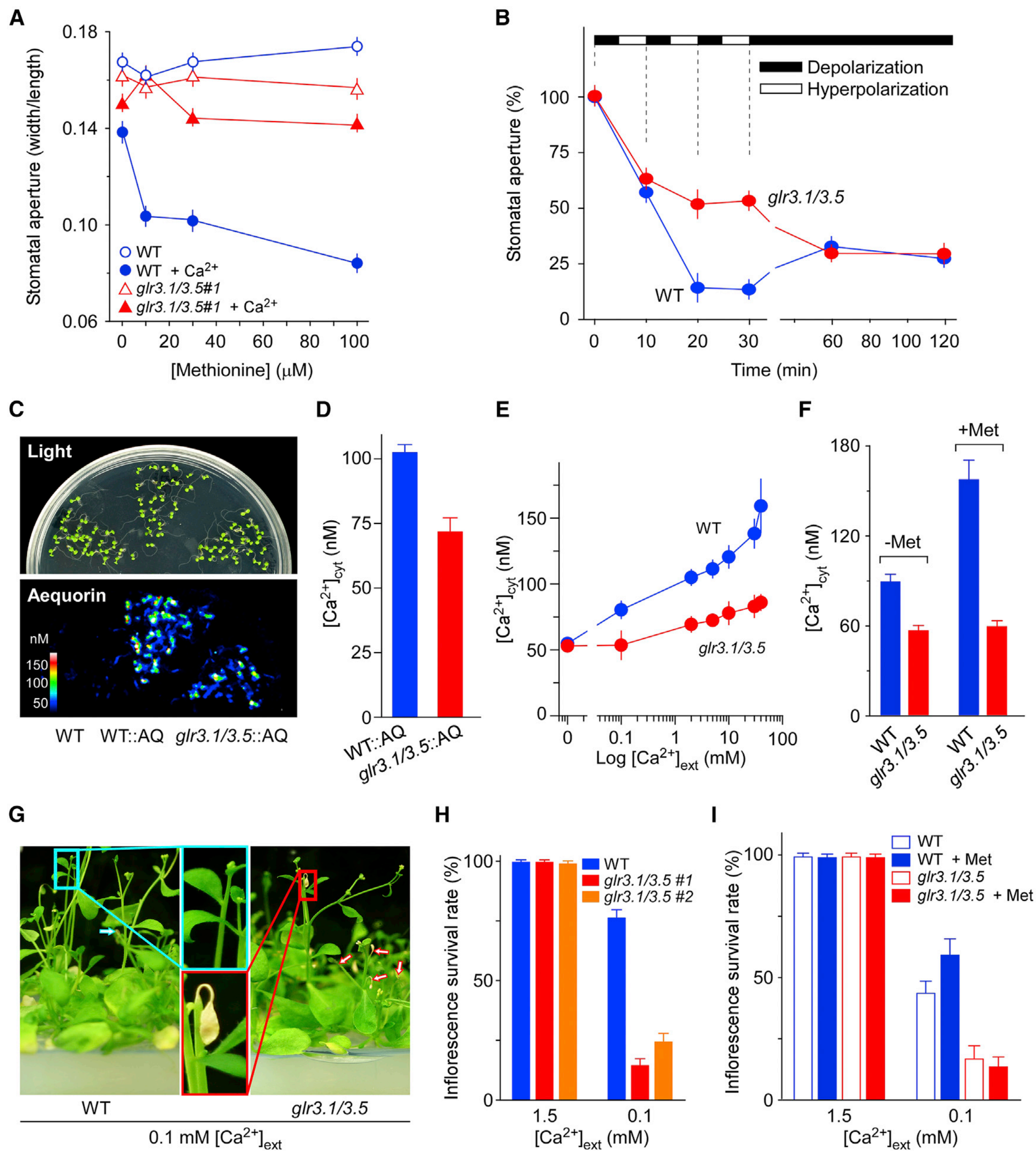

Figure 1. Role of GLR3.1/3.5 and Met in Ca^{2+}-Induced Stomatal Closure and Maintenance of Basal $[Ca^{2+}]_{cyt}$ and Normal Growth at the Tips of Inflorescences

(A) Met enhances Ca^{2+}-induced stomatal closure in a GLR3.1/3.5-dependent manner ($n > 91$; $p < 0.001$).

(B) Ca^{2+} transient-induced stomatal closure in WT and *glr3.1/3.5* plants. Closed and open bars indicate depolarization and hyperpolarization conditions, respectively (n = 50).

(C) Imaging of basal $[Ca^{2+}]_{cyt}$ in *Arabidopsis* wild-type plants and *glr3.1/3.5* mutants expressing aequorin. WT, wild-type; WT::AQ, wild-type expressing 35S::aequorin; *glr3.1/3.5*::AQ, a *glr3.1/3.5* line expressing aequorin.

(D) Quantification of basal $[Ca^{2+}]_{cyt}$ from experiments as in (C) (mean ± SD; n = 68).

(E) Defects in coupling basal $[Ca^{2+}]_{cyt}$ to media Ca^{2+} levels in the *glr3.1/3.5* mutants (mean ± SD; n = 68).

(F) $[Ca^{2+}]_{cyt}$ levels in WT and *glr3.1/3.5* mutants before and after 100 μM Met treatment (n = 90).

(G) *glr3.1/3.5* lines grown for 3 weeks in MS-based media containing 0.1 mM $CaCl_2$ show a typical Ca^{2+}-deficiency phenotype (arrowheads).

(H) Quantitative analysis of the Ca^{2+}-deficiency symptom from experiments as in (G) (n = 30).

(I) Quantification of the Ca^{2+}-deficiency symptom in 4-week-old WT and *glr3.1/3.5* mutants in response to Met at 1.5 and 0.1 mM media Ca^{2+} (n = 90).

Values in (A), (B), (F), (H), and (I) are mean ± SEM from at least three biological replicates. See also Figure S1.

elongating zone in the stem, where the Ca^{2+}-deficiency disorder was seen the earliest (Figure 1G). The inflorescence survival rate of *glr3.1/3.5* mutants was approximately 25%, whereas that of the WT inflorescence under the same conditions was more than 75% (Figure 1H), showing that *GLR3.1/3.5* are required for plant fitness at low Ca^{2+}. Because Met evoked cytosolic Ca^{2+} elevation and played a role in maintaining basal $[Ca^{2+}]_{cyt}$ in a GLR3.1/3.5-dependent manner (Figure 1F), we tested whether Met supply could alleviate the Ca^{2+}-deficiency symptom of *glr3.1/3.5* mutants. The survival rate of WT inflorescences was clearly increased by Met treatment, but not in the *glr3.1/3.5* mutants (Figure 1I; $p < 0.01$). The *glr3.1/3.5* mutant seedlings grown in media containing 1.5 mM Ca^{2+} contained lower $[Ca^{2+}]_{cyt}$ compared to the WT seedlings grown at 0.1 mM Ca^{2+}. However, the inflorescence survival rate of *glr3.1/3.5* was 100% in media containing 1.5 mM Ca^{2+}, whereas WT showed a 75% inflorescence survival rate in media containing 0.1 mM Ca^{2+}. The disparity between the inflorescence survival rate in the adult plants and the $[Ca^{2+}]_{cyt}$ level in the seedlings could also be due to a feedback mechanism that compensates for the loss of function in *GLR3.1* and *GLR3.5*, which may have arisen during the *glr3.1/3.5* plant's growth and development. Both *GLR3.1* and *GLR3.5* are also expressed in other cell types in seedlings (Figures S1C and S1D). Thus, the elevation in the cytosolic Ca^{2+} concentration (Figure 1F), as well as the partial rescue of the Ca^{2+}-deficiency symptom at the tips of inflorescence (Figure 1I) by Met treatment, suggests that GLR3.1/3.5 play a role not only in guard cells but also in other cell types. These results indicate that GLR3.1/3.5 are required to maintain basal cytosolic Ca^{2+} levels and are crucial for Ca^{2+} provision to rapidly growing cells; they also indicate that Met positively regulates Ca^{2+} supply via GLR3.1/3.5.

L-Met Specifically Activates GLR3.1/3.5 Ca^{2+} Channels in Guard Cells

The importance of Met in Ca^{2+}-induced stomatal closure, $[Ca^{2+}]_{cyt}$ elevation, and inflorescence growth prompted us to investigate whether GLR3.1/3.5 are Ca^{2+} channels activated by Met. Using the patch-clamp technique, we directly monitored Ca^{2+} currents across the plasma membrane in guard cells. In addition, to determine whether Met stereospecifically activates GLR3.1/3.5, we used stereospecific Met. The concentration of soluble Met in *Arabidopsis* leaf is approximately 14 pmol/mg fresh weight (FW) (Goto et al., 2002; Karchi et al., 1993); thus, we used L-Met at physiological concentrations in plants, which significantly triggered Ca^{2+} currents in WT guard cells in a dose-dependent manner (Figures 2A and 2B). In contrast, L-Met activation of Ca^{2+} currents was almost abolished not only in *glr3.1/3.5* double mutants (Figures 2C and 2D) but also in *glr3.1* and *glr3.5* single mutants (Figures 2E and 2F). We next tested D-Met, as well as D-Ser, which was previously shown to be implicated in activation of GLR proteins in pollen tube growth (Michard et al., 2011). D-Met, D-Ser, and L-Ser did not result in $[Ca^{2+}]_{cyt}$ elevation or activation of Ca^{2+} currents in WT guard cells (Figures S2A–S2F). These findings unequivocally show that L-Met activates guard cell Ca^{2+} channels consisting of GLR3.1/3.5.

To obtain an additional clue about the L-Met specificity to GLR3.1/3.5 Ca^{2+} channels, the binding poses of L-Met in the ligand-binding domains were modeled (Figure 2G). The topologies of the predicted ligand-binding domain structures are highly similar to each other (Figure S3A). The ligand-binding sites of GLR3.1/3.5 are also structurally similar, such that each protein could have similar affinity and specificity to L-Met, supporting the patch clamp data (Figures 2E and 2F). In contrast to the tight binding of L-Met to the GLR3.1/3.5 ligand-binding domains, its enantiomer D-Met has a configuration in which its side chain is not readily positioned in the pocket and there are ligand-receptor atom clashes, as well as loss of the sulfur-involving H bonds (Figure S3B). In addition, the short hydroxyl group of L-Ser and D-Ser results in energetically unfavorable binding to the pocket. These results support the specific activation of GLR3.1/3.5 Ca^{2+} channels by L-Met.

Using a heterologous expression system, GLR1.4 has been shown to function as a ligand-gated, nonselective, Ca^{2+}-permeable cation channel that binds to a range of amino acids, including Met as the most potent agonist (Tapken et al., 2013). To gain insight into structural differences between GLR3.1/3.5 and GLR1.4, we generated a ligand-binding model of L-Met in the predicted GLR1.4 ligand-binding domain (accession AEE74553, region 448–778) and compared it with that of GLR3.1. A major difference is that Ile676 on GLR1.4 is replaced with Gln729 and Ser732 in GLR3.1, which form strong interactions through H bonds with the sulfur atom in the Met side chain (Figure S3C). It is likely that the loss of these H bonds by the Met side chain in GLR1.4 could be compensated for by energetic stabilization between the Met sulfur and the aromatic group of Tyr719. In addition, the volume of the GLR1.4 ligand-binding pocket is larger than that of GLR3.1 (Figure S3D), suggesting the ability of GLR1.4 to accommodate bulkier amino acids. Because *GLR1.4* is expressed in guard cells (Leonhardt et al., 2004; Yang et al., 2008), we cannot rule out the involvement of GLR1.4 in Met-induced Ca^{2+} currents. Further studies are required to address this important issue. Nevertheless, the absence of L-Met activation of Ca^{2+} currents in the *glr3.1*, *glr3.5*, and *glr3.1/3.5* mutants (Figures 2E and 2F) suggests that GLR3.1/3.5 proteins are responsible for L-Met activation of Ca^{2+} currents in guard cells.

L-Met Activation of GLR3.1/3.5 Ca^{2+} Channels Regulates ROS Production and Stomatal Closure by ABA-Independent Regulation in Guard Cells

ROS activate plasma membrane Ca^{2+} channels, leading to $[Ca^{2+}]_{cyt}$ elevation and thus stomatal closure (Kwak et al., 2003; Pei et al., 2000). However, the molecular identity of ROS-activated Ca^{2+} channels is still unknown. Given that GLR3.1/3.5 Ca^{2+} channels mediate Ca^{2+} influx, leading to stomatal closure (Figures 1 and 2), we investigated whether GLR3.1/3.5 are ROS-activated Ca^{2+} channels in guard cells. We first tested stomatal response to H_2O_2 and found that, contrary to our expectations, H_2O_2 similarly elicited stomatal closure in *glr3.1/3.5* mutants and WT plants (Figure 3A). This result implied that the cellular events downstream of ROS, including the activation of the Ca^{2+} channel, are functional in *glr3.1/3.5* mutants. Time-point analyses of *glr3.1/3.5* and WT plants showed a comparable decrease in stomatal aperture in response to H_2O_2 (Figure 3B). Furthermore, patch clamping of

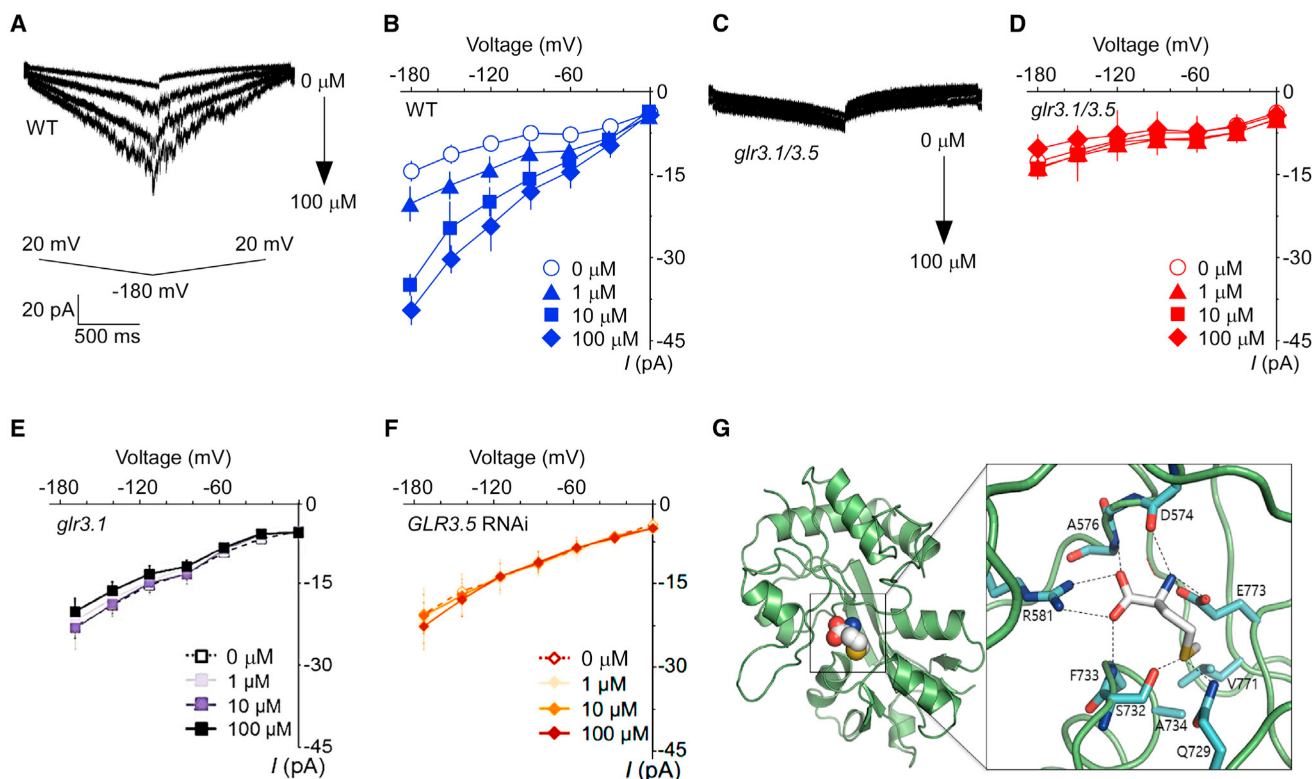

Figure 2. L-Met Acts as an Activator of GLR3.1/3.5 Ca^{2+} Channels in *Arabidopsis* Guard Cells

(A–F) Whole-cell patch clamp recordings of Ca^{2+} currents in WT (A and B), *glr3.1/3.5* mutants (C and D), and *glr3.1* (E) and *GLR3.5RNAi* (F) guard cells. Average current-voltage curves in WT (B), *glr3.1/3.5* mutants (D), *glr3.1* mutants (E), and *GLR3.5RNAi* plants (F). Data are mean ± SEM.

(G) Predicted structure of the ligand-binding domain of the GLR3.1 Ca^{2+} channel. A predicted binding pose of L-Met in GLR3.1 suggests that the carboxyl group of L-Met forms a bidentate salt bridge with Arg581 and H bonds with the main chain amide groups of Ala576 and Phe733. The L-Met amino group forms H bonds with the main chain oxygen of Asp574 and the side chain of Glu773. The sulfur atom of L-Met forms H bonds with the side chains of Gln729 and Ser732, and the side chain CH_3 group is adjacent to hydrophobic residues (Ala734 and Val771).

See also Figures S2 and S3.

guard cells showed no difference between WT and *glr3.1/3.5* in H_2O_2-activated Ca^{2+} currents (Figure 3C). Altogether, these results show that GLR3.1/3.5 Ca^{2+} channels act upstream of ROS production and are distinct from the previously characterized ROS-activated Ca^{2+} channels.

Cytosolic Ca^{2+} elevation occurs downstream of NADPH oxidase-originated ROS production in ABA signaling in guard cells (Kwak et al., 2003; Murata et al., 2001; Pei et al., 2000), as well as upstream of ROS in other cellular processes (Ranf et al., 2011). Because GLR3.1/3.5 Ca^{2+} channels act upstream of ROS, we investigated whether L-Met affects ROS production in guard cells. As shown in Figure 3D, L-Met clearly induced ROS production in WT, although the amount of ROS produced by L-Met was less than that produced by ABA. In contrast, L-Met failed to induce ROS production in *glr3.1/3.5* guard cells (Figure 3D), suggesting that activation of GLR3.1/3.5 Ca^{2+} channels by L-Met positively regulates ROS production. A considerable amount of ROS was produced in *glr3.1/3.5* upon ABA treatment, although it was less than that produced in WT ($p < 0.01$). The ROS produced in the *glr3.1/3.5* mutants appeared to be sufficient to elicit a stomatal response to ABA that was similar to that in the WT (Figure S4). The basal amount of steady-state ROS was reduced in *glr3.1/3.5* guard cells compared with that in the WT, further supporting the hypothesis that GLR3.1/3.5 Ca^{2+} channels act upstream of ROS and regulate cellular ROS homeostasis.

Next, we investigated the mechanism by which L-Met activation of GLR3.1/3.5 Ca^{2+} channels evoked ROS production in guard cells. Two NADPH oxidases, AtrbohD and AtrbohF, are positive regulators of ABA signaling and are responsible for ROS production in guard cells (Kwak et al., 2003, 2006). We accordingly monitored ROS production in guard cells and found that L-Met-induced ROS production was profoundly impaired in *atrbohD/F* double mutants compared with that in the WT (Figure 3D), indicating that AtrbohD and AtrbohF NADPH oxidases are responsible for ROS generation induced by L-Met via GLR3.1/3.5 Ca^{2+} channels. ABA-induced ROS production was also strongly impaired in *atrbohD/F* (Figure 3D), as previously shown (Kwak et al., 2003). These results, together with the data in Figure 1A, imply that activation of GLR3.1/3.5 Ca^{2+} channels by L-Met results in ROS generation via AtrbohD and AtrbohF, leading to regulation of stomatal aperture.

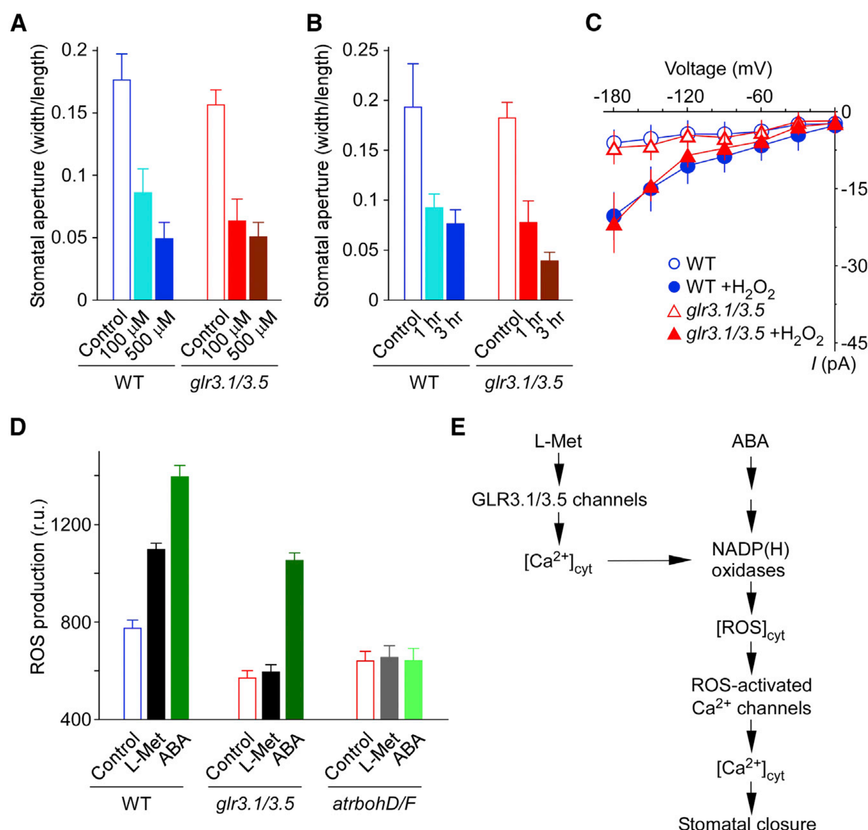

Figure 3. L-Met Activation of GLR3.1/3.5 Ca^{2+} Channels Closes Stomata through NADPH Oxidase-Mediated ROS Production in *Arabidopsis*

(A and B) H_2O_2-induced stomatal closure in *glr3.1/3.5* mutants at different concentrations (A) and different time points with 500 µM H_2O_2 (B).

(C) Whole-cell patch clamp recordings of 1 mM H_2O_2-activated Ca^{2+} currents in guard cells of WT and *glr3.1/3.5* mutants. Data are presented as mean ± SEM.

(D) Effects of 100 µM L-Met or 50 µM ABA on ROS production in WT, *glr3.1/3.5* mutants, and *atrbohD/F* mutants.

(E) A working model for L-Met-activated GLR3.1/3.5 Ca^{2+} channels in regulation of stomatal movement.

Data in (A), (B), (D), and (E) are presented as mean ± SEM from three independent experiments; n > 80 stomata for each treatment. See also Figure S4.

DISCUSSION

The identity of Ca^{2+} channels acting upstream of ROS has long been a hurdle impeding further understanding of cellular processes. Our study shows that GLR3.1/3.5 Ca^{2+} channels act upstream of ROS and may furnish initial $[Ca^{2+}]_{cyt}$ elevation that contributes to activation of NADPH oxidases, leading to subsequent cellular events, including ROS activation of previously identified Ca^{2+}-permeable channels (Figure 3E). Furthermore, we provide electrophysiological evidence that GLR3.1 and GLR3.5 form L-Met-activated Ca^{2+} channels that play an important role in plant growth and regulation of stomatal aperture. Identifying L-Met as a specific activator for the GLR channel that acts upstream of ROS production in guard cells provides an opportunity to identify the detailed regulatory mechanisms and underlying physiological functions of the entire GLR family in plant cells. Furthermore, it would be interesting to examine how these ligand-gated channels participate in the sensing processes in response to various environmental and intrinsic signals and coordinate these signals to mediate specific cellular reactions and physiological changes in plants. Further determination of the protein structure, accompanied by combined biochemical and electrophysiological studies of plant GLR channels, will also help to explain the genetic divergence in ligand-gated ion channels between animals and plants.

Met is the first amino acid of proteins during translation, and it serves as the precursor of S-adenosylmethionine, which not only acts as a methyl donor in various biological reactions by which proteins, lipids, and nucleic acids are methylated (Giovanelli et al., 1985; Ravanel et al., 2004) but also acts as the precursor in the biosynthesis of the polyamines, nicotianamine, phytosiderophores, and the gaseous plant hormone ethylene (Roje, 2006). Our guard cell patch clamp analyses show that perfusion of L-Met immediately activated GLR3.1/3.5 Ca^{2+} channels (Figure 2). It is possible that a compound metabolized from L-Met can activate GLR3.1/3.5 Ca^{2+} channels. It would be of interest to determine the L-Met source for GLR3.1/3.5 Ca^{2+} channel activation.

Accumulation of Met could provoke various physiological processes. Biosynthesis of Met is mediated by three enzymes: cystathionine γ-synthase, cystathionine β-lyase, and Met synthase. Cohen et al. (2014) have shown that overexpressing a feedback-insensitive mutant form of cystathionine γ-synthase led to an increased level of Met, which in turn induced upregulation of genes involved in drought, salt, and oxidative stress defense responses in *Arabidopsis*. This result hints at a link between ABA and L-Met. The gene encoding cystathionine γ-synthase is upregulated by light, sucrose, wounding, and ethylene (Hacham et al., 2013; Katz et al., 2006), but it remains unknown whether ABA regulates expression of the gene or activity of the enzyme.

ROS generated by NADPH oxidases are important signaling molecules that are extensively involved in various abiotic and biotic stress responses in plants (Kadota et al., 2015; Kurusu et al., 2015). In guard cells, ABA-induced ROS production is mediated by the RbohD and RbohF NADPH oxidases that are regulated by the SnRk2.6/OST1 protein kinase (Kwak et al., 2003; Sirichandra et al., 2009), leading to activation of Ca^{2+} channels in the plasma membrane (Hamilton et al., 2000; Mittler and Blumwald, 2015; Pei et al., 2000). Elevation in $[Ca^{2+}]_{cyt}$ in turn leads to stomatal closure by activating S-type anion channels or inhibiting inward-rectifying K^+ channels and H^+ ATPases (Hedrich, 2012;

Hedrich et al., 1990; Kwak et al., 2008; Vahisalu et al., 2008; Zhang et al., 2016). This ABA- and ROS-activated Ca^{2+} channel has been intensively studied, but little is known about the regulatory mechanisms of Ca^{2+} channels and Ca^{2+} signaling upstream ROS production. In this study, we showed that L-Met-triggered ROS accumulation by RbohD and RbohF NADPH oxidases is dependent on the GLR3.1/3.5 Ca^{2+} channels in the plasma membrane (Figure 3D). Steady-state ROS levels were significantly reduced in *glr3.1/3.5* guard cells compared with WT (Figure 3D). Furthermore, the level of ROS production induced by ABA was lower in *glr3.1/3.5* guard cells, suggesting that activation of NADPH oxidases by Ca^{2+} is impaired. In addition, in WT guard cells, L-Met induced ROS production, but not as much as ABA did (Figure 3D). Our results imply that L-Met activation of GLR3.1/3.5 channels contributes to activation of NADPH oxidases by ABA (Figure 3E). Given that the downstream NADPH oxidase-derived ROS accumulation has been shown to also contribute to Ca^{2+} signaling by a positive-feedback regulation loop (Kimura et al., 2012; Ranf et al., 2011), it will be of interest to explore the potential interplay and intrinsic characteristics between the redox-sensitive signaling pathways and the GLR3.1/3.5 channel-mediated Ca^{2+} signaling pathway in plants.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

All plant materials used in this study were of the *Arabidopsis thaliana* ecotype Col-0 background. The *glr3.1* transfer DNA (T-DNA) insertion line (SALK_063873) was obtained from the *Arabidopsis* Biological Resource Center (ABRC). The *glr3.1/3.5* double mutants, in which both *GLR3.1/3.5* transcripts were markedly suppressed, were generated by expression of a *GLR3.5*-specific RNAi construct in a *glr3.1* T-DNA disruption knockout mutant. The *glr3.1/3.5* double lines expressing aequorin were generated by crossing *glr3.1/3.5* double mutants to wild-type plants constitutively expressing aequorin. *Arabidopsis* plants were grown in the soil or in Petri dishes containing half-strength Murashige and Skoog (MS) media (Caisson Labs), 1.5% (w/v) sucrose (Sigma), and 0.8% (w/v) agar (Sigma) in controlled environmental chambers at 21 ± 2°C. The photoperiod was 16 hr light/8 hr dark (110 $\mu mol/m^2/s$).

Stomatal Movement Analysis

Detached rosette leaves from 4-week-old *Arabidopsis* plants were floated in an opening buffer containing 30 mM KCl, 10 mM MES/Tris (pH 6.0) for 2.5 hr under white light (120 mmol/m2/s), then further incubated for 1 hr after the application of various concentrations of L-methionine (L-Met) with or without 0.2 mM $CaCl_2$. To test ABA and H_2O_2 response, rosette leaves were incubated in an opening buffer containing 30 mM KCl, 10 mM MES/Tris, and 50 μM $CaCl_2$ (pH 6.0) under light for 2.5 hr and then transferred to an opening buffer containing 10 μM ABA or H_2O_2 at indicated concentrations for different periods.

Electrophysiological Recording in Guard Cells

Guard cell protoplasts were enzymatically isolated from *Arabidopsis* leaf epidermal strips of 4-week-old WT and *glr3.1/3.5* mutants. Whole-cell patch clamp recordings of guard cells were performed as described (Pei et al., 1998). L-Met (1, 10, and 100 μM) or H_2O_2 (1 mM) was applied by continuous bath perfusion during whole-cell recordings.

Measurement of ROS Production in Guard Cells

H_2DCF-DA was used to analyze ABA and Met-induced ROS production in guard cells as described previously (Kwak et al., 2003).

Aequorin Bioluminescence-Based Ca^{2+} Imaging

The *glr3.1/3.5* double mutants expressing the Ca^{2+} indicator protein aequorin were generated by a cross to plants harboring aequorin. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) was measured in plants harboring aequorin as described (Tang et al., 2007).

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.11.015.

AUTHOR CONTRIBUTIONS

Z.-M.P. and J.M.K. conceived the study, and J.M.K. designed the experiments. D.K., H.-C.H., E.O., H.S.L., S.M., D.C., L.P., B.R., J.W., and C.J. performed the experiments and analyzed data with Y.L., W.I., Y.M., Z.-M.P., and J.M.K. D.K. generated *glr3.1/3.5* mutants harboring aequorin and performed Ca^{2+} imaging, growth analysis, GUS analysis, and measurement of ROS production, together with L.P. H.-C.H. performed stomatal movement analyses in response to external Ca^{2+}, ABA, or amino acids, as well as Ca^{2+}-transient-induced stomatal closure analyses. B.R. and L.P. provided technical support. E.O. and S.M. performed electrophysiological analyses of GLR3.1/3.5 channels in guard cells. D.C. generated *glr3.1/3.5* mutants and determined the localization of GLR3.1/3.5 proteins. C.J. performed ROS-induced stomatal closure assays. D.K., Y.L., H.S.L., W.I., Z.-M.P., and J.M.K. wrote the manuscript.

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REFERENCES

Allen, G.J., Chu, S.P., Harrington, C.L., Schumacher, K., Hoffmann, T., Tang, Y.Y., Grill, E., and Schroeder, J.I. (2001). A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature *411*, 1053–1057.

Cho, D., Kim, S.A., Murata, Y., Lee, S., Jae, S.K., Nam, H.G., and Kwak, J.M. (2009). De-regulated expression of the plant glutamate receptor homolog AtGLR3.1 impairs long-term Ca2+-programmed stomatal closure. Plant J. *58*, 437–449.

Cohen, H., Israeli, H., Matityahu, I., and Amir, R. (2014). Seed-specific expression of a feedback-insensitive form of CYSTATHIONINE-γ-SYNTHASE in *Arabidopsis* stimulates metabolic and transcriptomic responses associated with desiccation stress. Plant Physiol. *166*, 1575–1592.

Cosgrove, D.J., and Hedrich, R. (1991). Stretch-activated chloride, potassium, and calcium channels coexisting in plasma membranes of guard cells of *Vicia faba* L. Planta *186*, 143–153.

Demidchik, V., Essah, P.A., and Tester, M. (2004). Glutamate activates cation currents in the plasma membrane of *Arabidopsis* root cells. Planta *219*, 167–175.

Dennison, K.L., and Spalding, E.P. (2000). Glutamate-gated calcium fluxes in *Arabidopsis*. Plant Physiol. *124*, 1511–1514.

Dodd, A.N., Kudla, J., and Sanders, D. (2010). The language of calcium signaling. Annu. Rev. Plant Biol. *61*, 593–620.

Giovanelli, J., Mudd, S.H., and Datko, A.H. (1985). Quantitative analysis of pathways of methionine metabolism and their regulation in lemna. Plant Physiol. *78*, 555–560.

Goto, D.B., Ogi, M., Kijima, F., Kumagai, T., van Werven, F., Onouchi, H., and Naito, S. (2002). A single-nucleotide mutation in a gene encoding

S-adenosylmethionine synthetase is associated with methionine over-accumulation phenotype in *Arabidopsis thaliana*. Genes Genet. Syst. *77*, 89–95.

Hacham, Y., Matityahu, I., and Amir, R. (2013). Light and sucrose up-regulate the expression level of *Arabidopsis* cystathionine γ-synthase, the key enzyme of methionine biosynthesis pathway. Amino Acids *45*, 1179–1190.

Hamilton, D.W., Hills, A., Kohler, B., and Blatt, M.R. (2000). Ca^{2+} channels at the plasma membrane of stomatal guard cells are activated by hyperpolarization and abscisic acid. Proc. Natl. Acad. Sci. USA *97*, 4967–4972.

Hedrich, R. (2012). Ion channels in plants. Physiol. Rev. *92*, 1777–1811.

Hedrich, R., Busch, H., and Raschke, K. (1990). Ca^{2+} and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. EMBO J. *9*, 3889–3892.

Kadota, Y., Shirasu, K., and Zipfel, C. (2015). Regulation of the NADPH oxidase RBOHD during plant immunity. Plant Cell Physiol. *56*, 1472–1480.

Karchi, H., Shaul, O., and Galili, G. (1993). Seed-specific expression of a bacterial desensitized aspartate kinase increases the production of seed threonine and methionine in transgenic tobacco. Plant J. *3*, 721–727.

Katz, Y.S., Galili, G., and Amir, R. (2006). Regulatory role of cystathionine-gamma-synthase and de novo synthesis of methionine in ethylene production during tomato fruit ripening. Plant Mol. Biol. *61*, 255–268.

Kim, S.A., Kwak, J.M., Jae, S.-K., Wang, M.-H., and Nam, H.G. (2001). Overexpression of the *AtGluR2* gene encoding an *Arabidopsis* homolog of mammalian glutamate receptors impairs calcium utilization and sensitivity to ionic stress in transgenic plants. Plant Cell Physiol. *42*, 74–84.

Kimura, S., Kaya, H., Kawarazaki, T., Hiraoka, G., Senzaki, E., Michikawa, M., and Kuchitsu, K. (2012). Protein phosphorylation is a prerequisite for the Ca^{2+}-dependent activation of *Arabidopsis* NADPH oxidases and may function as a trigger for the positive feedback regulation of Ca^{2+} and reactive oxygen species. Biochim. Biophys. Acta *1823*, 398–405.

Kong, D., Ju, C., Parihar, A., Kim, S., Cho, D., and Kwak, J.M. (2015). *Arabidopsis* glutamate receptor homolog3.5 modulates cytosolic Ca^{2+} level to counteract effect of abscisic acid in seed germination. Plant Physiol. *167*, 1630–1642.

Kurusu, T., Kuchitsu, K., and Tada, Y. (2015). Plant signaling networks involving $Ca^{(2+)}$ and Rboh/Nox-mediated ROS production under salinity stress. Front. Plant Sci. *6*, 427.

Kwak, J.M., Mori, I.C., Pei, Z.-M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D.G., and Schroeder, J.I. (2003). NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. EMBO J. *22*, 2623–2633.

Kwak, J.M., Nguyen, V., and Schroeder, J.I. (2006). The role of reactive oxygen species in hormonal responses. Plant Physiol. *141*, 323–329.

Kwak, J.M., Mäser, P., and Schroeder, J.I. (2008). The clickable guard cell, version II: interactive model of guard cell signal transduction mechanisms and pathway. In The *Arabidopsis* Book, R. Last, C. Chang, I. Graham, O. Leyser, R. McClung, and C. Weinig, eds. (American Society of Plant Biologists), pp. 1–17.

Lam, H.M., Chiu, J., Hsieh, M.H., Meisel, L., Oliveira, I.C., Shin, M., and Coruzzi, G. (1998). Glutamate-receptor genes in plants. Nature *396*, 125–126.

Leonhardt, N., Kwak, J.M., Robert, N., Waner, D., Leonhardt, G., and Schroeder, J.I. (2004). Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. Plant Cell *16*, 596–615.

MacRobbie, E.A.C. (2000). ABA activates multiple $Ca^{(2+)}$ fluxes in stomatal guard cells, triggering vacuolar $K^{(+)}(Rb^{(+)})$ release. Proc. Natl. Acad. Sci. USA *97*, 12361–12368.

McAinsh, M.R., Gray, J.E., Hetherington, A.M., Leckie, C.P., and Ng, C. (2000). Ca^{2+} signalling in stomatal guard cells. Biochem. Soc. Trans. *28*, 476–481.

Meyerhoff, O., Müller, K., Roelfsema, M.R., Latz, A., Lacombe, B., Hedrich, R., Dietrich, P., and Becker, D. (2005). AtGLR3.4, a glutamate receptor channel-like gene is sensitive to touch and cold. Planta *222*, 418–427.

Michard, E., Lima, P.T., Borges, F., Silva, A.C., Portes, M.T., Carvalho, J.E., Gilliham, M., Liu, L.H., Obermeyer, G., and Feijó, J.A. (2011). Glutamate receptor-like genes form Ca^{2+} channels in pollen tubes and are regulated by pistil D-serine. Science *332*, 434–437.

Mittler, R., and Blumwald, E. (2015). The roles of ROS and ABA in systemic acquired acclimation. Plant Cell *27*, 64–70.

Mousavi, S.A., Chauvin, A., Pascaud, F., Kellenberger, S., and Farmer, E.E. (2013). GLUTAMATE RECEPTOR-LIKE genes mediate leaf-to-leaf wound signalling. Nature *500*, 422–426.

Murata, Y., Pei, Z.-M., Mori, I.C., and Schroeder, J. (2001). Abscisic acid activation of plasma membrane $Ca^{(2+)}$ channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in *abi1-1* and *abi2-1* protein phosphatase 2C mutants. Plant Cell *13*, 2513–2523.

Niu, L., and Liao, W. (2016). Hydrogen peroxide signaling in plant development and abiotic responses: crosstalk with nitric oxide and calcium. Front. Plant Sci. *7*, 230.

Pei, Z.M., Ghassemian, M., Kwak, C.M., McCourt, P., and Schroeder, J.I. (1998). Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. Science *282*, 287–290.

Pei, Z.M., Murata, Y., Benning, G., Thomine, S., Klüsener, B., Allen, G.J., Grill, E., and Schroeder, J.I. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature *406*, 731–734.

Pinheiro, P.S., and Mulle, C. (2008). Presynaptic glutamate receptors: physiological functions and mechanisms of action. Nat. Rev. Neurosci. *9*, 423–436.

Qi, Z., Stephens, N.R., and Spalding, E.P. (2006). Calcium entry mediated by GLR3.3, an *Arabidopsis* glutamate receptor with a broad agonist profile. Plant Physiol. *142*, 963–971.

Ranf, S., Eschen-Lippold, L., Pecher, P., Lee, J., and Scheel, D. (2011). Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. Plant J. *68*, 100–113.

Ranty, B., Aldon, D., Cotelle, V., Galaud, J.P., Thuleau, P., and Mazars, C. (2016). Calcium sensors as key hubs in plant responses to biotic and abiotic stresses. Front. Plant Sci. *7*, 327.

Ravanel, S., Block, M.A., Rippert, P., Jabrin, S., Curien, G., Rébeillé, F., and Douce, R. (2004). Methionine metabolism in plants: chloroplasts are autonomous for de novo methionine synthesis and can import S-adenosylmethionine from the cytosol. J. Biol. Chem. *279*, 22548–22557.

Roje, S. (2006). S-Adenosyl-L-methionine: beyond the universal methyl group donor. Phytochemistry *67*, 1686–1698.

Rorison, I.H., and Robinson, D. (1984). Calcium as an environmental variable. Plant Cell Environ. *7*, 381–390.

Sanders, D., Pelloux, J., Brownlee, C., and Harper, J.F. (2002). Calcium at the crossroads of signaling. Plant Cell *14* (*Suppl*), S401–S417.

Schulz, P., Herde, M., and Romeis, T. (2013). Calcium-dependent protein kinases: hubs in plant stress signaling and development. Plant Physiol. *163*, 523–530.

Sirichandra, C., Gu, D., Hu, H.C., Davanture, M., Lee, S., Djaoui, M., Valot, B., Zivy, M., Leung, J., Merlot, S., and Kwak, J.M. (2009). Phosphorylation of the *Arabidopsis* AtrbohF NADPH oxidase by OST1 protein kinase. FEBS Lett. *583*, 2982–2986.

Tang, R.H., Han, S., Zheng, H., Cook, C.W., Choi, C.S., Woerner, T.E., Jackson, R.B., and Pei, Z.M. (2007). Coupling diurnal cytosolic Ca2+ oscillations to the CAS-IP3 pathway in Arabidopsis. Science *315*, 1423–1426.

Tapken, D., Anschütz, U., Liu, L.H., Huelsken, T., Seebohm, G., Becker, D., and Hollmann, M. (2013). A plant homolog of animal glutamate receptors is an ion channel gated by multiple hydrophobic amino acids. Sci. Signal. *6*, ra47.

Teardo, E., Carraretto, L., De Bortoli, S., Costa, A., Behera, S., Wagner, R., Lo Schiavo, F., Formentin, E., and Szabo, I. (2015). Alternative splicing-mediated

targeting of the *Arabidopsis* GLUTAMATE RECEPTOR3.5 to mitochondria affects organelle morphology. Plant Physiol. *167*, 216–227.

Vahisalu, T., Kollist, H., Wang, Y.F., Nishimura, N., Chan, W.Y., Valerio, G., Lamminmäki, A., Brosché, M., Moldau, H., Desikan, R., et al. (2008). SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. Nature *452*, 487–491.

Vincill, E.D., Clarin, A.E., Molenda, J.N., and Spalding, E.P. (2013). Interacting glutamate receptor-like proteins in Phloem regulate lateral root initiation in *Arabidopsis*. Plant Cell *25*, 1304–1313.

White, P.J., and Broadley, M.R. (2003). Calcium in plants. Ann. Bot. (Lond.) *92*, 487–511.

Yang, Y., Costa, A., Leonhardt, N., Siegel, R.S., and Schroeder, J.I. (2008). Isolation of a strong *Arabidopsis* guard cell promoter and its potential as a research tool. Plant Methods *4*, 6.

Zhang, A., Ren, H.M., Tan, Y.Q., Qi, G.N., Yao, F.Y., Wu, G.L., Yang, L.W., Hussain, J., Sun, S.J., and Wang, Y.F. (2016). S-type anion channels SLAC1 and SLAH3 function as essential negative regulators of inward K^+ channels and stomatal opening in *Arabidopsis*. Plant Cell *28*, 949–965.