

# Glutamate functions in stomatal closure in *Arabidopsis* and fava bean

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**Abstract** Guard cells are indispensable for higher plants because they control gas exchange and water balance to maintain photosynthetic activity. The signaling processes that govern their movement are controlled by several factors, such as abscisic acid (ABA), blue light, pathogen-associated molecular patterns (PAMPs), and carbon dioxide. Herein, we demonstrated that the amino acid glutamate (Glu), a well-known mammalian neurotransmitter, functions as a novel signaling molecule in stomatal closure in both *Arabidopsis* and fava bean (*Vicia faba* L.). Pharmacological and electrophysiological analyses provided important clues for the participation of Glu-receptors, Ca<sup>2+</sup>, and protein phosphorylation during the signaling process. Genetic analyses using *Arabidopsis* ABA-deficient (*aba2-1*) and ABA-insensitive (*abi1-1* and *abi2-1*) mutants showed that ABA is not required for Glu signaling. However, loss-of-function of the *Arabidopsis* gene encoding *Slow Anion Channel-Associated 1 (SLAC1)* and *Calcium-Dependent Protein Kinase 6 (CPK6)* impaired the Glu response. Moreover, T-DNA knockout mutations of the *Arabidopsis* Glu receptor-like gene (*GLR*), *GLR3.5*, lost their sensitivity to Glu-dependent stomatal closure. Our results strongly support functional Glu-signaling in

stomatal closure and the crucial roles of *GLRs* in this signaling process.

**Keywords** *Arabidopsis thaliana* · Glutamate · Glutamate receptor · Signal transduction · Stomatal closure · *Vicia faba*

## Introduction

The amino acid glutamate (Glu) plays pivotal roles in the functioning of the central nervous system in mammals (Watkins and Jane 2006). Glu-mediated signaling is initiated by the binding of Glu to several types of Glu receptors, including ionotropic Glu receptors (iGluRs) and metabotropic Glu receptors (mGluRs) (Mayer 2005). Plants do not have highly regulated nerve systems like animals, but families of Glu receptors homologous to mammalian iGluRs (GLRs) have been discovered in *Arabidopsis* (Lacombe et al. 2001; Lam et al. 1998), rice (Li et al. 2006), and tomato (Aouini et al. 2012). Since the discovery of *GLR* genes in plant cells, Glu-signaling has been studied intensively as a potential amino acid sensor, and Glu was found to cause rapid membrane depolarization and Ca<sup>2+</sup> flux in *Arabidopsis* roots (Dennison and Spalding 2000). Mutation of *GLR3.3*, one of 20 *Arabidopsis* genes, impaired both the membrane depolarization and the Ca<sup>2+</sup> rise triggered by Glu (Qi et al. 2006). Moreover, Glu has been found to have several roles in plant signaling, which include regulating hypocotyl elongation (Dubos et al. 2003; Lam et al. 1998), sensing mineral nutrient status (Kim et al. 2001), resisting aluminum toxicity (Sivaguru et al. 2003), and regulating the carbon/nitrogen balance (Kang and Turano 2003), abscisic acid (ABA) synthesis (Kang et al. 2004), cold (Meyerhoff et al. 2005), root meristem function (Li et al.

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2006; Walch-Liu et al. 2006), plant defense against pathogens (Vatsa et al. 2011), pollen tube development (Michard et al. 2011), and long-distance wound signaling (Mousavi et al. 2013).

Stomata are pores on the surface of leaves, and the opening and closing of these pores control the diffusion of gases into and out of plant tissues. Stomata are formed by pairs of guard cells that sense environmental signals such as light, humidity, carbon dioxide (CO<sub>2</sub>), and pathogens, and also respond to hormones including ABA, auxin, and ethylene (Melotto et al. 2006; Schroeder et al. 2001; Shimazaki et al. 2007; Shope et al. 2008). Numerous signaling components act in the induction of stomatal closure. Among them, Ca<sup>2+</sup> is the important signaling molecule in guard cell signaling. An increase in the cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>cyt</sub>) can transduce cellular responses to various biotic and abiotic stimuli, including light, gravity, oxidative stress, cold shock, drought, hormones, salt stress, and fungal elicitors (Berridge et al. 2003; Sanders et al. 2002). The plant hormone ABA causes increases in the [Ca<sup>2+</sup>]<sub>cyt</sub> in guard cells via Ca<sup>2+</sup> influx through plasma membrane Ca<sup>2+</sup>-permeable channels and Ca<sup>2+</sup> release from internal stores, resulting in stomatal closure (Kwak et al. 2003; MacRobbie 2000; Pei et al. 2000; Staxen et al. 1999). Cho et al. (2009) demonstrated that GLR3.1 participates in external Ca<sup>2+</sup> influx into the cytosol as well as external Ca<sup>2+</sup>-induced stomatal closure, but they did not show whether Glu induces stomatal closure through GLRs.

In the current study, we examined the possibility that Glu plays a role in guard cell signaling. We found that Glu functions as a signal for stomatal closure in both *Arabidopsis* and fava bean. This response required Glu receptors, activation of plasma membrane Ca<sup>2+</sup>-permeable channels, and protein phosphorylation, as revealed by pharmacological, electrophysiological and genetic analyses. Loss-of-function analyses demonstrated that one of the *Arabidopsis* GLR genes, *GLR3.5*, plays a pivotal role in this guard cell signaling. This is the first report demonstrating Glu-induced stomatal closure in higher plants.

## Materials and methods

### Plant materials and growth conditions

*Arabidopsis* Columbia (Col) and Landsberg *erecta* (Ler) ecotypes were used. *Arabidopsis* mutant lines *aba2-1*, *abi1-1*, *abi2-1*, *slac1-2*, *cpk6-1*, and *cpk6-2* were studied, and T-DNA insertion lines of *glr1.1*, *glr1.2*, *glr1.4*, *glr3.3*, *glr3.5*, and *glr3.7* in the Col background were obtained from the Arabidopsis Biological Research Center (ABRC). Plants were grown in soil (1:1 Metromix:vermiculite) in a controlled environment at 23 °C with a 11-h light:13-h dark

cycle. Seeds of fava bean (*Vicia faba* L. cv. House Ryou sai) were purchased from Kyouwa Seed Co. (Chiba, Japan). Plants were grown in a growth chamber at 23 °C with a 16-h light:8-h dark cycle.

### Stomatal aperture measurements

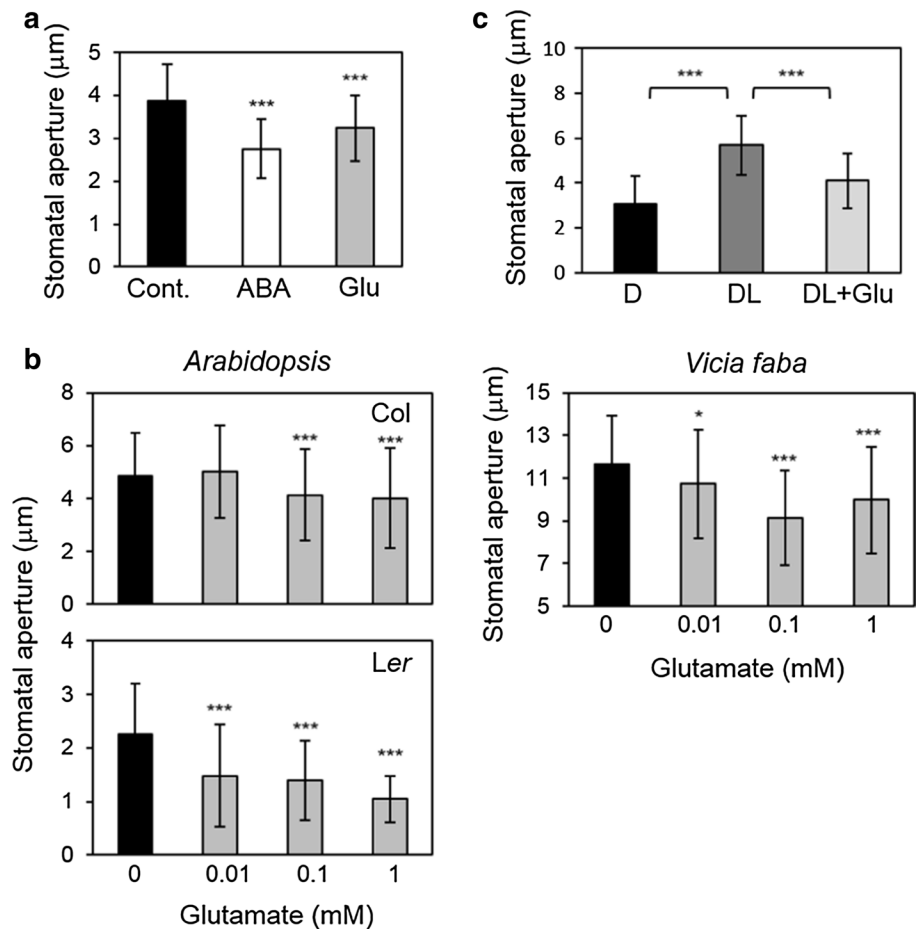
Epidermal strips were peeled from abaxial young, fully expanded leaves. Epidermal strips were floated in petri dishes (diameter, 9 cm) containing opening medium A [10 mM MES–KOH (pH 6.15), 50 mM KCl, 0.1 mM CaCl<sub>2</sub>] for *Arabidopsis*, or opening medium B [10 mM MES–KOH (pH 6.15), 50 mM KCl] for fava bean and were kept for 2 h at 23 °C under light (50 μmol m<sup>-2</sup> s<sup>-1</sup>). The strips were then transferred to opening medium containing sodium glutamate, glycine, and/or the pharmacological reagents (AP-5, EGTA, BAPTA-AM) and kept for 3 h at 23 °C under light irradiation. For light-induced stomatal opening, Glu was applied to the epidermal strips after the 2 h of dark period and subsequently exposed to light for 3 h. Following treatment, the stomata were photographed under a microscope (Eclipse E600; Nikon Corp. Tokyo, Japan) using a digital camera (Ds-L2, Nikon Corp.). Inner widths of stomatal pores were measured using a digital micro-analyzer (Japan Polaroid Digital Products, Tokyo). At least four strips containing 20 stomata were measured for each treatment. Experiments were repeated at least three times, and Student's *t* test was used to assess significant differences.

### Patch-clamp analysis

Calcium-permeable channel current (*I*<sub>Ca</sub>) was recorded essentially as published previously and described below (Ye et al. 2013). Patch-clamp data were recorded using a MultiClamp 700B amplifier equipped a CV-7B headstage and pClamp software 10.3, and analyzed with Clampfit 10.3 software (Molecular Devices, Sunnyvale, California, USA). Guard cell protoplasts for patch-clamping were isolated from *Arabidopsis* (Col) as described by Ye et al. (2013).

The pipette solution contained 10 mM BaCl<sub>2</sub>, 0.1 mM dithiothreitol, 4 mM EGTA and 10 mM MES-Tris (pH 7.1), and the bath solution contained 100 mM BaCl<sub>2</sub>, 0.1 mM dithiothreitol and 10 mM MES-Tris (pH 5.6). Osmolarity of the solutions was adjusted with sorbitol to 500 and 485 mmol kg<sup>-1</sup>, respectively. Recording was started 10 min after the establishment of whole cell configuration in the absence of glutamate in the bath solution and successively recorded in the presence of glutamate after perfusion with bath solution containing 10 mM Glu. The voltage was ramped from –18 to –218 mV (after liquid junction potential compensation) with a ramp speed of 0.1 V s<sup>-1</sup>. The ramp voltage protocol was applied 10 times to obtain an

**Fig. 1** Glu induces stomatal closure in both *Arabidopsis* and fava bean. **a** Stomatal aperture of *Arabidopsis* (Col) after incubation with ABA (10  $\mu$ M) and Glu (1 mM) for 3 h. **b** Stomatal response of *Arabidopsis* ecotypes (Col, *Ler*) and fava bean exposed to the indicated concentration of Glu for 3 h. **c** Effects of Glu (1 mM) on the light-induced stomatal opening in *Arabidopsis* (Col). Stomatal aperture after dark incubation (D) and subsequent light exposure without or with Glu (DL, DL + Glu) were measured. Each bar indicates the mean  $\pm$  S.D. of 80–120 measurements. Statistical differences were detected using a two-tailed *t* test (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001)



average of a cell. Significance of difference between data sets was assessed by Wilcoxon signed ranks test.

**Purification of guard cell protoplasts**

Guard cell protoplasts were isolated enzymatically from the abaxial epidermis of approximately 100 rosette *Arabidopsis* leaves, as described previously (Pandey et al. 2002; Ueno et al. 2005). Purified guard cells were visually inspected for purity using a light microscope, and RNA was isolated immediately.

**RT-PCR**

Total RNA was extracted using TRIzol<sup>®</sup> (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized from 1  $\mu$ g of total RNA using ReveTra Ace<sup>®</sup> (Toyobo, Osaka, Japan) in a 10- $\mu$ l reaction mixture. RT-PCR was performed for 30–35 cycles using GoTaq<sup>®</sup> (Promega, Fitchburg, WI, USA). The primer sequences used in RT-PCR analysis were *GLR3.5* Fw (GAGGAGCGAGGAGGCT), *GLR3.5* Rv (AGTTTCGTGATCTTTTCGACT), *ACTIN2*

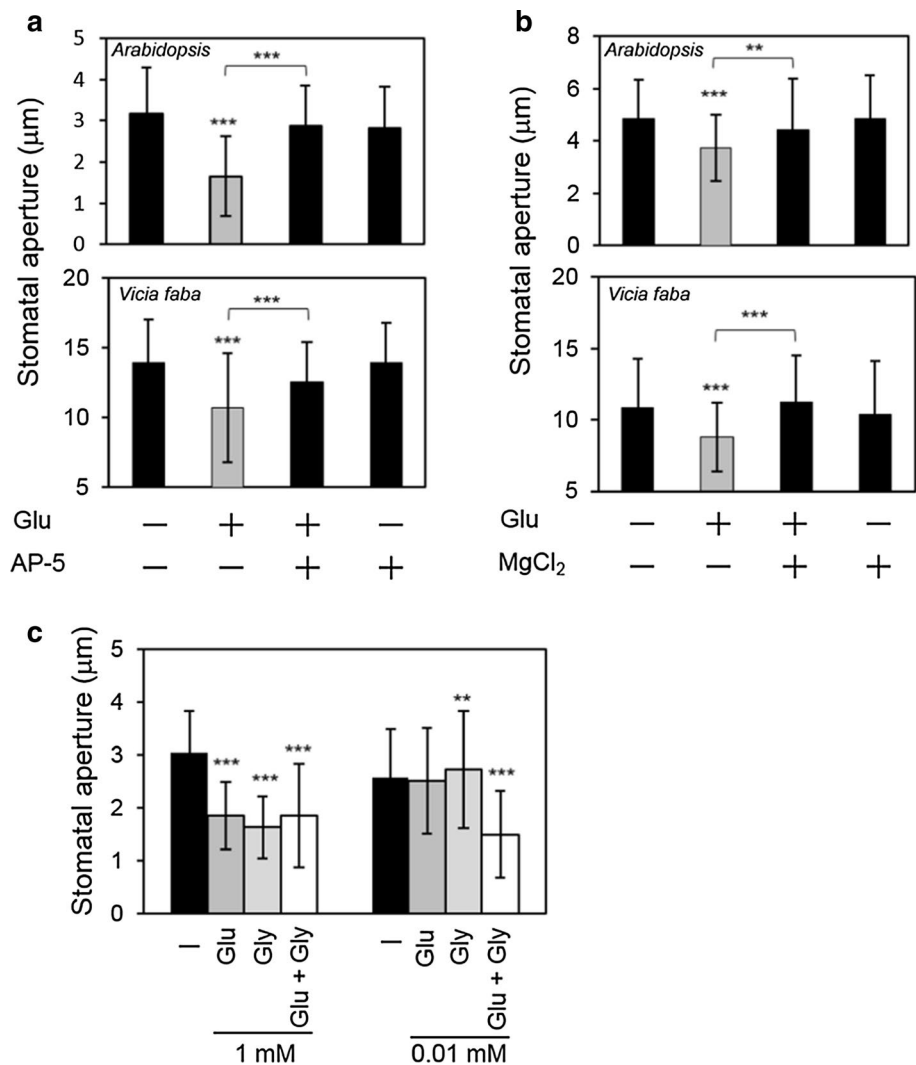
Fw (GCCATCCAAGCTGTTCTCTC) and *ACTIN2* Rv (GAACCACCGATCCAGACT).

**Results**

**Glu promotes stomatal closure in both *Arabidopsis* and fava bean**

To ascertain whether Glu promotes stomatal closure, we applied sodium L-glutamate monohydrate to epidermal strips prepared from *Arabidopsis* and fava bean. As observed in ABA treatments, 1 mM Glu potentiated stomatal closure in *Arabidopsis* (Fig. 1a). Next, we examined the effect of Glu on stomatal movement in *Arabidopsis* ecotypes and in fava bean. In the Columbia ecotype (Col), stomata started to close in response to 0.1 mM Glu, but did not respond to 0.01 mM Glu. In the Landsberg *erecta* ecotype (*Ler*), however, stomata closed even at 0.01 mM Glu. Glu-induced stomatal closure was also observed in fava bean (Fig. 1b). We also examined whether Glu inhibits light-induced stomatal opening or not in *Arabidopsis* (Col).

**Fig. 2** Involvement of iGluRs in Glu-induced stomatal closure. **a** Effect of the iGluR antagonist AP-5 on Glu-induced stomatal closure in *Arabidopsis* and fava bean; 1 mM and 0.1 mM Glu were used in *Arabidopsis* and fava bean, respectively. The concentration of AP-5 was 1 mM. **b** Effect of  $MgCl_2$  on Glu-induced stomatal closure in *Arabidopsis* and fava bean; 1 mM and 0.1 mM Glu were used in *Arabidopsis* and fava bean, respectively. The concentration of  $MgCl_2$  was 5 mM. **c** Effect of Gly on Glu-induced stomatal closure; amino acids were applied separately or concurrently at 1 and 0.01 mM. Each bar indicates the mean  $\pm$  S.D. of 80–120 measurements. Statistical differences were detected using a two-tailed *t* test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ )



We found Glu not only induce stomatal closure, but also inhibit light-induced stomatal opening (Fig. 1c).

### Participation of Glu receptors in Glu-induced stomatal closure

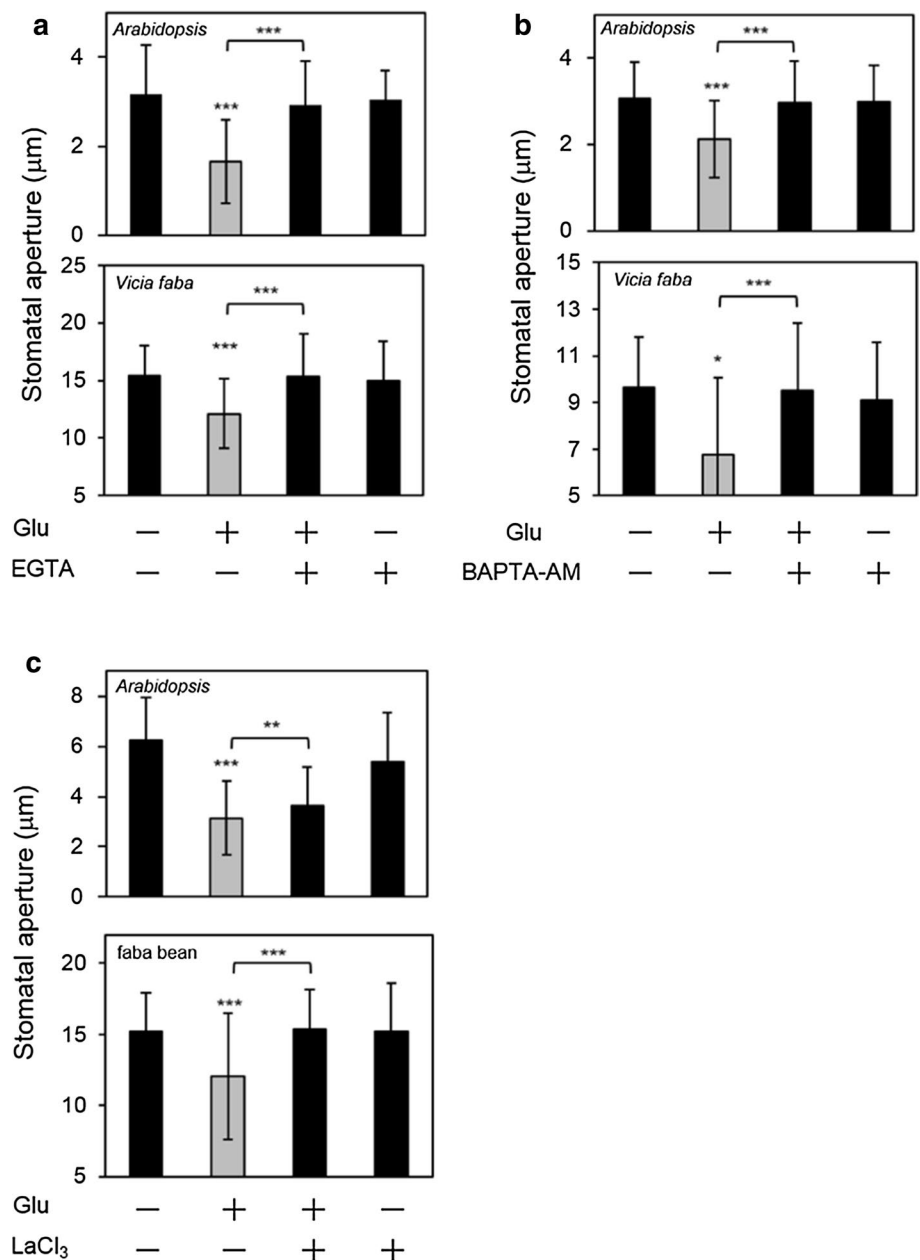
We used pharmacological reagents to probe the involvement of *N*-methyl-D-aspartate (NMDA)-type iGluRs in Glu-induced stomatal closure. Initially, we examined the effects of D(-)-2-amino-5-phosphonopentanoic acid (AP-5), a specific antagonist of NMDA GLRs, on Glu-induced stomatal closure. AP-5 inhibited Glu-induced stomatal closure in both *Arabidopsis* and fava bean (Fig. 2a). Magnesium chloride, which is known to block NMDA iGluR channels, suppressed the Glu-induced stomatal closure in both species (Fig. 2b). Since apoplasmic concentration of free  $Mg^{2+}$  was shown to be below 0.5 mM in fava bean (Mühling and Sattelmacher 1995), 5 mM  $Mg^{2+}$  used in this experiment was reasonable for assessing the inhibitory

effect of NMDA GLRs. Glycine (Gly), which functions as a iGluR agonist (Chatterton et al. 2002; Dubos et al. 2003; Ivanovic et al. 1998), induced stomatal closure at 1 mM (Fig. 2c), but no additive effect of Gly and Glu was observed. A low concentration of Gly (10  $\mu$ M) alone did not induce stomatal closure. However, when 10  $\mu$ M Glu was applied with 10  $\mu$ M Gly, the stomata closed (Fig. 2c). These results suggested the participation of NMDA iGluRs in Glu-induced stomatal closure.

### $Ca^{2+}$ is required for Glu-induced stomatal closure

To further elucidate the possible relevance of GLRs in Glu-induced stomatal closure, we examined whether  $Ca^{2+}$  affects this signaling process. EGTA (extracellular  $Ca^{2+}$ -chelator) and BAPTA-AM (intracellular  $Ca^{2+}$ -chelator) inhibited the Glu-induced stomatal closure in *Arabidopsis* and fava bean, indicating that  $Ca^{2+}$  influx to the cytosol is required for this effect (Fig. 3a, b). We also examined

**Fig. 3**  $\text{Ca}^{2+}$  is required for Glu-induced stomatal closure. **a** Effect of EGTA on Glu-induced stomatal closure in *Arabidopsis* and fava bean; 1 and 0.1 mM Glu were used in *Arabidopsis* and fava bean, respectively. EGTA was used at 0.1 and 1 mM in *Arabidopsis* and fava bean, respectively. **b** Effect of BAPTA-AM on Glu-induced stomatal closure in *Arabidopsis* and fava bean; 1 and 0.1 mM Glu were used in *Arabidopsis* and fava bean, respectively. The concentration of BAPTA-AM was 0.25 mM. **c** Effect of  $\text{LaCl}_3$  on Glu-induced stomatal closure in *Arabidopsis* and fava bean; 1 and 0.1 mM Glu were used in *Arabidopsis* and fava bean, respectively. The concentration of  $\text{LaCl}_3$  was 5 mM. Each bar indicates the mean  $\pm$  S.D. of 80–120 measurements. Statistical differences were detected using a two-tailed *t* test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ )



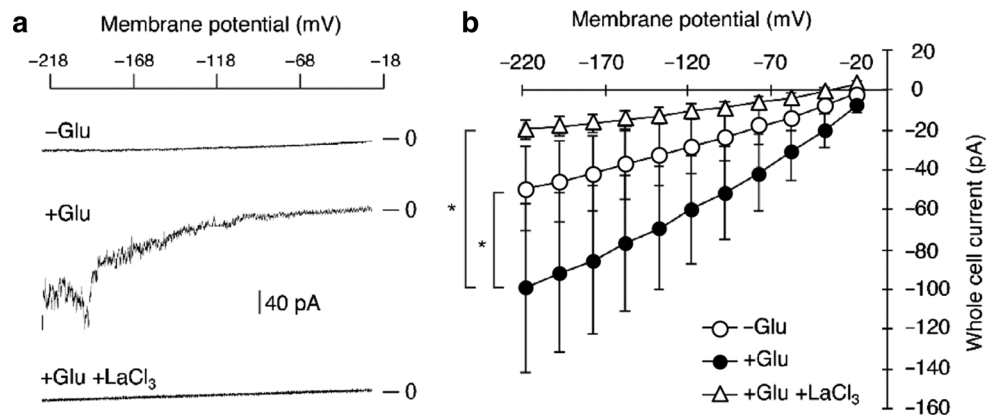
the effect of  $\text{LaCl}_3$  ( $\text{Ca}^{2+}$  channel blocker) on the Glu-induced stomatal closure.  $\text{La}^{3+}$  inhibited the Glu-induced stomatal closure in *Arabidopsis* and fava bean (Fig. 3c). These results indicated that  $\text{Ca}^{2+}$  influx occurred after Glu sensing.

**Glu activates plasma membrane  $\text{Ca}^{2+}$ -permeable channel currents ( $I_{\text{Ca}}$ ) in *Arabidopsis* guard cells**

It has been shown that  $I_{\text{Ca}}$  of guard cells is activated by ABA, MeJA and microbe-associated molecular patterns (Murata et al. 2015). Here we conducted patch-clamp analyses to examine activation of  $I_{\text{Ca}}$  by Glu application

(Fig. 4). In the absence of Glu small whole cell current ( $-46 \pm 20$  pA at  $-198$  mV) was observed. The current was significantly activated by perfusion with 10 mM Glu-containing bath solution to  $-92 \pm 40$  pA at  $-198$  mV ( $P = 0.046$ , Wilcoxon signed ranks test,  $n = 6$ ). These currents were substantially diminished by addition of 1 mM  $\text{La}^{3+}$  ( $-18 \pm 5$  pA at  $-198$  mV). These results indicate that Glu activated ion current of which charge carrier was  $\text{Ba}^{2+}$  and capable to be blocked with  $\text{La}^{3+}$  similarly to ABA-activated  $I_{\text{Ca}}$  (Pei et al. 2000). The current amplitude appeared fairly variable among cells, while the reason was unresolved in this study.





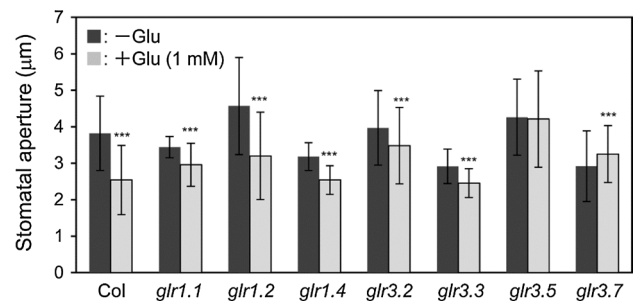
**Fig. 4** Glu activation of  $I_{Ca}$  in plasma membrane of *Arabidopsis* guard cells. **a** Typical raw current traces; *top horizontal scale* indicates membrane voltage, and *traces* indicate typical whole cell current without Glu, with Glu (10 mM) and with Glu (10 mM) and LaCl<sub>3</sub> (1 mM), respectively. *Vertical scale bar* indicates 40 pA. **b**

Averaged whole cell currents ( $n = 6$  cells). Current from a cell was an average of 10 repeated records from a cell with 1-s intervals. *Error bars* represent SEM. *Asterisks* indicates significance (Wilcoxon signed ranks test,  $\alpha = 0.05$ )

### Arabidopsis *GLR3.5* is involved in Glu-induced stomatal closure

The pharmacological experiments indicated the involvement of iGluRs in Glu-induced stomatal closure. To confirm the possibility, we used several T-DNA knockout lines of *Arabidopsis* *GLRs* and examined whether the mutations affect guard cell response to Glu. *Arabidopsis* has 20 *GLR* genes in its genome (Chiu et al. 2002; Roy et al. 2008). We obtained information on the expression of each *GLR* in *Arabidopsis* guard cells from the data published by Roy et al. (2008) and also from the *Arabidopsis* eFP Browser (<http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). We estimated that the genes *GLR1.1*, *GLR1.2*, *GLR1.4*, *GLR3.2*, *GLR3.3*, *GLR3.5*, and *GLR3.7* were expressed in guard cells, so we examined them further.

Among the seven lines tested, *glr3.5* and *glr3.7* impaired Glu-induced stomatal closure, whereas Glu induced stomatal closure in *glr1.1*, *glr1.2*, *glr1.4*, and *glr3.3* (Fig. 5). We focused on *GLR3.5*. To ascertain whether *GLR3.5* functions in guard cells, we prepared cDNA from guard cell protoplasts (GCPs) and confirmed its expression in these cells. The purity of GCPs was examined according to the expression of phosphoenolpyruvate carboxylase 2 (*ATPPC2*, At2g42600), which shows low-level expression in guard cells and high-level expression in mesophyll cells (Leonhardt et al. 2004), and hydroxyproline-rich protein (*HPRP*, At2g21140), which shows high-level expression in guard cells (Cho et al. 2009). We found a strong *ATPPC2* signal in mesophyll cell cDNA compared with GCP cDNA (Fig. 6a). In contrast, *HPRP* was highly expressed in GCPs, but was scarce in mesophyll cells. As RT-PCR reflects the purity and specificity of GCP cDNA, we performed RT-PCR analyses on *GLR3.5* using GCP cDNA and examined



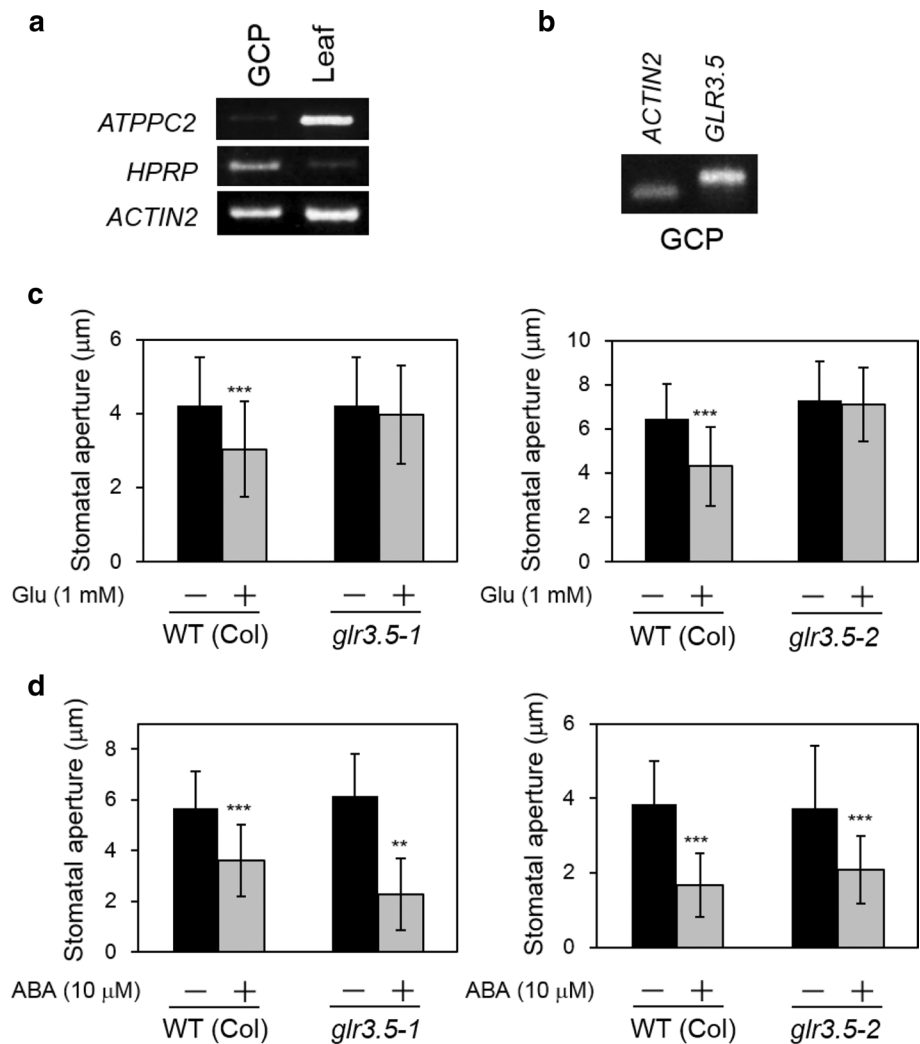
**Fig. 5** Stomatal response of T-DNA *GLR1.1*, *GLR1.2*, *GLR1.4*, *GLR3.2*, *GLR3.3*, *GLR3.5*, and *GLR3.7* knockout mutants. Expression of these genes was reported in guard cells (eFP Browser). These mutants were exposed to 1 mM Glu. *Each bar* indicates the mean  $\pm$  S.D. of 80–120 measurements. Statistical differences were detected using a two-tailed *t* test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ )

the expression in GCPs (Fig. 6b). We used two independent T-DNA lines, *glr3.5-1* and *glr3.5-2* (Fig. S1), and confirmed the disruption of this gene in these two lines via RT-PCR. Both lines showed impaired Glu-induced stomatal closure (Fig. 6c), whereas ABA and darkness induced stomatal closure (Fig. 6d, Fig. S2). These results suggest that *GLR3.5* is expressed in guard cells and contributes to Glu-induced stomatal closure.

### Glu response does not mediate ABA signaling, but requires CPK6 and SLAC1 in an ABA-independent manner

To clarify the signaling pathway leading to Glu-induced stomatal closure, we examined Glu-sensitivity in several *Arabidopsis* mutants having impaired guard cell responses.

**Fig. 6** Loss-of-function of the *Arabidopsis* GLR gene *GLR3.5* prevented Glu-induced stomatal closure. **a** RT-PCR analysis of the mesophyll cell marker gene *AtPPC2* and guard cell marker gene *HPRP* in guard cell protoplasts (GCPs) and leaf tissue. Total RNA (1 µg) was prepared from GCPs and entire leaves for cDNA synthesis. **b** RT-PCR analysis of the *GLR3.5* gene in GCPs. **c** Stomatal response of *glr3.5-1* and *glr3.5-2* mutants exposed to Glu (1 mM). **d** Stomatal response of *glr3.5-1* and *glr3.5-2* mutants exposed to ABA (10 µM). Each bar indicates the mean ± S.D. of 80–120 measurements

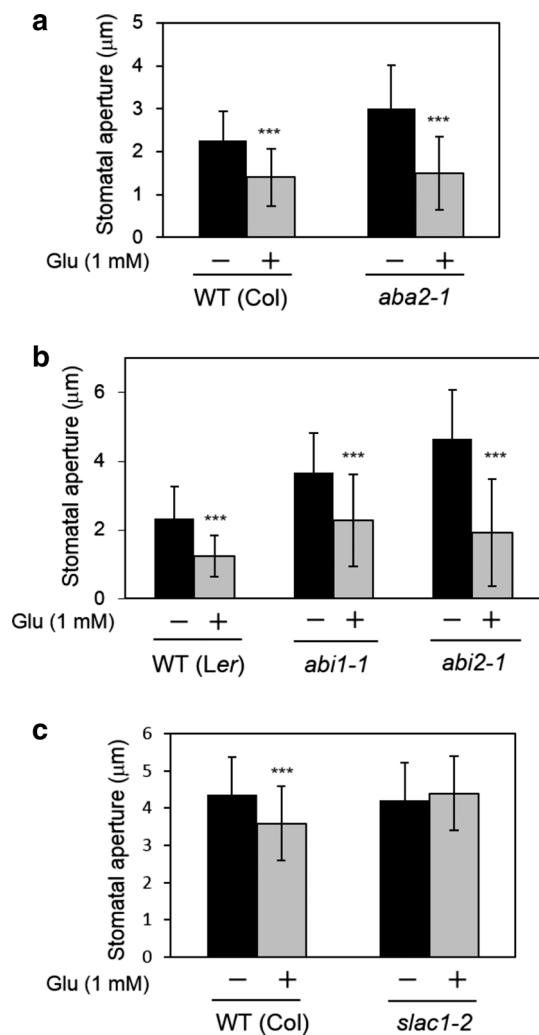


First, we tested the ABA-deficient mutant *aba2-1* and the ABA-insensitive mutants *abi1-1* and *abi2-1*. Glu induced stomatal closure in these mutants (Fig. 7a, b), indicating that Glu signaling operates independently from ABA signaling in guard cells. We also used the *slac1-2* mutant to ascertain whether this S-type anion channel gene, which has been shown to be involved in CO<sub>2</sub> and ABA signaling (Xue et al. 2011), acts downstream of Glu-signaling. We found that *slac1-2* lost its sensitivity for Glu-induced stomatal closure (Fig. 7c). Next, we examined whether protein phosphorylation is involved in Glu-dependent guard cell signaling. To address this question, we used protein kinase inhibitors of K252a and staurosporine. These reagents concurrently inhibited Glu-induced stomatal closure (Fig. 8a). The same concentration of solvent (dimethyl sulfoxide) used for dissolving these two inhibitors had no effect on Glu-dependent stomatal movement (Fig. S3). We also tested a loss-of-function mutation in *Calcium Dependent Protein Kinase 6* (*CPK6*), which is a positive regulator of ABA-signaling (Mori et al. 2006). *CPK6* has been shown

to phosphorylate and activate SLAC1 protein (Brandt et al. 2012). We used two independent T-DNA knockout lines, *cpk6-1* and *cpk6-2*. These mutants impaired Glu-induced stomatal closure (Fig. 8b), indicating that *CPK6* protein kinase is involved in Glu-induced stomatal closure in an ABA-independent manner.

### Discussion

Since the discovery of GLRs in the *Arabidopsis* genome (Lam et al. 1998), numerous studies have investigated Glu as a plant signaling molecule. Glu plays roles in, for example, the nitrogen to carbon balance (Kang and Turano 2003), regulation of ABA synthesis (Kang et al. 2004), control of root growth (Li et al. 2006; Miller et al. 2010), and leaf-to-leaf wounding response (Mousavi et al. 2013). We assessed the possibility that Glu functions in guard cell signaling. In this study, we found that Glu induced stomatal closure in both *Arabidopsis* and fava bean in a



**Fig. 7** Glu does not mediate ABA-signaling but requires SLAC1 in stomatal closure. **a** Stomatal response of the ABA-deficient mutant *aba2-1* exposed to Glu (1 mM). **b** ABA-insensitive *abi1-1* and *abi2-1* mutants exposed to Glu (1 mM). Stomatal response of *slac1-2* mutant exposed to Glu (1 mM). Each bar indicates the mean  $\pm$  S.D. of 80–120 measurements. Statistical differences were detected using a two-tailed *t* test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ )

dose-dependent manner. Pretreatment with a  $\text{Ca}^{2+}$  chelator and a  $\text{Ca}^{2+}$  channel blocker weakened Glu-induced stomatal closure. Furthermore, an antagonist and channel blocker of the NMDA receptor (AP-5,  $\text{Mg}^{2+}$ ) also inhibited Glu-induced stomatal closure. In contrast, glycine, which acts as an agonist of the NMDA receptor, promoted stomatal closure. Moreover, we have obtained direct evidence of Glu-dependent  $\text{Ca}^{2+}$  channel activity in guard cells by patch-clamp analysis. These results suggested that Glu indeed functions in guard cells and promotes stomatal closure through GLR-type  $\text{Ca}^{2+}$  channels in higher plants.

Previously, *Arabidopsis* GLR1.1 was shown to participate in stomatal movements (Kang et al. 2004). We showed that Glu normally induced stomatal closure in

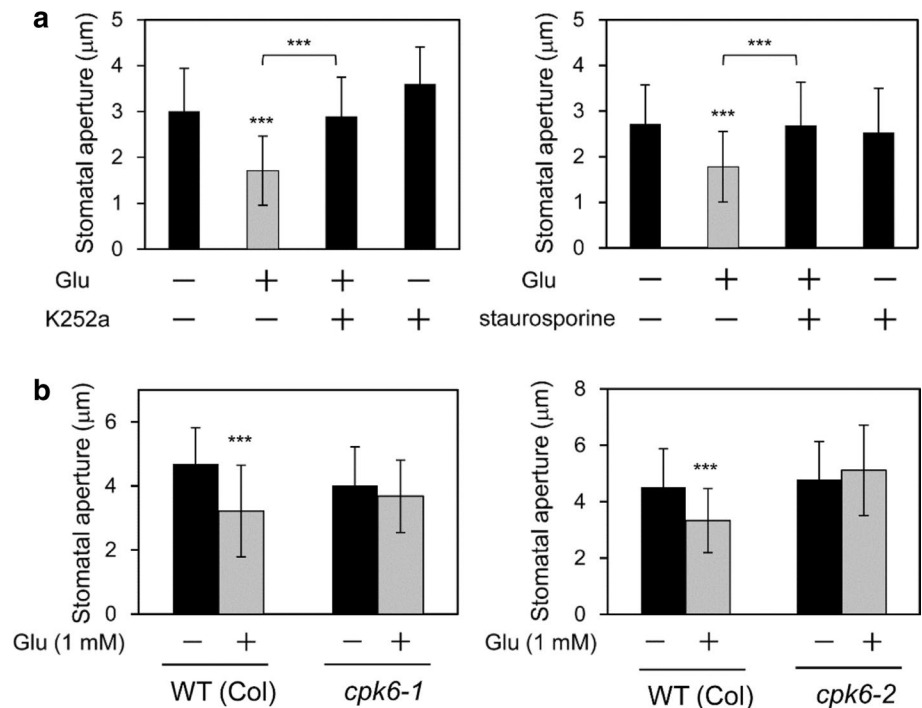
*GLR1.1* knockout mutant *glr1.1*, indicating that GLR1.1 is not involved in Glu-induced stomatal closure. In this study, we showed that another *Arabidopsis* GLR, *GLR3.5*, was expressed in guard cells and that its loss-of-function mutant impaired Glu-induced stomatal closure. These results indicated that *GLR3.5* participates in Glu-induced stomatal closure, and we confirmed *GLR3.7*'s involvement as well. Transcripts of *GLR3.7* were found in guard cells, as revealed by RT-PCR analysis (data not shown). However, we could not prepare alternative knockout lines of this gene. We also tried to prepare *glr3.5;glr3.7* double knockout mutant, however, these genes are tandemly located in chromosome II (*GLR3.5*: At2g32390; *GLR3.7*: At2g32400), and this made it difficult to produce double mutant. GLR channel may function as a heteromer and different combination of GLR subunit may specify its specific roles in different tissues or cells (Nicholas et al. 2008). It must be interesting if *GLR3.5* and *GLR3.7* form complex as a channel in guard cells. Future work will include experiments to identify other possible GLRs that function in Glu-dependent stomatal signaling.

Drought elicits the synthesis of ABA, which induces stomatal closure. Kang et al. (2004) reported that GLR1.1 regulates ABA synthesis, leading to the possibility that other GLRs also regulate ABA synthesis. We tested whether Glu affects stomata movements via ABA synthesis. In the ABA-deficient mutant *aba2-1*, Glu still induced stomatal closure. We also confirmed that Glu-induced stomatal closure was not abolished in the ABA-insensitive mutants *abi1-2* and *abi2-1*. Based on these results, we concluded that Glu elicits stomatal closure in an ABA-independent manner.

Calcium Dependent Protein Kinase 6 (CPK6) is a positive regulator in ABA-signaling, and it also regulates SLAC1 activity (Brandt et al. 2012; Mori et al. 2006). Once we had established that Glu-induced stomatal closure requires the activation of  $\text{Ca}^{2+}$  channels, protein phosphorylation, and SLAC1, we examined whether CPK6 controls Glu-induced stomatal response. We prepared two T-DNA knockout mutants of *CPK6* and observed an impaired response to Glu-induced stomatal closure. This protein kinase phosphorylates and activates SLAC1 to trigger stomatal closure in a  $\text{Ca}^{2+}$ -dependent manner (Brandt et al. 2012). Furthermore, CPK6 has been demonstrated to interact weakly with SLAC1 (Geiger et al. 2011). Given that SLAC1 was also involved in Glu-induced stomatal closure, Glu-dependent elevation of the cytosolic  $\text{Ca}^{2+}$  may activate CPK6 to promote SLAC1 activity. SLAC1 is also involved in cryptogam induced ion fluxes, ROS production, defense-related gene expression, and hypersensitive cell death in tobacco BY-2 cells (Kurusu et al. 2013), which may also support the presence of the functional Glu-signaling in guard cells.

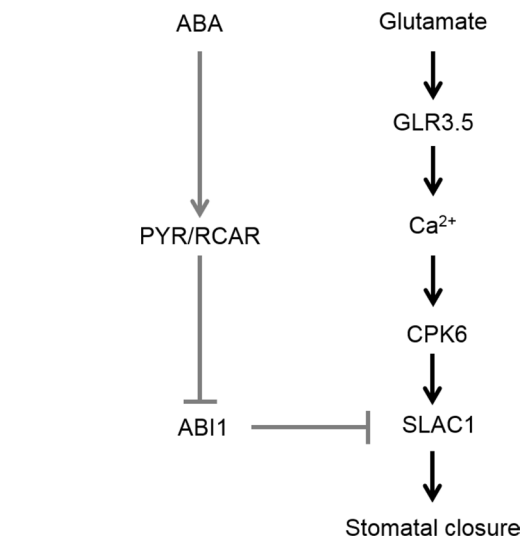


**Fig. 8** Protein phosphorylation or calcium-dependent protein kinase 6 (CPK6) is prerequisite for Glu-induced stomatal closure. **a** Effect of protein kinase inhibitor K252a and staurosporine on Glu-induced stomatal closure in *Arabidopsis*; 1 mM Glu and 1 μM K252a and 1 μM staurosporine were used. K252a and staurosporine were applied 30 min before Glu treatment. **b** Stomatal response of the wild type (Col), and *cpk6-1* and *cpk6-2* mutants exposed to Glu (1 mM). Each bar indicates the mean ± S.D. of 80–120 measurements. Statistical differences were detected using a two-tailed *t* test (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001)



We propose a possible signaling cascade in Glu-induced stomatal closure (Fig. 9). Glu may activate GLR3.5 and trigger an increase in cytosolic Ca<sup>2+</sup>, which would then activate CPK6 in an ABA-independent manner, and then active CPK6 would elevate SLAC1 activity to induce stomatal closure. Several reports have indicated that protein phosphorylation, especially MAP kinase, is involved in Glu signaling. Application of Glu modulates MAMP-triggered MAP kinase activity and also affects MAMP-induced accumulation of defense gene transcripts in *Arabidopsis* (Kwaaitaal et al. 2011). *Arabidopsis* MAP kinase kinase of MEKK1 plays a key role in Glu-induced root architecture (Forde et al. 2013). It must be interesting to examine whether or not MAP kinases are also involved in Glu-induced stomatal closure. Recently, Teardo et al. (2015) found that two isoforms of GLR3.5, isoform1 and isoform2, whose are derived from splicing variants are localized to mitochondria and chloroplast, respectively. Since mitochondria and chloroplast are known to play important roles in stomatal movement through modulating ROS, NO and Ca<sup>2+</sup> signaling (Cvetkovska et al. 2014; Nomura et al. 2008; Weinl et al. 2008), Glu may changes the some physiological status of these two organelles through activating GLR3.5 (see Fig. 9).

Although we do not yet understand the precise biological roles of Glu-induced stomatal closure, some clues may support or explain the physiological relevance of Glu-signaling in guard cells. Vatsa et al. (2011) demonstrated that the plant pathogen elicitor cryptogein induces the release of Glu from plant cells by exocytosis and also increases



**Fig. 9** Possible model of Glu signaling in stomatal closure. Glu activates *Arabidopsis* GLR3.5 and increases cytoplasmic Ca<sup>2+</sup>. Increased Ca<sup>2+</sup> may activate CPK6 and subsequently activate anion channel SLAC1 to induce stomatal closure. ABA signaling does not participate in Glu signaling

cytosolic Ca<sup>2+</sup>. They also indicated that the Glu concentration in the apoplast is increased by 2.6 mM after 5 min in cryptogein treatment. In addition to Glu, Gly was also found to present in the tomato leaf apoplast at a concentration around 0.2 mM (Solomon and Oliver 2001). We tried to ascertain whether 0.2 mM of Gly induces stomatal closure in *Arabidopsis*, however, any changes in stomatal

movement were observed (Fig. S4). In contrast, we found that low concentration of Glu (0.1 mM) did induce stomatal closure in both *Arabidopsis* and fava bean (Fig. 1). We did not observe any additive effects of Glu and Gly on stomatal closure at the concentration of 1 mM (Fig. 2). These results may reflect some physiological aspect of plant iGluRs in ligand efficacy. We do not have any information about whether the apoplastic concentration of Gly is increased or not by certain stimuli. Nevertheless, application of 1 mM Gly was shown to trigger the elevation of cytosolic  $\text{Ca}^{2+}$  in *Arabidopsis* seedlings (Dubos et al. 2003). These studies may support the functional roles of Glu in guard cell signaling.

Stomata are one of the pores that plant pathogens can invade inside the leaf tissues, and several studies have demonstrated that pathogen elicitors trigger stomatal closure (Koers et al. 2011; Melotto et al. 2006). In addition, bacteria are capable of secreting Glu or Gly during their growth (Park et al. 2003). The Glu secreted from plant cells or bacteria may act on the epidermis and play a key role in the regulation of stomatal closure to restrict pathogen invasion. We will further examine whether exogenous Glu confers stomatal defense in higher plants.

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