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, Ca²⁺, 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 Ca²⁺ flux in *Arabidopsis* roots (Dennison and Spalding 2000). Mutation of GLR3.3, one of 20 *Arabidopsis* genes, impaired both the membrane depolarization and the Ca²⁺ 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.
and T-DNA insertion lines of \( \text{glr1.1} \), \( \text{glr3.5} \), and \( \text{glr3.7} \) in the Col background were obtained (Mousavi et al. 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 \((\text{CO}_2)\), 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, \( \text{Ca}^{2+} \) is the important signaling molecule in guard cell signaling. An increase in the cytosolic \( [\text{Ca}^{2+}]_{\text{cyt}} \) 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 \( [\text{Ca}^{2+}]_{\text{cyt}} \) in guard cells via \( \text{Ca}^{2+} \) influx through plasma membrane \( \text{Ca}^{2+} \)-permeable channels and \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \)-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 \textit{Arabidopsis} and fava bean. This response required Glu receptors, activation of plasma membrane \( \text{Ca}^{2+} \)-permeable channels, and protein phosphorylation, as revealed by pharmacological, electrophysiological and genetic analyses. Loss-of-function analyses demonstrated that one of the \textit{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**

\textit{Arabidopsis} Columbia (Col) and Landsberg \textit{erecta} (Ler) ecotypes were used. \textit{Arabidopsis} mutant lines \textit{aba2-1}, \textit{abi1-1}, \textit{abi2-1}, \textit{slac1-2}, \textit{cpk6-1}, and \textit{cpk6-2} were studied, and T-DNA insertion lines of \textit{glr1.1}, \textit{glr1.2}, \textit{glr1.4}, \textit{glr3.3}, \textit{glr3.5}, and \textit{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 16-h light:8-h dark cycle. Seeds of fava bean (\textit{Vicia faba} L. cv. House Ryousai) 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\(_2\)] for \textit{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 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)). 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 \((I_{\text{Ca}})\) 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 \textit{Arabidopsis} (Col) as described by Ye et al. (2013).

The pipette solution contained 10 mM BaCl\(_2\), 0.1 mM dithiothreitol, 4 mM EGTA and 10 mM MES-Tris (pH 7.1), and the bath solution contained 100 mM BaCl\(_2\), 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\(^{-1}\), 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\(^{-1}\). The ramp voltage protocol was applied 10 times to obtain an
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® (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized from 1 μg of total RNA using ReveTra Ace® (Toyobo, Osaka, Japan) in a 10-μl reaction mixture. RT-PCR was performed for 30–35 cycles using GoTaq® (Promega, Fitchburg, WI, USA). The primer sequences used in RT-PCR analysis were GLR3.5 Fw (GAGGAGCGAGGAGGCT) and GLR3.5 Rv (AGTTTCGTGATCTTTCGACT), ACTIN2 Fw (GCCATCCAAGCTTTCTTCTC) and ACTIN2 Rv (GAACCACCGATCCAGACACT).

**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 (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 ± 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-δ-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 *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 apoplastic concentration of free Mg²⁺ was shown to be below 0.5 mM in fava bean (Mühling and Sattelmacher 1995), 5 mM Mg²⁺ 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 μM) alone did not induce stomatal closure. However, when 10 μM Glu was applied with 10 μM Gly, the stomata closed (Fig. 2c). These results suggested the participation of NMDA iGluRs in Glu-induced stomatal closure.

**Ca²⁺ is required for Glu-induced stomatal closure**

To further elucidate the possible relevance of GLRs in Glu-induced stomatal closure, we examined whether Ca²⁺ affects this signaling process. EGTA (extracellular Ca²⁺-chelator) and BAPTA-AM (intracellular Ca²⁺-chelator) inhibited the Glu-induced stomatal closure in *Arabidopsis* and fava bean, indicating that Ca²⁺ influx to the cytosol is required for this effect (Fig. 3a, b). We also examined
the effect of LaCl$_3$ (Ca$^{2+}$ channel blocker) on the Glu-induced stomatal closure. La$^{3+}$ inhibited the Glu-induced stomatal closure in Arabidopsis and fava bean (Fig. 3c). These results indicated that Ca$^{2+}$ influx occurred after Glu sensing.

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

It has been shown that $I_{Ca}$ of guard cells is activated by ABA, MeJA and microbe-associated molecular patters (Murata et al. 2015). Here we conducted patch-clamp analyses to examine activation of $I_{Ca}$ by Glu application (Fig. 4). In the absence of Glu small whole cell current ($-46 \pm 20 \text{ pA at } -198 \text{ mV}$) was observed. The current was significantly activated by perfusion with 10 mM Glu-containing bath solution to $-92 \pm 40 \text{ pA at } -198 \text{ mV}$ ($P = 0.046$, Wilcoxon signed ranks test, $n = 6$). These currents were substantially diminished by addition of 1 mM La$^{3+}$ ($-18 \pm 5 \text{ pA at } -198 \text{ mV}$). These results indicate that Glu activated ion current of which charge carrier was Ba$^{2+}$ and capable to be blocked with La$^{3+}$ similarly to ABA-activated $I_{Ca}$ (Pei et al. 2000). The current amplitude appeared fairly variable among cells, while the reason was unresolved in this study.
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 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.
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 CO₂ 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
dose-dependent manner. Pretreatment with a Ca\(^{2+}\) chelator and a Ca\(^{2+}\) channel blocker weakened Glu-induced stomatal closure. Furthermore, an antagonist and channel blocker of the NMDA receptor (AP-5, 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 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 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 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 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 Ca\(^{2+}\) may activate CPK6 to promote SLAC1 activity. SLAC1 is also involved in cryptogein 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. 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 ± 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$^{2+}$, 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$^{2+}$ 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 cytosolic Ca$^{2+}$. 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 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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