De-regulated expression of the plant glutamate receptor homolog AtGLR3.1 impairs long-term Ca\textsuperscript{2+}-programmed stomatal closure

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Summary

Cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{cyt}) mediates diverse cellular responses in both animal and plant cells in response to various stimuli. Calcium oscillation amplitude and frequency control gene expression. In stomatal guard cells, [Ca\textsuperscript{2+}]\textsubscript{cyt} has been shown to regulate stomatal movements, and a defined window of Ca\textsuperscript{2+} oscillation kinetic parameters encodes necessary information for long-term stomatal movements. However, it remains unknown how the encrypted information in the cytosolic Ca\textsuperscript{2+} signature is decoded to maintain stomatal closure. Here we report that the Arabidopsis glutamate receptor homolog AtGLR3.1 is preferentially expressed in guard cells compared to mesophyll cells. Furthermore, over-expression of AtGLR3.1 using a viral promoter resulted in impaired external Ca\textsuperscript{2+}-induced stomatal closure. Cytosolic Ca\textsuperscript{2+} activation of S-type anion channels, which play a central role in Ca\textsuperscript{2+}-reactive stomatal closure, was normal in the AtGLR3.1 over-expressing plants. Interestingly, AtGLR3.1 over-expression did not affect Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} oscillation kinetics, but resulted in a failure to maintain long-term ‘Ca\textsuperscript{2+}-programmed’ stomatal closure when Ca\textsuperscript{2+} oscillations containing information for maintaining stomatal closure were imposed. By contrast, prompt short-term Ca\textsuperscript{2+}-reactive closure was not affected in AtGLR3.1 over-expressing plants. In wild-type plants, the translational inhibitor cyclohexamide partially inhibited Ca\textsuperscript{2+}-programmed stomatal closure induced by experimentally imposed Ca\textsuperscript{2+} oscillations without affecting short-term Ca\textsuperscript{2+}-reactive closure, mimicking the guard cell behavior of the AtGLR3.1 over-expressing plants. Our results suggest that over-expression of AtGLR3.1 impairs Ca\textsuperscript{2+} oscillation-regulated stomatal movements, and that de novo protein synthesis contributes to the maintenance of long-term Ca\textsuperscript{2+}-programmed stomatal closure.

Keywords: anion channel, calcium oscillation, gene expression, glutamate receptor homolog, guard cells.

Introduction

Cytosolic Ca\textsuperscript{2+} functions as a second messenger in both animal and plant cells (Sanders et al., 2002; Berridge et al., 2003). In plant cells, an increase in the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{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 (Sanders et al., 2002; Berridge et al., 2003). For instance, the plant hormone abscisic acid (ABA) causes increases in the [Ca\textsuperscript{2+}]\textsubscript{cyt} concentration in guard cells via Ca\textsuperscript{2+} influx through plasma membrane Ca\textsuperscript{2+}-permeable channels and Ca\textsuperscript{2+} release from internal stores, resulting in stomatal closure (Staxen et al., 1999; MacRobbie, 2000; Pei et al., 2000; Kwak et al., 2003).

It was previously shown that stimuli for stomatal closure, including ABA, oxidative stress, cold and external calcium, induce [Ca\textsuperscript{2+}]\textsubscript{cyt} oscillations with various frequencies and amplitudes in Arabidopsis guard cells (Allen et al., 2000). This finding is supported by studies of the Arabidopsis V-ATPase mutant det3, in which external calcium and oxidative stress elicted prolonged [Ca\textsuperscript{2+}]\textsubscript{cyt} elevations rather
than \([Ca^{2+}]_{cyt}\) oscillations in wild-type guard cells. This elevation resulted in the failure of stomatal closure, indicating that stimulus-specific \([Ca^{2+}]_{cyt}\) oscillations are important for long-term stomatal closure (Allen et al., 2000). Furthermore, a study using sphingosine-1-phosphate showed that the \([Ca^{2+}]_{cyt}\) oscillation frequency and amplitudes were dependent on the sphingosine-1-phosphate concentration, resulting in different extents of stomatal closure, further supporting the importance of stimulus-specific \([Ca^{2+}]_{cyt}\) oscillations in stomatal movements (Ng et al., 2001). Combined use of the calcium clamp technique (De Koninck and Schulman, 1998; Dolmetsch et al., 1998) and the \(Ca^{2+}\) indicator protein yellow cameleon (Miyawaki et al., 1997, 1999) made it feasible to monitor and experimentally induce \([Ca^{2+}]_{cyt}\) oscillations in guard cells (Allen et al., 1999b, 2000), leading to the finding that \([Ca^{2+}]_{cyt}\) regulates stomatal closure by two mechanisms, short term calcium-reactive closure, which does not depend on the \(Ca^{2+}\) elevation pattern, and long-term calcium-programmed closure (Allen et al., 2001). Long-term maintenance of stomatal closure is programmed by \([Ca^{2+}]_{cyt}\) oscillations within a defined range of parameters, including frequency, amplitude and the number and duration of transients, whereas short-term calcium-reactive closure occurs when \([Ca^{2+}]_{cyt}\) is elevated, irrespective of the kinetics of the \([Ca^{2+}]_{cyt}\) transients (Allen et al., 2000; Li et al., 2004). Further genetic evidence supporting this finding was obtained from the \(gca2\) mutant, in which external calcium and \(ABA\) induced \([Ca^{2+}]_{cyt}\) oscillations with a faster period than wild-type, resulting in impairment of long-term calcium-programmed closure (Allen et al., 2001). Despite these recent exciting findings, it remains largely unknown how \(Ca^{2+}\) signatures are decoded in order to elicit various cellular responses in plant or animal cells, although a few molecules have been suggested to function in decoding of the \([Ca^{2+}]_{cyt}\) oscillations (De Koninck and Schulman, 1998; Oancea and Meyer, 1998; Allen and Schroeder, 2001; Sanders et al., 2002; Tomida et al., 2003; Walker et al., 2004).

In plants, it has been shown that \(ABA\)-induced \([Ca^{2+}]_{cyt}\) elevations are important for \(ABA\) induction of \(RAB18\) gene expression in Arabidopsis suspension cells (Ghelis et al., 2000). Studies involving the use of calcium dyes and transgenic Arabidopsis expressing the GUS reporter under the control of the \(ABA\)-responsive \(CdeT6-19\) gene promoter have shown that \([Ca^{2+}]_{cyt}\) is involved in both \(ABA\)-induced stomatal movements and \(ABA\)-induced gene expression (Webb et al., 2001). Furthermore, \(ABA\)-induced stomatal movements and \(ABA\)-induced gene expression differed with respect to their sensitivity to alterations in \(Ca^{2+}\), suggesting that these pathways are not completely convergent (Webb et al., 2001). However, it remains unknown how \(Ca^{2+}\) oscillations specifically control gene expression by differing amplitude and frequency, and how \(Ca^{2+}\) oscillations are decoded to regulate long-term stomatal movements.

In animals, ionotropic glutamate receptors act as ligand-gated ion channels and function as neurotransmitter receptors that mediate synaptic excitation in the central nervous system, and thus play a crucial role in long-term memory (Hollmann and Heinemann, 1994; Mayer and Armstrong, 2004). Mammalian glutamate receptors assemble to form functional hetero-tetrameric channel proteins that are permeable to \(Ca^{2+}\) and monovalent cations when activated by agonists (Hollmann and Heinemann, 1994; Mayer and Armstrong, 2004). Completion of sequencing of the Arabidopsis genome revealed that 20 glutamate receptor homologs are present in Arabidopsis, and this gene family is divided into three phylogenetically distinct subfamilies, \(AtGLR1\), \(AtGLR2\) and \(AtGLR3\) (Lacombe et al., 2001; Chiu et al., 2002; Davenport, 2002). A pharmacological study showed that Arabidopsis seedlings grown on medium containing an antagonist of ionotropic glutamate receptors mimicked the phenotype of Arabidopsis long-hypocotyl mutants, suggesting that plant glutamate receptor homologs may be involved in transduction of light signals (Lam et al., 1998). A study utilizing antisense suppression of \(AtGLR1.1\) suggested that \(AtGLR1.1\) plays a role in carbon/nitrogen metabolism, \(ABA\) biosynthesis and the control of seed germination (Kang and Turano, 2003). Another possible role for \(AtGLR\) genes was identified independently in a transgenic plant study and a physiological study. Over-expression of \(AtGLR3.2\) in Arabidopsis resulted in \(Ca^{2+}\) deficiency symptoms, and also caused sensitivity to other ions that could be rescued by applying external \(Ca^{2+}\), suggesting that \(AtGLR3.2\) may function in calcium translocation in plants (Kim et al., 2001). In Arabidopsis roots, glutamate was shown to induce \([Ca^{2+}]_{cyt}\) transients and plasma membrane depolarization that are inhibited by the \(Ca^{2+}\) blocker \(La^{3+}\) (Dennison and Spalding, 2000). Furthermore, the glutamate-triggered membrane depolarization was reduced under certain conditions in \(atglr3.3\) mutants, suggesting a role for \(AtGLR3.3\) in the regulation of membrane potential (Qi et al., 2006). A physiological study using \(atglr3.3\) and \(atglr3.4\) null mutants suggests that the \(AtGLR\) subtypes, depending on whether they consist of \(AtGLR3.3\) or \(AtGLR3.4\), are differently activated and desensitized by different amino acids (Stephens et al., 2008). A rice mutant carrying a T-DNA insertion in \(GLR3.1\) shows a short-root phenotype due to enhanced programmed cell death in the root apical meristem, suggesting that rice \(GLR3.1\) plays a role in cell division and survival (Li et al., 2006). Although visualization of protein localization of GFP-fused \(AtGLR\)s has not been successful to date (Davenport, 2002), a study with polyclonal antibodies raised to the C-terminal region of \(AtGLR3.2\) has suggested that \(AtGLR3.2\) is an integral membrane protein (Turano et al., 2002).

In this paper, we report that over-expression of the \(AtGLR3.1\) glutamate receptor homolog led to impairment in external \(Ca^{2+}\)-induced stomatal closure and imposed
Ca\textsuperscript{2+} oscillation-induced long-term stomatal closure without affecting the anion channel activity or the Ca\textsuperscript{2+} oscillation kinetics, which have been suggested to play an important role in long-term steady-state stomatal closure. Furthermore, wild-type guard cells subjected to a short-term treatment with the translational inhibitor cyclohexamide phenocopied the \textit{AtGLR3.1} over-expressing plants, suggesting the involvement of gene expression in long-term steady-state stomatal closure.

### Results

**\textit{AtGLR3.1} over-expression impairs Ca\textsuperscript{2+}-induced stomatal closure**

A \textit{Brassica rapa} cDNA clone showing a significant similarity to animal glutamate receptors was previously isolated from a cDNA sequencing study of a \textit{Brassica} guard cell cDNA library (Kwak \textit{et al.}, 1997). In order to isolate Arabidopsis orthologs, a guard cell-enriched cDNA library (Kwak \textit{et al.}, 2002; Mori \textit{et al.}, 2006; Lee \textit{et al.}, 2007) was used as a template for PCR, and \textit{AtGLR3.1} cDNA was isolated. To test whether \textit{AtGLR3.1} is also expressed in guard cells, RT-PCR was performed using guard-cell cDNA that was independently prepared from guard-cell protoplasts (Leonhardt \textit{et al.}, 2004). Mesophyll-cell cDNA was also used for comparison. As shown in Figure 1a, RT-PCR revealed that \textit{AtGR3.1} is preferentially expressed in guard cells relative to mesophyll cells. This result is consistent with data obtained from Affymetrix Arabidopsis whole-genome chips hybridized with guard-cell or mesophyll-cell RNA (Yang \textit{et al.}, 2008; Figure S1). A previous study identified a guard-cell marker gene encoding hydroxyproline-rich protein (HPRP, \textit{At2g21140}) and a mesophyll-cell marker gene encoding calmodulin-binding protein (CBP, \textit{At4g33050}) (Leonhardt \textit{et al.}, 2004). In order to determine whether the guard-cell and mesophyll-cell preparations were free of contaminating cells, these cell type-specific marker genes were used as controls. The RT-PCR results showed that CBP mRNA was not detected in guard cells whereas HPRP mRNA was not detected in mesophyll cells, indicating that the guard-cell and mesophyll-cell preparations did not contain contaminating cells (Figure 1a). In order to obtain further information on \textit{AtGLR3.1} expression, we amplified a 1961 upstream promoter region of \textit{AtGLR3.1} and cloned into pBI101 containing the GUS gene. As shown in Figure 1b, GUS reporter activity was detected in guard cells. In addition, GUS activity was also detected in vascular tissues in leaves, roots and stems (Figure S2), consistent with a previous finding by Chiu \textit{et al.} (2002).

We generated transgenic plants in which \textit{AtGLR3.1} was either over-expressed or antisensed by expression of \textit{AtGLR3.1} cDNA under the control of the CaMV 35S promoter.

![Figure 1](image-url)

**Figure 1.** External Ca\textsuperscript{2+}-induced stomatal closure is impaired in \textit{AtGLR3.1} over-expressing plants.  
(a) RT-PCR confirms that \textit{AtGLR3.1} is preferentially expressed in guard cells. \textit{Actin2} cDNA was amplified as a control. A guard-cell (GC) marker gene, hydroxyproline-rich protein (HPRP, \textit{At2g21140}), and a mesophyll-cell (MC) marker gene, calmodulin-binding protein (CBP, \textit{At4g33050}) were amplified to indicate the purity of RNA from each cell type.  
(b) The \textit{AtGLR3.1} promoter drives GUS expression in guard cells. Scale bar = 20 μm.  
(c) Northern blot analysis shows over-expression of \textit{AtGLR3.1} in the two independent transgenic plants. \textit{AtGLR3.1} was not detected in wild-type (WT) due to the low expression level of the endogenous transcript under these experimental conditions. Ethidium bromide-stained RNA is shown for loading controls.  
(d) Over-expression of \textit{AtGLR3.1} disrupts external Ca\textsuperscript{2+}-induced stomatal closure. Error bars represent the standard error for 9 or 10 experiments (total of 180–200 stomata) for WT, 6 or 7 experiments for \textit{AtGLR3.1OE#4-2} (total of 120–140 stomata), and three experiments for \textit{AtGLR3.1OE#8-3} (total of 60 stomata). If not shown, the error bars are smaller than the symbols.
promoter. Two transgenic over-expression lines 4–2 and 8–3 with a single T-DNA insertion were selected because these lines showed a higher transcript level of transgenic AtGLR3.1 (Figure 1c) and a Mendelian segregation ratio of 3:1 for kanamycin resistance in the T2 generation \{(ratio kanamycinR:kanamycinS = 141:35 for line 4–2 (χ² = 2.45, P > 0.05) and 155:49 for line 8–3 (χ² = 0.10, P > 0.05))\}. We first examined whether these transgenic plants have any altered stomatal responses, as the preferential expression in guard cells suggested a role for AtGLR3.1 in guard-cell signal transduction and/or development. Unexpectedly, external Ca²⁺, which induces stomatal closure by increasing cytosolic Ca²⁺ concentrations in guard cells (McAinsh et al., 1995; Allen et al., 2000, 2001; Han et al., 2003), did not induce stomatal closure in the two independent AtGLR3.1 over-expressing transgenic plants at all concentrations of external calcium tested (Figure 1d; P < 0.001 at 0.01, 0.1, 1 and 10 mM Ca²⁺). As AtGLR3.2 is expressed in guard cells (Figure S1) and shares the highest homology with AtGLR3.1, we also tested whether AtGLR3.2 over-expression has any effect on external calcium-induced stomatal closure. Two independent AtGLR3.2 over-expression lines (Kim et al., 2001) did not show any altered stomatal response to external calcium (data not shown). Transgenic plants in which antisense AtGLR3.1 transcripts were expressed did not show any impairment in Ca²⁺-induced stomatal closure (data not shown). This might be due to the possible functional redundancy in the AtGLR gene family, as there are 20 AtGLR genes in the Arabidopsis genome (Lacombe et al., 2001). These results suggest the involvement of AtGLR3.1 in regulation of stomatal movements by external Ca²⁺.

Ca²⁺-induced Ca²⁺ oscillation kinetics parameters are normal in AtGLR3.1 transgenic guard cells

Parameters of [Ca²⁺]_{cyt} oscillation kinetics contain necessary information for stomatal movements (Allen et al., 2001). For example, external Ca²⁺-induced stomatal closure is abolished in gca2 and det3 mutants because of altered Ca²⁺ oscillation kinetics (Allen et al., 2000, 2001). In order to examine whether impaired Ca²⁺-induced stomatal closure in AtGLR3.1 over-expressing transgenic guard cells is due to altered external Ca²⁺-induced [Ca²⁺]_{cyt} oscillations, ratiometric calcium imaging experiments were performed using wild-type and AtGLR3.1 over-expressing transgenic plants expressing yellow cameleon 2.1 (YC2.1) (Miyawaki et al., 1997; Allen et al., 1999b, 2001; Jung et al., 2002; Kwak et al., 2002, 2003; Mori et al., 2006). Interestingly, AtGLR3.1 transgenic guard cells showed wild-type Ca²⁺-induced [Ca²⁺]_{cyt} oscillation kinetics at both 1 and 10 mM external Ca²⁺ (Figure 2). Wild-type guard cells showed a period of 4.58 ± 2.02 mins and 8.35 ± 1.81 transients per recording at 1 mM external Ca²⁺ (n = 17 cells), and a period of 3.59 ± 1.84 mins and 12.15 ± 2.85 transients per recording at 10 mM external Ca²⁺ (n = 26 cells). AtGLR3.1 transgenic guard cells showed a mean duration of transients of 5.14 ± 2.13 mins and 6.94 ± 2.53 transients per recording at 1 mM external Ca²⁺ (n = 19 cells) and a mean duration of transients of 3.27 ± 1.68 mins and 14.25 ± 3.25 transients per recording at 10 mM external Ca²⁺ (n = 40 cells). These results indicate that the mechanisms responsible for external Ca²⁺-induced [Ca²⁺]_{cyt} oscillations are not affected by over-expression of AtGLR3.1 in transgenic guard cells. To our knowledge, no knockout or transgenic plants have been reported so far that show altered stomatal movements when the parameters of endogenous Ca²⁺ oscillation kinetics are normal (Allen et al., 1999b, 2000, 2001, 2002; Hugouvieux et al., 2001; Kwak et al., 2002, 2003; Vahisalu et al., 2008).
AtGLR3.1 over-expression inhibits the long-term Ca\textsuperscript{2+}-programmed stomatal closure induced by imposed Ca\textsuperscript{2+} oscillations

To further investigate the underlying mechanisms of impaired external Ca\textsuperscript{2+}-induced stomatal closure in AtGLR3.1 transgenic guard cells, the calcium clamp technique (De Konink and Schulman, 1998; Dolmetsch et al., 1998; Allen et al., 2000, 2001) was performed using AtGLR3.1 transgenic plants expressing YC2.1. Experimentally imposed cytosolic Ca\textsuperscript{2+} oscillations were induced by incubating cells in a solution containing 10 mM CaCl\textsubscript{2} and 0.1 mM KCl, and caused repetitive hyperpolarization-mediated calcium influx in AtGLR3.1 transgenic guard cells and wild-type guard cells (Figure 3a,c and Movie S1). The trace of the [Ca\textsuperscript{2+}]\textsubscript{cyt} transients in AtGLR3.1 transgenic guard cells showed no significant difference compared to that in the wild-type guard cells (Figure 3a). These experimentally imposed [Ca\textsuperscript{2+}]\textsubscript{cyt} oscillations contained the necessary information for the two components of stomatal closure, short-term calcium-reactive closure and long-term calcium-programmed closure (Allen et al., 2001; Mori et al., 2006). Calcium-reactive closure occurs rapidly when [Ca\textsuperscript{2+}]\textsubscript{cyt} is elevated, but steady-state stomatal aperture is maintained by calcium-programmed closure even without further elevations in [Ca\textsuperscript{2+}]\textsubscript{cyt} (Figure 3b) (Allen et al., 2001). In the wild-type, short-term calcium-reactive stomatal closure was immediately elicited upon Ca\textsuperscript{2+} transients (WT, Figure 3b). After the third Ca\textsuperscript{2+} transient was induced, epidermal strips were kept for a period of 2 h 30 mins in a solution containing 100 mM KCl and 0 mM CaCl\textsubscript{2} in order to open stomata. However, long-term calcium-programmed stomatal closure was maintained in wild-type plants (Figure 3b; 65.5 ± 2.7% for WT at 180 mins). In contrast, the experimentally imposed [Ca\textsuperscript{2+}]\textsubscript{cyt} oscillations failed to 'program' long-term steady-state stomatal closure in the two independent AtGLR3.1 transgenic lines, but short-term calcium-reactive closure did occur (Figure 3b; 82.6 ± 5.1% for line 4–2, 96.5 ± 2.1% for line 8–3 at 180 mins). This is a possible explanation as to why external Ca\textsuperscript{2+} failed to induce stomatal closure measured 3 h after addition of calcium in AtGLR3.1 transgenic plants (Figure 1d). A previous study demonstrated that a defined range of parameters of [Ca\textsuperscript{2+}]\textsubscript{cyt} oscillation kinetics encode specific information for stomatal movements (Allen et al., 2001). As the AtGLR3.1 transgenic guard cells failed to maintain long-term Ca\textsuperscript{2+}-programmed closure when low-frequency Ca\textsuperscript{2+} oscillations (Figure 3a) were applied, we wished to test whether AtGLR3.1 transgenic guard cells differentially respond to [Ca\textsuperscript{2+}]\textsubscript{cyt} oscillations with different parameters. In contrast to the low-frequency [Ca\textsuperscript{2+}]\textsubscript{cyt} oscillations (10 mins period, 5 mins Ca\textsuperscript{2+} transient duration) used in the experiments in Figure 3a, high-frequency [Ca\textsuperscript{2+}]\textsubscript{cyt} oscillations (4 mins period, 2 mins Ca\textsuperscript{2+} transient duration) elicited wild-type stomatal movements in AtGLR3.1 transgenic plants (Figure 3d), suggesting that de-regulation of AtGLR3.1 expression specifically caused a defect in the long-term calcium-programmed closure induced by low-frequency [Ca\textsuperscript{2+}]\textsubscript{cyt} oscillations.

Anion channels are not affected in AtGLR3.1 transgenic guard cells

S-type anion channels play an important role in stomatal closure in response to ABA and increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} (Schroeder and Hagiwara, 1989; Grabov et al., 1997; Allen et al.,
imposed Ca$^{2+}$ oscillations (Figure 3b), suggesting that consistent with short-term stomatal closure induced by vated by calcium, as in the wild-type (Mori et al., 2006). Anion channel currents were measured in the presence of 2 μM Ca$^{2+}$ in the pipette solution from guard-cell protoplasts that were pre-incubated in 40 mM external Ca$^{2+}$. As shown in Figure 4, anion channels in AtGLR3.1 transgenic guard cells (n = 14 cells) were acti-vated by calcium, as in the wild-type (n = 9 cells), which is consistent with short-term stomatal closure induced by imposed Ca$^{2+}$ oscillations (Figure 3b), suggesting that de-regulation of AtGLR3.1 does not alter anion-channel responses to cytosolic Ca$^{2+}$ and Ca$^{2+}$-reactive stomatal closure. This result also supports the previous finding by Mori et al. (2006) that short-term Ca$^{2+}$-reactive and long-term Ca$^{2+}$-programmed closure can be mechanistically separated. Together with the results from Ca$^{2+}$-induced stomatal closure assays (Figure 1d), Ca$^{2+}$ imaging experiments (Figure 2), and time-course measurements of stomatal closure induced by imposed Ca$^{2+}$ oscillations (Figure 3), these data suggest that AtGLR3.1 transgenic guard cells are likely to be defective in maintaining the long-term Ca$^{2+}$ stomatal closure programed by Ca$^{2+}$-induced [Ca$^{2+}$]$_{cyt}$ oscillations with parameters that lead to stomatal closure in wild-type.

**Gene expression contributes to long-term stomatal movements**

Cytosolic calcium levels are increased in response to the plant hormone ABA in guard cells via Ca$^{2+}$ influx and Ca$^{2+}$ release, and mediates ABA signaling (Staxen et al., 1999; MacRobbie, 2000; Pei et al., 2000; Schroeder et al., 2001a; Kwak et al., 2003). In addition, the mRNA cap binding protein ABH1 and an Sm-like protein have come to represent a novel mechanism for ABA signal transduction regulation (Hugouvieux et al., 2001; Xiong et al., 2001; Li et al., 2002). Transcription factors including MYB61, MYB44 and AtERF7 function in ABA signaling (Liang et al., 2005; Song et al., 2005; Jung et al., 2008). Thus further exploratory studies were performed to test whether RNA transcription and protein translation processes contribute to ABA- or external Ca$^{2+}$-induced stomatal closure. Interestingly, both the transcriptional inhibitor actinomycin D and the translational inhibitor cyclohexamide partially inhibited ABA-induced stomatal closure (Figure S3). Moreover, as shown in Figure 5a, external Ca$^{2+}$-induced stomatal closure was partially inhibited in the presence of cyclohexamide (P < 0.01 at 1 and 10 mM Ca$^{2+}$). Further experiments were performed to test

**Figure 5.** The translational inhibitor cyclohexamide partially blocks Ca$^{2+}$-induced stomatal closure and long-term Ca$^{2+}$-programmed stomatal closure induced by imposed Ca$^{2+}$ oscillations.

(a) Cyclohexamide (CH, 5 μM) partially inhibits external Ca$^{2+}$-induced stomatal closure at both Ca$^{2+}$ concentrations. Error bars represent the standard error of four experiments (8 stomates at each data point).

(b) Cyclohexamide (+CH, 5 μM) partially impairs long-term Ca$^{2+}$-programmed closure but not short-term Ca$^{2+}$-reactive closure. Error bars represent the standard error of three experiments (15 stomates at each data point). If not shown, the error bars are smaller than the symbols.
the effect of cyclohexamide on imposed calcium oscillations. Long-term Ca\(^{2+}\)-programmed closure was partially inhibited by cyclohexamide, whereas short-term Ca\(^{2+}\)-reactive closure was not affected (Figure 5b; \(P < 0.03\) at 150 mins), which is similar to the phenotype of AtGLR3.1 over-expressing plants (Figure 3b). The results in Figure 5 suggest that de novo protein synthesis is required to maintain long-term steady-state stomatal closure.

**Discussion**

Since the presence of the glutamate receptor homologs was reported in plants (Lam et al., 1998), several studies have suggested that these animal neurotransmitter receptor homologs play a role in light signaling, calcium fluxes, cell division and survival in roots, carbon and nitrogen metabolism, ABA biosynthesis, responses to aluminum or environmental stress, calcium homeostasis and resource allocation (Lam et al., 1998; Dennison and Spalding, 2000; Kim et al., 2001; Kang and Turano, 2003; Sivaguru et al., 2003; Kang et al., 2004; Dubos et al., 2005; Meyerhoff et al., 2005; Li et al., 2006; Qi et al., 2006; Stephens et al., 2008). In this paper, we provide evidence that AtGLR3.1 plays a role in stomatal movements. Transgenic Arabidopsis plants over-expressing AtGLR3.1 are impaired in external Ca\(^{2+}\)-induced stomatal closure due to re-opening of stomata during long-term steady-state stomatal closure (Figures 1d and 3b). In wild-type plants, this long-term steady-state stomatal closure is induced and maintained by experimentally imposed Ca\(^{2+}\) transients that encode information for stomatal closure (Figure 3a,b). Cytosolic Ca\(^{2+}\) transients with the same kinetics parameters were also experimentally induced in AtGLR3.1 over-expressing guard cells but did not maintain long-term stomatal closure (Figure 3a,b). Furthermore, the parameters of Ca\(^{2+}\) oscillations, including the duration of the transients and the number of transients per recording in response to external Ca\(^{2+}\), were not significantly different in AtGLR3.1 over-expressing guard cells from those in wild-type guard cells (Figure 2). This stomatal behavior of AtGLR3.1 over-expressing plants is different from that of det3 and gca2 mutants, in which external calcium elicited prolonged calcium increases (det3 guard cells) or faster Ca\(^{2+}\) oscillations (gca2 guard cells), resulting in impaired Ca\(^{2+}\)-induced stomatal closure (Allen et al., 2000, 2001). Moreover, the experimentally imposed \([Ca^{2+}]_{cyt}\) oscillations with the same kinetics rescued long-term Ca\(^{2+}\)-programmed closure in gca2 and det3 mutants in which \([Ca^{2+}]_{cyt}\) oscillation kinetics is altered (Allen et al., 2000, 2001). Together, these results suggest that the mechanisms affected in det3 and gca2 mutants are different from those affected in AtGLR3.1 over-expressing guard cells.

AtGLR3.1 is one of seven AtGLR3 sub-family members, and shares 78, 55 and 53% identity with AtGLR3.2, AtGLR3.3 and AtGLR3.4, respectively, at the peptide level. In addition to its preferential expression in guard cells relative to mesophyll cells (Figure 1a,band Figure S1), analysis of the AtGLR3.1 promoter–GUS reporter activity shows that AtGLR3.1 is also expressed in vascular tissues and other organs such as roots (Figure S2) (Chiu et al., 2002). This suggests that AtGLR3.1 may function in other pathways or cellular responses. The rice ortholog of AtGLR3.1, OsGLR3.1, is expressed in roots and more highly in shoots (Li et al., 2006), which is different to the AtGLR3.1 expression (Chiu et al., 2002). Indeed, a rice T-DNA insertion mutant for GLR3.1 displays a short-root phenotype, distorted root meristematic activity, and increased cell death in root meristems (Li et al., 2006), although how a OsGLR3.1 loss-of-function mutation can cause such phenotypes was not addressed at the molecular level. Because of the functions of their animal homologs, it has been suggested that AtGLR proteins provide a calcium-permeable pathway across the plasma membrane and participate in cytosolic Ca\(^{2+}\)-mediated signaling (Sanders et al., 2002). In fact, previous studies have suggested that AtGLR3.2, AtGLR3.3 and AtGLR3.4 are all implicated in Ca\(^{2+}\) signaling (see Introduction) (Kim et al., 2001; Meyerhoff et al., 2005; Qi et al., 2006; Stephens et al., 2008). The results of the present paper also suggest a role for AtGLR3.1 in Ca\(^{2+}\)-mediated signaling in guard cells.

The CaMV 35S promoter not only drives high gene expression in guard cells (Allen et al., 1999b; Hugouvieux et al., 2001; Kwak et al., 2003; Young et al., 2006) but also in other cell types. The epidermal strips used in calcium imaging and time-course measurements of stomatal apertures contained guard cells and epidermal cells, as other leaf cells including mesophyll cells were removed. Furthermore, guard cells respond cell-autonomously to signals (Schröeder et al., 2001b). For example, when cytosolic calcium levels were measured in the two guard cells of single stomates, Ca\(^{2+}\) oscillations induced by various stimuli were not necessarily synchronized in the two guard cells (Allen et al., 1999b; Klüsener et al., 2002; Kwak et al., 2003). Therefore, it is unlikely that the stomatal movement phenotype of the AtGLR3.1 over-expressing plants is primarily caused by AtGLR3.1 expression in other cell types, although we cannot exclude the possibility that AtGLR3.1 expressed in the neighboring cells may contribute to the phenotype of the AtGLR3.1 over-expressing plants.

Recently, four Ca\(^{2+}\)-dependent protein kinases, CPK3, CPK4, CPK6 and CPK11, have been reported to function in ABA signaling in guard cells. Both the cpk3 cpk6 and cpk4 cpk11 double mutants showed an ABA-insensitive stomatal response, indicating that these CPK genes positively regulate guard-cell ABA signaling (Mori et al., 2006; Zhu et al., 2007). The guard cells of cpk3 cpk6 mutants are also insensitive to external Ca\(^{2+}\) (Mori et al., 2006). Experimentally imposed Ca\(^{2+}\) oscillations were induced in cpk3 cpk6 mutant guard cells and in wild-type by the repetitive hyperpolarizing Ca\(^{2+}\) influx; however, the Ca\(^{2+}\)-reactive short-term stomatal
closure was significantly reduced in the \textit{cpk3 cpk6} mutant, whereas the longer-term Ca\textsuperscript{2+}-programmed stomatal closure was not impaired (Mori \textit{et al.}, 2006). ABA activation of S-type anion channels was abolished in \textit{cpk3 cpk6} mutants (Mori \textit{et al.}, 2006). Moreover, S-type anion channels in the \textit{cpk3 cpk6} mutant were less activated by 2 \textmu M cytosolic Ca\textsuperscript{2+} (Mori \textit{et al.}, 2006). Anion channels have been suggested to provide a major regulatory mechanism for stomatal closure (Schroeder and Hagiwara, 1989; Schroeder \textit{et al.}, 1993). Therefore, impaired stomatal closure in response to ABA and the reduced short-term Ca\textsuperscript{2+}-reactive stomatal closure in \textit{cpk3 cpk6} mutants are, in part at least, due to impairment in the ABA and cytosolic Ca\textsuperscript{2+} activation of S-type anion channels. In contrast, use of 2 \textmu M cytosolic Ca\textsuperscript{2+} in the pipette activated S-type anion channels in \textit{AtGLR3.1} over-expressing guard cells and in wild-type guard cells (Figure 4). In \textit{AtGLR3.1} over-expressing plants, short-term Ca\textsuperscript{2+}-reactive stomatal closure was not altered, but long-term Ca\textsuperscript{2+}-programmed stomatal closure was not maintained (Figure 3). Thus, it might be possible that S-type anion channels provide a major control mechanism for short-term Ca\textsuperscript{2+}-reactive stomatal closure.

Very recently, \textit{SLAC1}, which encodes a plasma membrane protein that has distant similarity to a bacterial malate transporter, was shown to be required for S-type anion channels (Negi \textit{et al.}, 2008; Vahisalu \textit{et al.}, 2008). Stomatal closure in \textit{slac1} mutants was much less sensitive to ABA, CO\textsubscript{2}, humidity and H\textsubscript{2}O\textsubscript{2} (Negi \textit{et al.}, 2008; Vahisalu \textit{et al.}, 2008). Interestingly, \textit{slac1} also showed reduced short-term Ca\textsuperscript{2+}-reactive stomatal closure in response to experimentally imposed Ca\textsuperscript{2+} oscillations, whereas long-term Ca\textsuperscript{2+}-programmed stomatal closure was not impaired (Vahisalu \textit{et al.}, 2008). This result also indicates that S-type anion channels play a central role in short-term Ca\textsuperscript{2+}-reactive stomatal closure (Mori \textit{et al.}, 2006; Vahisalu \textit{et al.}, 2008). However, we do not exclude the possibility that S-type anion channels could contribute to long-term Ca\textsuperscript{2+}-programmed stomatal closure. Further studies are required to address this important issue. Furthermore, it would be interesting to determine whether the endogenous Ca\textsuperscript{2+} oscillation kinetics of \textit{cpk3 cpk6} and \textit{slac1} mutant guard cells is altered in response to ABA and Ca\textsuperscript{2+}.

Ca\textsuperscript{2+} signatures, consisting of differences in spatial and temporal Ca\textsuperscript{2+} signal characteristics (Ca\textsuperscript{2+} oscillation frequency, amplitude and localization), have been suggested to contain the specificity for cellular responses (Sanders \textit{et al.}, 2002; Berridge \textit{et al.}, 2003; Hetherington and Brownlee, 2004). In animal cells, cytosolic and nuclear Ca\textsuperscript{2+} function distinctly to control gene expression (Hardingham \textit{et al.}, 1997). Ca\textsuperscript{2+} oscillations function in the control of gene expression by activating transcription factors in the cytoplasm or in the nucleus (Dolmetsch \textit{et al.}, 1998; Li \textit{et al.}, 1998; Hardingham and Bading, 1999; West \textit{et al.}, 2001; Hogan \textit{et al.}, 2003; Tomida \textit{et al.}, 2003). In plants, stomatal conductance has been suggested to control the phase and period of diurnal Ca\textsuperscript{2+} oscillations that are regulated by the Ca\textsuperscript{2+}-sensing receptor CAS and inositol-1,4,5-trisphosphate (Tang \textit{et al.}, 2007). A recent study showed that circadian [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillations are modulated by circadian oscillations of cytosolic cyclic adenosine diphosphate ribose, which regulates transcriptional feedback loops of circadian oscillators (Dodd \textit{et al.}, 2007; Xu \textit{et al.}, 2007). Furthermore, a defined range of parameters of [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillation kinetics encodes information for stomatal movements (see Introduction) (Allen \textit{et al.}, 2001; Yang \textit{et al.}, 2003; Li \textit{et al.}, 2004; Mori \textit{et al.}, 2006). Previous studies demonstrated that all Ca\textsuperscript{2+} elevations above a threshold, and independent of the oscillation pattern, cause closure of stomatal pores (‘Ca\textsuperscript{2+}-reactive’ stomatal closure) (Allen \textit{et al.}, 2001; Yang \textit{et al.}, 2003; Li \textit{et al.}, 2004; Mori \textit{et al.}, 2006). Interestingly, however, imposed Ca\textsuperscript{2+} oscillation patterns affect the ability of stomatal pores to re-open once Ca\textsuperscript{2+} has closed them (‘Ca\textsuperscript{2+}-programmed’ stomatal closure) (Allen \textit{et al.}, 2001; Yang \textit{et al.}, 2003; Li \textit{et al.}, 2004). For this Ca\textsuperscript{2+}-programmed response, re-opening of stomata depends on the preceding Ca\textsuperscript{2+} pattern (Allen \textit{et al.}, 2001; Yang \textit{et al.}, 2003; Li \textit{et al.}, 2004). One interesting question regarding Ca\textsuperscript{2+}-induced stomatal closure is how calcium ‘programs’ or ‘maintains’ long-term steady-state closure without further increases in the cytosolic Ca\textsuperscript{2+} concentration; in other words, how the information encoded in [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillations is translated into long-term steady-state stomatal closure. Our data show that the translational inhibitor cycloheximide partially inhibits Ca\textsuperscript{2+}-programmed long-term stomatal closure but not Ca\textsuperscript{2+}-reactive short-term stomatal closure (Figure 5). This result suggests that short-term stomatal closure in response to increases in cytosolic Ca\textsuperscript{2+} utilizes the pre-existing cellular machinery including signaling molecules, ion channels and transporters. Long-term stomatal closure programmed by [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillations is likely to require the pre-existing proteins and also \textit{de novo} synthesis of proteins that are necessary to maintain long-term stomatal closure. Previously, cytosolic Ca\textsuperscript{2+} was shown to regulate gene expression in plants (Clayton \textit{et al.}, 1999; Ghelis \textit{et al.}, 2000; Webb \textit{et al.}, 2001). Thus, it is possible that [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillations regulate transcriptional factor(s) that play a central role in \textit{de novo} synthesis of ion channels and transporters that are required to maintain long-term stomatal closure. It is also possible that [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillations lead to regulation of the expression of proteins involved in signal transduction. For example, in animal cells, protein kinase C, CaM kinase II and a Ras GTPase-activating protein have been suggested to be regulated by [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillations and involved in cellular responses encoded in [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillations (De Koninck and Schulman, 1998; Oancea and Meyer, 1998; Walker \textit{et al.}, 2004). Thus, it would be interesting to perform microarray analyses to examine changes in gene expression caused by the cytosolic calcium transients or oscillations that elicit Ca\textsuperscript{2+}-programmed stomatal closure.
The phenotypic similarity between AtGLR3.1 over-expressing guard cells and wild-type guard cells in the presence of cyclohexamide suggests that the effects of AtGLR3.1 on 
$[Ca^{2+}]_{cyt}$ oscillation-controlled stomatal movements require de novo protein synthesis. A working model for $[Ca^{2+}]_{cyt}$ oscillation-mediated stomatal movements and the contribution of AtGLR3.1 is shown in Figure 6.

Unlike the gca2 and det3 mutants, in which parameters of $[Ca^{2+}]_{cyt}$ oscillation kinetics are altered, over-expression of AtGLR3.1 resulted in impairment in stomatal closure induced by external $Ca^{2+}$ and experimentally imposed $[Ca^{2+}]_{cyt}$ oscillations without affecting the parameters of $[Ca^{2+}]_{cyt}$ oscillation kinetics (Figures 1–3). It is still unclear how de-regulated expression of AtGLR3.1 leads to such phenotypes. A possible scenario is that AtGLR3.1 over-expression may alter the spatial characteristics of $Ca^{2+}$ transients. In addition to the specificity encoded in the kinetics of cytosolic $Ca^{2+}$ oscillations, the subcellular localization of $Ca^{2+}$ signals and the internal $Ca^{2+}$ stores mobilized can also influence the subsequent cellular response (Tsien and Tsien, 1990; Berridge et al., 2000). What we analyzed in guard cells expressing YC2.1 is the change in the whole cytosolic $Ca^{2+}$ concentration, because 35S–YC2.1 only reports changes in $Ca^{2+}$ concentration in the cytoplasm (Allen et al., 1999b). Therefore, it was not possible for us to determine the specific localization of possible $Ca^{2+}$ increases and gradients in the cytoplasm caused by AtGLR3.1 over-expression. Moreover, it is also possible that AtGLR3.1 over-expression may have affected nuclear $Ca^{2+}$ transients that could contribute to the regulation of gene expression (Hardingham et al., 1997; van Der Luit et al., 1999; Hardingham and Bading, 1999). It has been shown in tobacco protoplasts expressing aequorin in the nucleus that different stimuli can separately regulate nuclear $Ca^{2+}$, and that the resulting changes in gene expression are specific for either nuclear or cytosolic $Ca^{2+}$ signals (van Der Luit et al., 1999). It was also shown that regulation of nuclear $Ca^{2+}$ signals occurs separately from that of cytosolic $Ca^{2+}$ signals in Arabidopsis guard cells (Allen and Schroeder, 2001).

Another possible scenario is that AtGLR3.1 over-expression may have affected the $Ca^{2+}$ oscillation decoding machinery that deciphers the $Ca^{2+}$ signatures. In animal cells, for example, the frequency of $Ca^{2+}$ oscillation is known to regulate transcription by NFAT (nuclear factor for activated T cells) (Dolmetsch et al., 1998; Li et al., 1998). NFAT is de-phosphorylated by the $Ca^{2+}$-dependent protein phosphatase calcineurin, leading to translocation of NFAT to the nucleus (Klee et al., 1998; Graef et al., 2001; Tomida et al., 2003). $Ca^{2+}$ oscillation is more effective in inducing the translocation of NFAT than continuous $Ca^{2+}$ increases, and high-frequency $Ca^{2+}$ oscillations are more effective than low-frequency $Ca^{2+}$ oscillations, suggesting that the decoding machinery of $Ca^{2+}$ oscillation regulates the function of NFAT (Tomida et al., 2003). In plants, Rhizobium infection of legume root hairs induces the formation of nodules, which involves triggering of $Ca^{2+}$ oscillations by nod factors. The Medicago truncatula mutant dmi3 shows normal $Ca^{2+}$ transients, whereas the dmi1 and dmi2 mutants are impaired in nod factor induction of $Ca^{2+}$ transients, although all three mutants showed the same phenotype (Wais et al., 2000). DMI3 encodes a $Ca^{2+}$- and calmodulin-dependent protein kinase that functions downstream of $Ca^{2+}$ transients and induces symbiotic gene expression via transcriptional factors (Levy et al., 2004; Mitra et al., 2004; Smit et al., 2005). It might therefore be possible that de-regulated AtGLR3.1 expression has affected the activity of signaling molecules, such as NFAT and DMI3, that comprise the cellular calcium decoding machinery. This could result in altered interpretation of $Ca^{2+}$ oscillations, thus affecting the de novo synthesis of proteins that are required to maintain long-term stomatal closure.

Further studies on the function of AtGLR proteins, along with development of calcium-imaging tools such as yellow cameleons that can be specifically targeted to sub-organelles of plant cells allowing monitoring of spatio-temporal changes in $Ca^{2+}$ concentration in intracellular organelles, will enable identification of the underlying mechanisms of AtGLR3.1 function in $Ca^{2+}$ signaling in plant cells.
Experimental procedures

**Generation of transgenic AtGLR3.1 plants**

A full-length AtGLR3.1 cDNA fragment in either the antisense or sense orientation was cloned into the pBIN-JIT plant expression vector (Kwak et al., 2001) containing dual CaMV 35S promoters; 6-week-old Arabidopsis plants (ecotype Ws-0) were transformed with Agrobacterium tumefaciens LBA4404 strain harboring AtGLR3.1 in pBIN-JIT or harboring the vector alone (Kwak et al., 2003). Several transformants were selected from MS plates containing 50 µg ml⁻¹ kanamycin. Three transgenic lines with a single T-DNA insertion were selected that showed a Mendelian segregation ratio of 3:1 for kanamycin resistance in the T₂ generation. The selected lines were homozgyous, as indicated by the 100% kanamycin resistance in the T₃ generation. Lines 4–2 and 8–3 were selected for further study based on their higher transcript levels of transgenic AtGLR3.1.

**RT-PCR and RNA blot analysis**

Guard-cell and mesophyll-cell protoplasts were prepared as previously described (Lemoine et al., 2004). Total RNA was extracted from the guard-cell and mesophyll-cell protoplasts using Trizol reagent (Invitrogen, http://www.invitrogen.com/), and then further purified using a plant RNeasy kit (Qiagen, http://www.qiagen.com/). Total RNA was converted to cDNA using a first-strand cDNA synthesis kit (GE Healthcare, http://www.gehealthcare.com/usen/index.html) as described by the manufacturer. Actin2 cDNA was amplified to ensure that the same amount of guard-cell and mesophyll-cell cDNA template was used for PCR, and the cDNA was further diluted accordingly. Guard-cell and mesophyll-cell cDNA (1 µl) were used to amplify AtGLR3.1, calmodulin-binding protein (CBP, At4g33050), hydroxyproline-rich protein (HPRP, At2g11140) and actin2 in PCR reactions prepared in 25 µl mixture [primers at 200 nM, 1 x ExTaq polymerase buffer, each dNTP at 200 µM and 2.5 units of ExTaq polymerase (Takara, http://www.takara.bio.com)]. The PCR mixture was denatured at 94°C for 3 mins, followed by 35 cycles (AtGLR3.1), 30 cycles (CBP and HPRP) or 27 cycles (actin2) of amplification (94°C for 30 sec, 56°C (AtGLR3.1, CBP and actin2) or 58°C (HPRP) for 30 sec, and 72°C for 3 mins (AtGLR3.1) or 1.5 mins (HPRP, CBP, actin2). PCR was repeated at least three times. The primer sequences used in the PCR analysis were AtGLR3.1-51 (5’-GGAGGT-3’), AtGLR3.1-31 (5’-TCGATGGACCTGACTCATCGTACTCACTC-3’), actin2-51 (5’-ATGGGATCCTTTGAGTCTGACTCTTATGC-3’), CBP-31 (5’-ATGGTCTGAAGTTGGGTCCTTATGC-3’), HPRP-31 (5’-ATGTTGAAGTTGGGTCCTTATGC-3’), actin2-31 (5’-TGTAGTGAAGTTGGGTCCTTATGC-3’) and CBB-31 (5’-GGAGGT-3’). Total RNA (18 µg) was separated on a denaturing 1.2% w/v agarose gel, and then transferred to a Hybond N+ (GE Healthcare) nylon membrane. The membrane was hybridized with a radiolabeled AtGLR3.1 cDNA fragment, and then washed as described previously (Kwak et al., 2002).

**Promoter–GUS reporter construction and GUS activity analysis**

A 1861 bp promoter region of AtGLR3.1 was amplified by PCR using a genomic DNA clone as a template, and then cloned into the pGEM-T Easy vector (Promega, http://www.promega.com/). The plasmid was then digested with XbaI and Smal to clone the promoter sequence into pBI101 (Stratagene, http://www.stratagene.com/).

The resulting pAtGLR3.1-GUS101 vector was used to transform Arabidopsis, and transformants were selected based on resistance to kanamycin. GUS activity was assayed on transgenic plants grown on MS medium as described previously (Kwak et al., 2002). The primer sequences used to amplify the promoter sequence were AtGLR3.1-P5 (5’-TCTAGAGAAAAAGGTAGTACGCCACATTCTTACAG-3’) and AtGLR3.1-P3 (5’-CCCCGGGAATTTGAGCTTAAACATTGGAAC-3’).

**Stomatal movement assays**

Leaves of 4–5-week-old wild-type and transgenic plants expressing AtGLR3.1 were incubated under white light with a fluence rate of 120 µmol m⁻² sec⁻¹ for 3 h in stomatal opening solution containing 5 mM KCl and 10 mM MES/Tris, pH 6.15. In each experiment, 20 stomatal apertures per plant line and each concentration were measured 2.5 h after calcium was added. In order to measure changes in stomatal aperture in response to imposed calcium oscillations over a period of time, epidermal strips from excised rosette leaves of 4–5-week-old plants were mounted onto a cover slip that was sealed onto a custom microscope slide with a hole in the middle and then repeatedly incubated in depolarization buffer (100 mM KCl, 10 mM MES/Tris, pH 5.6) and hyperpolarization buffer (0.1 mM KCl, 10 mM CaCl₂, 10 mM MES/Tris, pH 5.6) as previously described (Allen et al., 2001; Mori et al., 2006). Calcium oscillations with a 10 or 2 mins period were experimentally induced to induce stomatal movements, and 4–6 stomata were monitored at the indicated times in each experiment. Wild-type and AtGLR3.1 over-expressing plants expressing 35S-YC2.1-Bar (Allen et al., 1999b) were used to monitor changes in [Ca²⁺]cyt in response to the imposed calcium oscillations (see below). Ca²⁺-induced and imposed Ca²⁺ oscillation-induced stomatal movements assays were performed by two or three researchers, respectively, and blind experiments in which the researchers did not know the genotype of the samples were also performed and are included in the results. Student’s t-test (two-tailed distribution, two-sample assuming equal variance) was used to determine the statistical significance of the data.

**Cytosolic Ca²⁺ imaging analysis**

Calcium imaging was performed as described previously using 4–5-week-old wild-type (two independent lines) and AtGLR3.1 over-expressing plants (two independent lines 4–2 and 8–3) expressing the yellow cameleon construct p35SYC2.1-Bar (Allen et al., 1999b, 2000, 2001, 2002; Kwak et al., 2002, 2003). To open stomata, abaxial epidermal strips were incubated in white light with a fluence rate of 125 µmol m⁻² sec⁻¹ for 2.5 h in a solution containing 5 mM KCl, 50 µM CaCl₂ and 10 mM MES/Tris pH 6.15 before recordings. In all plant lines analyzed, guard cells that exhibited spontaneous oscillations in [Ca²⁺]cyt were not included in the analysis. Cells showing stable [Ca²⁺]cyt ratios during the first 10 mins were used to analyze external calcium-induced [Ca²⁺]cyt oscillations. The YFP of YC2.1 bleaches at a faster rate than the CFP does, leading to a slow decline in baseline, which was later corrected by a linear correction factor calculated from the rate of decrease in baseline ratio. [Ca²⁺]cyt transients were counted when the change in [Ca²⁺]cyt ratios was ±0.1 units above the baseline. MetaFluor of the software package Meta Imaging Series 6.1 (Molecular Devices, http://www.moleculardevices.com) was used to analyze the fluorescence ratio of YC2.1.

**Anion channel recordings**

Arabidopsis guard cell protoplasts were isolated from leaves of 5-week-old wild-type and AtGLR3.1 over-expressing plants as...
previously described (Kwak et al., 2002). Whole-cell configuration was used to measure S-type anion channel currents of guard cells as described previously (Kwak et al., 2002). The pipette solution contained 150 mM CsCl, 5.87 mM CaCl2, 2 mM MgCl2, 6.7 mM EGTA, 5 mM Mg-ATP, 10 mM HEPES/Tris pH 7.1. The bath solution contained 30 mM CsCl, 1 mM CaCl2, 2 mM MgCl2 and 10 mM MES/Tris, pH 5.6. The osmolality was adjusted to 485 mmol/kg for the bath solution and 500 mmol/kg for the pipette solution. Guard cells were pre-incubated in the bath solution supplemented with 39 mM CaCl2 for 30 mins (Allen et al., 2002). The standard voltage protocol stepped the voltage from a holding potential of +30 mV, and subsequent voltage steps were reduced by 30 mV per pulse. Leak currents were not subtracted.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Figure S1. Microarray results from Arabidopsis whole-genome chip (ATH1) hybridizations with guard-cell and mesophyll-cell RNA identified several AtGLR3 sub-family members that are highly and/or preferentially expressed in guard cells.
Figure S2. GUS activity assays showing that AtGLR3.1 is expressed in vascular tissues in the leaf, root and stem.
Figure S3. Actinomycin D and cyclohexamid inhibit ABA-induced stomatal closure in wild-type plants.
Movie S1. Cytosolic Ca2+ transients in response to imposed Ca2+ oscillations in AtGLR3.1 transgenic guard cells expressing YC2.1. Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

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