running title: Ca$^{2+}$ entry mediated by a plant glutamate receptor

Edgar P. Spalding
Department of Botany
University of Wisconsin
430 Lincoln Drive
Madison, WI 53706

spalding@wisc.edu
tel: 608-265-5294
fax: 608-262-7509

Category: Signal Transduction and Hormone Action
Calcium Entry Mediated by GLR3.3, an Arabidopsis Glutamate Receptor with a Broad Agonist Profile

Zhi Qi, Nicholas R. Stephens, and Edgar P. Spalding

Department of Botany
University of Wisconsin
430 Lincoln Drive
Madison, Wisconsin 53706
USA
This work was supported by DOE grant 04ER15527 to E.P.S.

Present address: Donald Danforth Plant Science Center
975 North Warson Road
St. Louis, Missouri 63132
USA

*Corresponding author: e-mail spalding@wisc.edu
fax 608-262-7509

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the Journal policy described in the Instructions for Authors (http://www.plantphysiol.org) is: E.P. Spalding (spalding@wisc.edu).
Abstract
The amino acids glutamate and glycine trigger large, rapid rises in cytosolic Ca\textsuperscript{2+} concentration and a concomitant rise in membrane potential (depolarization) in plants. The possibility that plant homologs of neuronal ionotropic glutamate receptors mediate these neuron-like ionic responses was tested in Arabidopsis thaliana seedlings using a combination of Ca\textsuperscript{2+} measurements, electrophysiology, and reverse genetics. The membrane depolarization triggered by glutamate was greatly reduced or completely blocked in some conditions by mutations in GLR3.3, one of the 20 GLR genes in Arabidopsis. The same mutations completely blocked the associated rise in cytosolic Ca\textsuperscript{2+}. These results genetically demonstrate the participation of a glutamate receptor in the rapid ionic responses to an amino acid. The GLR3.3-independent component of the depolarization required glutamate concentrations above 25 µM, did not display desensitization, and was strongly suppressed by increasing extracellular pH. It is suggested to result from H\textsuperscript{+}-amino acid symport. Six amino acids commonly present in soils (glutamate, glycine, alanine, serine, asparagine, and cysteine) as well as the tripeptide glutathione (\(\gamma\)-glutamyl-cysteiny1-glycine) were found to be strong agonists of the GLR3.3-mediated responses. All other amino acids induced a small depolarization similar to the non-GLR, putative symporter component and in most cases evoked little or no Ca\textsuperscript{2+} rise. From these results it may be concluded that sensing of six amino acids in the rhizosphere and perhaps extracellular peptides is coupled to Ca\textsuperscript{2+} signaling through a GLR-dependent mechanism homologous to a fundamental component of neuronal signaling.
INTRODUCTION

A transient rise in the cytosolic concentration of Ca^{2+} is an early step in the process by which many stimuli are transduced into a physiological or developmental response. In plants such stimuli include light, hormones, temperature, microbes, and touch (Sanders et al., 2002; Hetherington and Brownlee, 2004; Hepler, 2005). Calcium transients may result from Ca^{2+} influx across the plasma membrane and/or Ca^{2+} release to the cytoplasm from internal stores. Recently, the TPC1 channel of Arabidopsis was shown to conduct Ca^{2+} from the vacuole to the cytoplasm (Peiter et al., 2005). However, almost nothing is known at the molecular level about Ca^{2+} entry across the plasma membrane, a fundamental issue in plant cell physiology. Cyclic-nucleotide-gated channels are candidates for Ca^{2+}-influx channels at the plasma membrane (Véry and Sentenac, 2002; White et al., 2002; Lemtiri-Chlieh and Berkowitz, 2004) but no evidence to date demonstrates a role for them in the generation of a cytosolic Ca^{2+} signal. Glutamate receptors are also candidates for an influx pathway (Lacombe et al., 2001; Véry and Sentenac, 2002; White et al., 2002). The Arabidopsis genome contains a family of 20 GLR genes homologous with the ionotropic (ion-conducting) glutamate receptors that mediate synaptic transmission and generate Ca^{2+} signals in the mammalian central nervous system (Chiu et al., 1999; Lacombe et al., 2001; Davenport, 2002). At synapses, glutamate released by the presynaptic cell opens glutamate-receptor channels in the postsynaptic cell, causing influx of Ca^{2+}, K^{+}, and Na^{+} (Dingledine et al., 1999; Madden, 2002). The resulting membrane depolarization propagates the impulse and the rise in Ca^{2+} influences many postsynaptic processes including the synaptic conditioning that underpins learning (Ghosh and Greenberg, 1995).

In Arabidopsis, glutamate and glycine trigger very large and fast changes in cytosolic Ca^{2+} (Dennison and Spalding, 2000; Dubos et al., 2003; Meyerhoff et al., 2005). The rise in Ca^{2+} triggered by glutamate is accompanied by a large, transient membrane depolarization that is due at least in part to Ca^{2+} influx across the plasma membrane (Dennison and Spalding, 2000; Meyerhoff et al., 2005). A patch clamp study concluded that glutamate activated non-specific cation channels in root cells, which may be the conductances responsible for these ionic responses (Demidchik et al., 2004). Overexpression of GLR3.2 led to poor plant health that was ameliorated by treatment with Ca^{2+}, and to hypersensitivity to K^{+} and Na^{+} (Kim et al., 2001). Glutamate was shown to depolarize the membrane, depolymerize cortical microtubules, and slow root growth within minutes (Sivaguru et al., 2003). All of these observations are consistent
with the action of ionotropic glutamate receptors at the plant plasma membrane. However, no evidence more direct than the effects of animal iGluR inhibitors links the Arabidopsis GLR genes to the transport of Ca\(^{2+}\) or any electrophysiological response (Dennison and Spalding, 2000; Dubos et al., 2003; Meyerhoff et al., 2005). Needed is a genetic test of the connection between the GLR genes and the ionic responses that quickly follow treatment with amino acids.

The GLRs may not be the molecules responsible for the Ca\(^{2+}\) rises or electrical responses triggered by amino acids. When depolarizations in response to amino acids in plants were first described by Etherton and colleagues, the results were interpreted in terms of multiple amino acid transporters and consequent active proton extrusion (Etherton and Rubinstein, 1978; Kinraide and Etherton, 1980; Kinraide and Etherton, 1982). No genetic or molecular evidence to date precludes this interpretation of ionic responses to amino acids. It is possible that plant glutamate receptors have evolved a different physiological function and that they do not form ligand-gated ion channels similar to their neuronal counterparts. Indeed, the amino acid sequence in the predicted pore region of the plant GLRs differs significantly from that of animal ionotropic glutamate receptors, which may be evidence of a very different or even null ion transport function (Davenport, 2002). If, on the other hand, it could be shown that a GLR gene is responsible for a component of the ionic responses to glutamate in plants, a fundamental aspect of neuronal signaling will have been demonstrated to operate in a complex, multicellular, but aneural organism. Any link between a plant GLR gene and the Ca\(^{2+}\) transient triggered by glutamate would add much-needed molecular information to the topic of how Ca\(^{2+}\) signals are generated in plants. The present research addresses the function of plant GLR molecules with a combination of reverse genetic and cell physiological techniques.

**RESULTS**

*Desensitization of ionic responses to glutamate*

Desensitization is an important mode of regulation of ligand-gated channels that participate in Ca\(^{2+}\) signaling at synapses in the central nervous system (Jones and Westbrook, 1996). Glutamate-induced membrane depolarizations in leaf mesophyll cells display desensitization (Meyerhoff et al., 2005), consistent with the possibility that ligand-gated channels are responsible for the electrical effects of amino acids. If the electrical responses to glutamate and the cytosolic Ca\(^{2+}\) rise triggered by glutamate are
manifestations of the same mechanism (e.g. the activation of ligand-gated channels) then both should display desensitization. This was investigated in Arabidopsis seedlings by experiments employing aequorin-expressing seedlings to measure Ca\(^{2+}\) changes and intracellular microelectrodes to measure membrane potential. Figure 1A shows that glutamate triggered a large, rapid, transient membrane depolarization in the cells of the Arabidopsis root apex similar to previous reports (Dennison and Spalding, 2000; Sivaguru et al., 2003). After switching the flowing bathing solution to a glutamate-free medium, the membrane potential returned to near its initial potential. A second application of glutamate produced a smaller depolarization; the large initial component was desensitized. The second depolarization was repeatedly reversible, i.e. it did not desensitize. When the experiment was performed at an external pH of 7.7, the initial depolarization was similar to the response obtained at pH 5.7. However, at the higher pH, responses to subsequent applications of glutamate were greatly suppressed. The effect of pH on the non-desensitized component receives a possible explanation below.

The Ca\(^{2+}\) rise also displayed desensitization. Figure 1B demonstrates that pretreatment with 10 µM glutamate caused a 25% reduction in the Ca\(^{2+}\) response to a subsequent application of 1000 µM glutamate, while 100 µM glutamate desensitized responsiveness by 56%. The reduced responsiveness was not due to a decrease in the capacity of the aequorin to report Ca\(^{2+}\) because even a 2-h pretreatment with 1000 µM glutamate did not affect the Ca\(^{2+}\) response to cold shock (data not shown). The above data indicate that both the Ca\(^{2+}\) rise and the membrane depolarization display the phenomenon of desensitization as would be expected if both were the result of glutamate activating GLR ion channels.

*Genetic link between ionic responses to amino acids and GLR3.3*

A glutamate-gated Ca\(^{2+}\) rise involving influx across the plasma membrane and desensitization is only circumstantial evidence of a GLR-mediated Ca\(^{2+}\)-influx mechanism operating in plants. A more formal link between the ionic phenomena and the GLR gene family would be established if a mutation in one or more family member affected the ionic response to glutamate. T-DNA insertion (knockout) mutants for 18 members of the family were obtained from the Salk collection (http://signal.salk.edu/cgi-bin/tdnaexpress) and glr homozygotes were screened for aberrant electrophysiological responses to glutamate. One of the knockouts, glr3.3, responded very differently than wild type to certain amino acid applications. While 10 µM glutamate induced a
membrane depolarization of $34 \pm 4$ mV ($n = 3$) in wild-type roots (Fig. 2A), the response was completely absent in two independent glr3.3 knockout plants (Fig. 2B,C).

Increasing glutamate to 100 µM increased the peak magnitude of the wild-type voltage change to $65 \pm 4$ mV ($n = 9$), but the average responses of glr3.3-1 and glr3.3-2 roots were only $20 \pm 4$ mV and $22 \pm 5$ mV, respectively ($n = 9$ for each). The residual depolarization in the glr3.3 mutants increased substantially when the glutamate treatment was increased to 1000 µM (Figure 2B,C). The GLR3.3-independent component of the depolarization may be the result of H⁺-coupled amino acid symport (Fischer et al., 1998) because changing external pH from 5.7 to 7.7 almost completely abolished it (Fig. 2D), as was observed for the non-desensitizing component of the wild-type response (Fig. 1A). The onset of the putative amino acid symport activity at a glutamate concentration between 10 and 100 µM agrees well with previous studies of electrogenic glutamate uptake by an Arabidopsis amino-acid transporter (Boorer et al., 1996).

To determine if the Ca²⁺ rise also depended on the GLR3.3 glutamate receptor, a fluorescent Ca²⁺ reporter (yellow cameleon YC2.1; Allen et al., 1999) was introduced into the two glr3.3 lines by crossing. Figure 2E shows that even the high concentration of 1000 µM glutamate did not produce a rise in Ca²⁺ in either mutant line, while the wild-type response was robust. Glycine, a co-agonist of some animal glutamate receptors, has been shown to elicit a glutamate-like Ca²⁺ response in Arabidopsis (Dubos et al., 2003; Meyerhoff et al., 2005). The glr3.3 mutations blocked the Ca²⁺ response to glycine as well (Fig. 2F). The YC2.1 reporter was functional in the glr3.3 lines because cold shock produced a Ca²⁺ rise in the mutant similar to wild type (data not shown). Photobleaching the YFP acceptor decreased the FRET ratio and almost completely suppressed the response to glycine (data not shown), indicating that the recorded signal was a bona fide measure of cytosolic Ca²⁺ concentration. These results unequivocally demonstrate that the rise in cytoplasmic Ca²⁺ concentration and concomitant membrane depolarization triggered by glutamate or glycine depend on GLR3.3. These results constitute the first formal genetic connection between the GLR gene family and the rapid ionic responses triggered by amino acids.

*Regulation of Ca²⁺ influx by micromolar glutamate*

The above results are consistent with GLR3.3 mediating an inward Ca²⁺ flux across the plasma membrane. If so, then the magnitude of the depolarization and cytosolic Ca²⁺
responses to glutamate should depend on extracellular Ca\(^{2+}\) concentration. Figure 3A shows that increasing external Ca\(^{2+}\) from 1 to 10 mM increased the peak membrane depolarization induced by 25 µM glutamate (from 41 ± 6 mV; n = 4 to 86 ± 6 mV; n = 5). The Ca\(^{2+}\) rise triggered by 25 µM glutamate was also enhanced by increasing external Ca\(^{2+}\) concentration (Fig. 3B). Both Ca\(^{2+}\)-dependent responses were abolished by the glr3.3-1 mutation (Fig. 3A,B). These results indicate that GLR3.3 is required for Ca\(^{2+}\) transport across the plasma membrane from the apoplast in response to glutamate.

By stimulating GLR activity without causing major desensitization, low concentrations of glutamate in the rhizosphere may regulate the Ca\(^{2+}\) permeability of the root plasma membrane. This was tested by determining the effect of changes in extracellular Ca\(^{2+}\) on cytosolic Ca\(^{2+}\) in the continuous presence or absence of 10 µM glutamate. Seedlings expressing aequorin were used for this assay of the effect of glutamate on Ca\(^{2+}\) entry driven by a change in the Ca\(^{2+}\) electrochemical potential gradient. In the absence of exogenous glutamate, a 10-fold increase in extracellular Ca\(^{2+}\) concentration (from 1 to 10 mM) had little effect on cytoplasmic Ca\(^{2+}\) concentration (Fig. 3C). Either the membrane was not very permeable to Ca\(^{2+}\) or the influx resulting from this change was managed efficiently by a homeostatic efflux mechanism, or both. However, in the continuous presence of 10 µM glutamate, the same shift in extracellular Ca\(^{2+}\) caused a substantial rise in cytoplasmic Ca\(^{2+}\) (Fig. 3C). Thus, chronic exposure to 10 µM glutamate increased the ease with which Ca\(^{2+}\) entered the cytoplasm.

**Broad agonist profile of GLR3.3**

Although glutamate is often the most abundant amino acid found in soils, several others are frequently present in appreciable quantities (Abuarghub and Read, 1988; Kielland, 1994; Jones et al., 2005). To determine if others may trigger GLR3.3-dependent activity in roots, the effectiveness of all twenty L-amino acids as well as D-glutamate, D-serine, D-alanine, NMDA, and \(\gamma\)-aminobutyric acid (GABA) was determined. Of the tested compounds, alanine, asparagine, cysteine and serine triggered large, transient membrane depolarizations that were GLR3.3-dependent and in all respects similar to glutamate or glycine responses (Table 1; Fig. S1A). Each of these effective amino acids also triggered a rise in Ca\(^{2+}\) as assayed by aequorin luminescence (Fig. S1A, Table S1). This result is surprising because alanine, asparagine, and cysteine are not known to be agonists of animal glutamate receptors and are structurally dissimilar. Of these, cysteine, was selected for further study. The large rise in cytosolic Ca\(^{2+}\) triggered by
1000 µM cysteine in the wild type was completely absent in cameleon-expressing glr3.3 mutants, but normal in a line heterozygous for the glr3.3 mutation (data not shown). Thus, six amino acids (glu, gly, ser, ala, asn, and cys) may be considered agonists of a GLR3.3-dependent Ca²⁺ influx mechanism. The ineffective compounds (the remainder of the amino acids, the D-isomers, NMDA, and GABA) produced smaller, persistent depolarizations similar to the responses of glr3.3 mutants to glutamate or glycine. Some of these responses are shown in Figure S1B and Table S1. The small, persistent depolarizations induced by glutamine and aspartate were further investigated and found to be both pH-sensitive and independent of GLR3.3 (data not shown). The small responses evoked by the ineffective amino acids may reflect proton-coupled uptake of the compounds.

Given that six different amino acids were each found to induce Ca²⁺ transients and large membrane depolarizations, it was hypothesized that short peptides composed of one or more of these agonists would have similar effects. Glutathione, an abundant tripeptide in plants, is composed of three of the six effective amino acids: glutamate, cysteine and serine. Glutathione plays a number of important roles in plant metabolism and responses to stress, partly because of its ability to be reversibly oxidized and reduced (Noctor et al., 2002; Ogawa, 2005). The reduced form of glutathione (GSH) depolarized root cells by approximately 100 mV in a GLR3.3-dependent manner (Table 1; Fig. 4A). GSH also caused a substantial Ca²⁺ transient (Fig. 4C). The oxidized form of glutathione (GSSG) was much less effective than GSH, though its action was also dependent on GLR3.3 (Table 1; Fig. 4B,C).

DISCUSSION
In soils, glutamate originates from decomposing organic matter and exudates produced by living roots (Lynch and Whipps, 1990; Nguyen, 2003; Jones et al., 2004). Its concentration is typically in the low micromolar range (Abuarghub and Read, 1988; Kielland, 1994; Jones et al., 2005). A study of tomato root exudation estimated rhizosphere glutamate concentration to be 9 µM (Simons et al., 1997). The other five effective amino acids are also among the most prevalent in root exudates and in soils. For example, glutamate, alanine, and glycine were among the four most abundant amino acids detected in maize root exudates (Krafczyk et al., 1984). Metabolic interplay between microbes and roots (Somers et al., 2004; Phillips et al., 2004; Singh et al., 2004) may dynamically change amino acid concentrations in the rhizosphere, thereby
producing chemical signals potentially useful to the root. Figure 2 shows that 10 µM glutamate activated GLR3.3 and Figure 1B demonstrates that this same low level of agonist partially desensitized the Ca$^{2+}$-signaling mechanism. Thus, GLR3.3 would naturally encounter regulatory concentrations of effective amino acids. This argues in favor of GLR3.3-dependent Ca$^{2+}$ influx being physiologically relevant rather than a spurious pharmacological effect. The ability of GLR3.3 to distinguish between oxidized and reduced forms of glutathione raises the possibility that it could also sense the redox poise of the rhizosphere.

Amino acids are not only released to the rhizosphere but are present in the apoplast throughout the plant. One study of Arabidopsis reported that the GLR3.3 activators glutamate, serine, and asparagine are among the four most abundant amino acids in root xylem and leaf exudates (Pilot et al., 2004). In the leaf exudate, glutamate accounts for approximately 12% of the total amino acids (Pilot et al., 2004). Therefore, GLR-mediated ionic signaling mediated by different amino acids could be occurring between cells throughout the plant.

The surprisingly broad agonist profile of GLR3.3 may reflect the unusual structure of its extracellular amino terminus, relative to neuronal glutamate receptors (Turano et al., 2001). A recent bioinformatic analysis of amino acid binding motifs in prokaryotic and eukaryotic genomes (Acher and Bertrand, 2005) found that the Arabidopsis GLR genes were unique in containing two distinct amino acid-binding domains homologous to those found in bacterial periplasmic binding proteins. Together these domains may be responsible for the activating effect of six different amino acids and a tripeptide on GLR3.3-dependent Ca$^{2+}$-influx.

The breadth of the agonist profile found here is difficult to reconcile with the conclusion that glutamate binds only to GLR1.1, and that glycine is probably the exclusive ligand of the other 19 GLRs (Dubos et al., 2003). Homology modeling of plant GLR ligand-binding domains using animal glutamate receptor structures as templates (Dubos et al., 2003) may not be precise enough to predict ligands with accuracy. Alternatively, the logic used here to infer agonists may be overextended. For example, it is formally possible that treatment with any of the six effective amino acids causes the immediate release of the true GLR3.3 ligand, so that in effect what is measured is a secondary response to the initial treatment. The data presented here do not provide good evidence that the effective amino acids actually bind to GLR3.3. They may interact with another protein that acts through GLR3.3 to open a Ca$^{2+}$ conductance.
Nonetheless, it is clear that GLR3.3-dependent ion fluxes are triggered directly or indirectly by multiple amino acids that naturally occur in effective concentrations in the rhizosphere.

GLR3.3 mediates amino-acid-gated Ca\(^{2+}\) influx in the root, but it does not necessarily form a channel. The possibility that membrane depolarization due to glutamate receptor activation triggers Ca\(^{2+}\) influx via separate, voltage-dependent Ca\(^{2+}\) channels (Courtney et al., 1990; White et al., 2002), was investigated by an experiment not presented here. Extracellular K\(^+\) concentration was raised from 10 to 1000 µM to depolarize the membrane, but this did not result in a detectable Ca\(^{2+}\) increase as reported by aequorin (data not shown). Despite minimal sequence similarity in the pore regions of Arabidopsis and mammalian glutamate receptors, GLRs (Davenport, 2002), the currently preferred hypothesis is that GLR3.3 plays a direct role in the conduction of Ca\(^{2+}\) across the plasma membrane in response to amino acids.

A growth or developmental phenotype that would help connect GLR3.3-mediated ion fluxes to a higher-level biological function has not yet been identified in the knockout mutants reported here. Functional redundancy among members of gene families is a common explanation of phenotype absence but it does not easily explain the present case because glr3.3 mutations essentially eliminated the ionic responses in the cells studied. Another family member does not appear to compensate at the cell physiology level for the glr3.3 mutation in the root apex. In contrast to the lack of obvious outward phenotype in glr3.3, mutation of the OsGLR3.1 gene in rice (Li et al., 2006) very obviously slowed root growth by impairing meristematic activity and cell viability at the root apex. Perhaps a similar phenotype will be observed in Arabidopsis glr3.3 mutants when they are cultured in the presence of microorganisms or exudates that naturally activate the GLR3.3-dependent Ca\(^{2+}\) signaling mechanism.

The GLR3.3-mediated Ca\(^{2+}\) signaling system may participate in a number of physiological processes indicated by previous studies. One is the balancing of carbon and nitrogen metabolism, which was affected in certain conditions by antisense suppression of GLR1.1 (Kang and Turano, 2003). Also, the amount of lateral root development in proportion to primary root growth is influenced by micromolar levels of glutamate and therefore may be a GLR-mediated process (Walch-Liu et al., 2006). However, of the effective amino acids in Table 1, glutamate was the only one capable of causing the root architecture changes (Walch-Liu et al., 2006), which argues against GLR3.3 playing a role in this developmental response. Pharmacological data indicated
a role for GLRs in light-induced inhibition of hypocotyl growth (Lam et al., 1998). Signal transduction chains linking photoreceptors to some stages of this process are thought to involve fluxes of ions including Ca\(^{2+}\) (Spalding, 2000). Therefore, detailed studies of photomorphogenesis in \textit{glr3.3} seedlings may uncover a phenotype. A role for GLRs in Ca\(^{2+}\) nutrition was suggested by (Kim et al., 2001) because overexpression of \textit{GLR3.2} led to Ca\(^{2+}\) deficiency symptoms but not lower Ca\(^{2+}\) levels. Perhaps GLR3.3-mediated Ca\(^{2+}\) signaling plays a role in regulating Ca\(^{2+}\) nutrition. Lastly, the responses to harmful elements in the rhizosphere such as Al\(^{3+}\) may also depend on Ca\(^{2+}\) signals mediated by glutamate receptors (Sivaguru et al., 2003). One attractive hypothesis has Al\(^{3+}\) triggering the release of amino acids that activate GLR3.3. The resulting rise in Ca\(^{2+}\) and associated change in microtubules would result in the observed reduction in root growth rate (Sivaguru et al., 2003).

Establishing that GLR3.3 mediates ion fluxes including a large Ca\(^{2+}\) influx across the Arabidopsis plasma membrane prompts some intriguing questions. Has a chemosensing mechanism with prokaryotic origins (Kuner et al., 2003) been molded by evolution to serve the chemosensing needs of roots and the communication needs of a central nervous system? Are the signaling components that function downstream of glutamate-triggered Ca\(^{2+}\) signals in neurons, such as CREB transcription factors (Ghosh and Greenberg, 1995), conserved in plants? Do plants have a Ca\(^{2+}\)-based, cell-to-cell communication system that is molecularly homologous with the mechanism underpinning learning? Do GLRs mediate inter- and intraspecific signaling between roots? Further work on the Arabidopsis GLR molecules and \textit{glr} mutants can be expected to provide answers.

MATERIALS AND METHODS

Plant Growth

\textit{Arabidopsis thaliana} seeds (Columbia ecotype) were surface sterilized with 75\% ethanol and sown on vertical 0.7\% agar plates containing 1 mM KCl, 1 mM CaCl\(_2\), 5 mM MES, pH 5.7 adjusted with Bis-Tris Propane. The plates were maintained at 4°C in darkness for 48 to 72 h before transfer to a growth chamber with a 16/8 h light/dark photoperiod cycle and grown vertically for 4-6 d.

Membrane Potential Measurement
Seedlings were lifted from the growth plates, mounted horizontally on a thin layer of growth media gelled with 0.6% agar at the bottom of a 3.5-cm diameter recording chamber, then covered with a thin layer of agar-free growth medium and allowed to recover for 3 h. Measurements of membrane potential were made as previously described (Dennison and Spalding, 2000) except that data acquisition in this study was achieved with a PCI-MIO-16XE-10 analog to digital converter (National Instruments, Austin, TX) controlled by custom software written in the Labview computer language (National Instruments). The sample rate was 20 Hz. Perfusion of the recording chamber (7 mL min\(^{-1}\)) was driven by a peristaltic pump (Dynamax RP-1, Rainin Co.) and controlled by electronic valves. The initial (control) perfusion solution was the same as the growth medium (minus the agar) and could be switched to one supplemented with an amino acid via the data acquisition software. It was important to position the inflow tube as close as possible to the site of impalement because reproducible electrophysiological responses required abrupt (within approximately 2 s) exchange of solutions. Experiments proceeded only if a stable membrane potential more negative than -120 mV was obtained.

Measurement of intracellular Ca\(^{2+}\) with Aequorin

Seeds of Arabidopsis aequorin-expressing plants described previously (Lewis et al., 1997) were sown and grown on plates as described above. To reconstitute the aequorin with its substrate, four-day-old seedlings were transferred to a darkroom and under green safelight placed in growth media (minus agar) except for containing 10 µM coelenterazine hcp (Molecular Probes, Eugene, OR) and allowed to soak overnight. For each trial, five seedlings were loaded into a luminometer cuvette containing 200 µL of the growth medium (minus agar). The seedlings were allowed to recover from the handling in darkness at 25°C for 2 to 4 h. Programmable, rapid delivery of agonist solutions into the cuvette was performed by the onboard injectors of the luminometer used to record the aequorin signal (TD-20/20, Turner Designs, Sunnyvale, CA). A computer collected data from the luminometer at a rate of 5 Hz. While resting in the dark, the level of aequorin luminescence emitted from five seedlings varied but was generally less than 100 relative light units (RLU). The variability was probably due to different degrees of coelenterazine incorporation and different amounts of tissue in the cuvettes. Treatment with 1000 µM glutamate caused a rapid luminescence increase, typically into the thousands of RLU. A statistical analysis showed that there was a
strong, direct correlation between the level of pretreatment aequorin luminescence and the peak response to agonist. Those tubes with higher aequorin levels gave larger responses and the correlation was near perfect ($R^2 = 0.98$ for glutamate, 0.99 for glycine). Therefore, for each individual trace, the data were divided by the average background (pretreatment) luminescence, producing a fold-increase in aequorin luminescence trace. Recordings normalized in this fashion are shown as traces (e.g. Fig. S1), averaged traces (e.g. Fig. 1B and 4C), or were integrated over time and then averaged (e.g. Fig 1B inset).

To test the effect of glutamate pretreatments on a subsequent response to high levels of glutamate, seedlings expressing aequorin reconstituted with coelenterazine were pretreated with the indicated glutamate concentration for 3 h and then their responses to 1000 µM glutamate were recorded as described above. Four independent trials were performed for each glutamate pretreatment concentration. To test the effects of increasing extracellular Ca$^{2+}$ on the response to low glutamate levels (Fig. 3C), the seedlings were pretreated for 3-4 h with either 1 mM Ca$^{2+}$ (n=9) or 10 mM Ca$^{2+}$ (n=8) before being treated with the same Ca$^{2+}$ concentration plus 25 µM glutamate.

**Measurement of intracellular Ca$^{2+}$ with YC2.1**

Seeds of transgenic plants expressing yellow-cameleon YC2.1 (Allen et al., 1999) were kindly provided by Simon Gilroy, Penn State University. Seedlings grown for 4 d were lifted from the growth plates, mounted horizontally on a thin layer of growth medium gelled with 0.6% agar at the bottom of a recording chamber, bathed in agar-free growth medium and allowed to recover for at least 1 h. Unless stated otherwise, media contained 1 mM CaCl$_2$. The recording chamber was mounted horizontally on the stage of a Zeiss LSM 510 Meta confocal microscope. Root tips were imaged with an Olympus LUMPlanFl x40W objective immersed in the solution bathing the root. Excitation with 458 nm light was provided by a 200-mW argon laser. ECFP fluorescence and the FRET-based EYFP signal were collected using the Meta detector using bandwidths of 462.6–484.0 nm and 526.8–548.2 nm, respectively. Patches of epidermal and cortical cells in the elongating zone were selected as the ROI and scanned at approximately 600 ms intervals. For each time point, 4 separate scans were averaged to minimize noise. Agonist solution was introduced into and removed from the chamber at a rate of approximately 2 mL min$^{-1}$ using a syringe pump (SP120p, World Precision Instruments,
Mean ECFP and EYFP signals were determined for the ROI for each scan and used to produce the calcium-dependent EYFP:ECFP ratio.

**Mutant Genotyping**

Seeds of plant lines containing a T-DNA insertion in the gene of interest were obtained from the Salk Institute ([http://signal.salk.edu/cgi-bin/tdnaexpress](http://signal.salk.edu/cgi-bin/tdnaexpress)). The lines used here were Salk_040458 (glr3.3-1, second exon insertion) and Salk_066009 (glr3.3-2, first intron insertion). To isolate homozygous mutant individuals, DNA was isolated from leaf samples and evaluated for the presence or absence of allele-specific PCR products. Whole leaves were placed into a 96 well plate containing chloroform and extraction buffer (400 mM Tris, pH 8.0; 480 mM NaCl; 50 mM EDTA, pH 8.0; 1% SDS) and ground using a tissue grinder (GenoGrinder 2000, Spex CertiPrep Inc). Following a 1200 x g spin, the DNA-containing fraction was spun twice at 6100xg, first in isopropanol and then in an 80% ethanol wash. The DNA pellet was then resuspended in distilled water after drying. A left border T-DNA primer 5’-TGGTTCACGTAGTGGGCCATCG-3’) was used in combination with gene-specific primers to test for the presence or absence of the T-DNA insertion alleles in segregating populations using PCR and agarose gel electrophoresis. The following are gene-specific primers used to confirm the genotype of both of the glr3.3 mutant lines (SALK_040458 and SALK_066009):

**Acknowledgments**

The authors are grateful for the plant materials supplied by the Arabidopsis Biological Resource Center and to Nathan Miller, University of Wisconsin, for constructing the data acquisition software and perfusion control apparatus. This work was supported by a Department of Energy grant (04ER15527) and by a National Science Foundation Major Research Instrumentation grant (DBI-0421266).
LITERATURE CITED


FIGURE LEGENDS

Figure 1. Desensitization of ionic responses to glutamate. A, Glutamate application-washout cycles indicated by the alternating black and white bar show that a large, rapid membrane depolarization is observed only in response to the first exposure to glutamate (1000 µM, black). The initial response was pH-independent but the smaller subsequent responses to glutamate were strongly suppressed by raising the pH from 5.7 to 7.7. Both traces are the averages of four independent experiments with SEM shown at arbitrarily-selected time points. B, Pretreatment of aequorin-expressing wild-type seedlings with the indicated concentration of glutamate caused a reduction in the response to a subsequent application of 1000 µM glutamate made 3 h later. The traces shown are the averages of at least 3 trials. inset A plot of Ca^{2+} response versus glutamate pretreatment made by integrating each of the trials and then averaging the integrals. Data points are the mean ± SEM. Desensitization of the Ca^{2+} response occurred over the range of glutamate concentrations that roots are expected to experience in soils.

Figure 2. Comparison of ionic responses induced by glutamate in roots of wild type and glr mutants. A, Depolarizations increased in magnitude and changed shape as glutamate concentration was increased from 10 to 1000 µM in the wild type. The external medium was pH 5.7. B, Responses of glr3.3-1 to 10 µM glutamate were not detectable. A sustained depolarization was induced in the mutant by glutamate concentrations at or greater than 100 µM. C, The dependence of the depolarization on GLR3.3 is confirmed with the independent glr3.3-2 allele. D, At pH 7.7, the wild-type response to 1000 µM glutamate (black) was large and the mutant response was nearly absent (red). All membrane potential traces shown are representative of between 3 and 9 independent trials. Average peak responses are stated in the text. E, The change in cytoplasmic Ca^{2+} induced by 1000 µM glutamate in the wild type (n=12) was abolished in two independent glr3.3 mutant alleles (n=6,7). F, The change in cytoplasmic Ca^{2+} induced by 1000 µM glycine in the wild type (n=12) was absent in two independent glr3.3 mutant alleles (n=7,8).

Figure 3. Effects of increasing extracellular Ca^{2+} on GLR3.3-mediated responses to micromolar glutamate. A, Increasing external Ca^{2+} from 1 mM to 10 mM increased the
magnitude of the membrane depolarization induced by 25 µM glutamate. Even at the higher Ca\(^{2+}\) concentration, glr3.3-1 root cells did not depolarize in response to this low level of glutamate. The glr3.3-2 allele also failed to respond (data not shown). See the text for mean peak values. B, A rise in cytosolic Ca\(^{2+}\) in response to 25 µM glutamate was detected in wild-type root cells bathed in 10 mM Ca\(^{2+}\) (n=7) but not when external Ca\(^{2+}\) was 1 mM (n=4). Even in the presence of 10 mM external Ca\(^{2+}\), glr3.3-1 mutants did not show a detectable response to 25 µM glutamate (n=6). C, A rise in cytosolic Ca\(^{2+}\) (assayed by aequorin luminescence) in response to shifts of external Ca\(^{2+}\) from 1 mM to 10 mM was greatly enhanced by pretreatment with 10 µM glutamate. (n=8 for the 0 Glu trials and n=13 for the 10 µM glu trials)

**Figure 4.** Glutathione activates GLR3.3 activity in roots. A, The reduced form of glutathione (GSH, 1000 µM) triggered a large, transient membrane depolarization in the wild type but not in glr3.3 mutants. B, The oxidized form of glutathione (GSSG, 1000 µM) triggered a smaller, broader depolarization that was also GLR3.3-dependent. All membrane potential traces shown are representative of 3 to 5 independent trials. C, GSH triggered a large, transient change in cytosolic Ca\(^{2+}\) (n=5). Consistent with the depolarization measurements, GSSG triggered a slower, smaller Ca\(^{2+}\) response than GSH (n=4). SEM values are shown at arbitrarily-selected time points.

**Figure S1.** Survey of changes in membrane potential and cytosolic Ca\(^{2+}\) in response to L isomers of the 20 common amino acids and GABA. A, Example recordings of membrane potential showing large, transient depolarizations (black lines) typical of the responses induced by the six potent amino acids and the accompanying changes in cytoplasmic Ca\(^{2+}\) as assayed by aequorin luminescence (red lines). B, Small, stable membrane depolarizations and minimal changes in Ca\(^{2+}\) typical of 14 other amino acids and GABA. Vertical scale bars correspond to 60 mV (black) and a 380-fold increase in aequorin luminescence over background (red). Average responses of multiple trials are shown in Table S1.
Table 1. Peak changes in membrane potential induced by 1000 µM of the six potent amino acids or glutathione in wild type and glr3.3 mutants. Values shown are the mean peak change in mV ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Ala</th>
<th>Asn</th>
<th>Cys</th>
<th>Glu</th>
<th>Gly</th>
<th>Ser</th>
<th>GSH</th>
<th>GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>wt</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>117 ± 11</td>
<td>87 ± 7</td>
<td>91 ± 1</td>
<td>83 ± 5</td>
<td>98 ± 8</td>
<td>90 ± 12</td>
<td>104 ± 15</td>
<td>43 ± 3</td>
<td></td>
</tr>
<tr>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=4)</td>
<td>(n=8)</td>
<td>(n=7)</td>
<td>(n=3)</td>
<td>(n=7)</td>
<td>(n=10)</td>
<td></td>
</tr>
<tr>
<td><strong>glr3.3-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 ± 13</td>
<td>41 ± 3</td>
<td>45 ± 3</td>
<td>38 ± 2</td>
<td>47 ± 4</td>
<td>47 ± 4</td>
<td>19 ± 6</td>
<td>22 ± 3</td>
<td></td>
</tr>
<tr>
<td>(n=4)</td>
<td>(n=5)</td>
<td>(n=3)</td>
<td>(n=7)</td>
<td>(n=6)</td>
<td>(n=3)</td>
<td>(n=4)</td>
<td>(n=5)</td>
<td></td>
</tr>
<tr>
<td><strong>glr3.3-2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41 ± 3</td>
<td>50 ± 4</td>
<td>40 ± 2</td>
<td>44 ± 6</td>
<td>38 ± 5</td>
<td>43 ± 6</td>
<td>31 ± 4</td>
<td>18 ± 4</td>
<td></td>
</tr>
<tr>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=7)</td>
<td>(n=6)</td>
<td>(n=3)</td>
<td>(n=4)</td>
<td>(n=4)</td>
<td></td>
</tr>
</tbody>
</table>
Table S1. Mean peak changes in membrane potential and integrated aequorin signals in response to each of the common amino acids.

The concentration of each compound was 1000 µM. Values shown are means ± SEM.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Change in membrane potential (mV)</th>
<th>Integrated aequorin luminescence (fold over background)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>117 ± 11 (n = 3)</td>
<td>1805 ± 169 (n = 5)</td>
</tr>
<tr>
<td>Asn</td>
<td>87 ± 7 (n = 3)</td>
<td>1490 ± 133 (n = 7)</td>
</tr>
<tr>
<td>Cys</td>
<td>91 ± 1 (n = 4)</td>
<td>2645 ± 233 (n = 5)</td>
</tr>
<tr>
<td>Glu</td>
<td>83 ± 5 (n = 8)</td>
<td>2206 ± 163 (n = 5)</td>
</tr>
<tr>
<td>Gly</td>
<td>98 ± 8 (n = 7)</td>
<td>2378 ± 82 (n = 5)</td>
</tr>
<tr>
<td>Ser</td>
<td>90 ± 12 (n = 3)</td>
<td>1720 ± 103 (n = 5)</td>
</tr>
<tr>
<td>Arg</td>
<td>30 ± 4 (n = 3)</td>
<td>490 ± 112 (n = 5)</td>
</tr>
<tr>
<td>Asp</td>
<td>54 ± 8 (n = 4)</td>
<td>89 ± 12 (n = 3)</td>
</tr>
<tr>
<td>Gln</td>
<td>62 ± 7 (n = 4)</td>
<td>787 ± 73 (n = 3)</td>
</tr>
<tr>
<td>His</td>
<td>41 ± 3 (n = 3)</td>
<td>123 ± 6 (n = 3)</td>
</tr>
<tr>
<td>Ile</td>
<td>33 ± 4 (n = 3)</td>
<td>120 ± 10 (n = 3)</td>
</tr>
<tr>
<td>Leu</td>
<td>50 ± 4 (n = 3)</td>
<td>858 ± 29 (n = 3)</td>
</tr>
<tr>
<td>Lys</td>
<td>46 ± 8 (n = 2)</td>
<td>245 ± 51 (n = 4)</td>
</tr>
<tr>
<td>Met</td>
<td>43 ± 4 (n = 3)</td>
<td>1535 ± 54 (n = 3)</td>
</tr>
<tr>
<td>Phe</td>
<td>42 ± 3 (n = 3)</td>
<td>94 ± 17 (n = 3)</td>
</tr>
<tr>
<td>Pro</td>
<td>48 ± 3 (n = 3)</td>
<td>154 ± 37 (n = 3)</td>
</tr>
<tr>
<td>Thr</td>
<td>44 ± 2 (n = 3)</td>
<td>154 ± 2 (n = 3)</td>
</tr>
<tr>
<td>Trp</td>
<td>9 ± 1 (n = 3)</td>
<td>105 ± 10 (n = 3)</td>
</tr>
<tr>
<td>Tyr</td>
<td>14 ± 2 (n = 3)</td>
<td>72 ± 3 (n = 4)</td>
</tr>
<tr>
<td>Val</td>
<td>40 ± 9 (n = 3)</td>
<td>89 ± 20 (n = 3)</td>
</tr>
</tbody>
</table>
A

- wt, 10 mM Ca$^{2+}$
- wt, 1 mM Ca$^{2+}$
- glr3.3-1, 10 mM Ca$^{2+}$

B

![Graph showing EYFP/ECFP over time for different Ca$^{2+}$ concentrations.](graph.png)

C

Shift of external Ca$^{2+}$ from 1 to 10 mM

- Integrated Ca$^{2+}$ signal (10$^9$)

0 Glu

10 μM Glu