Glutamate Receptor-Like Channel3.3 Is Involved in Mediating Glutathione-Triggered Cytosolic Calcium Transients, Transcriptional Changes, and Innate Immunity Responses in Arabidopsis[1][W][OA]

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The tripeptide reduced glutathione (GSH; γ-glutamate [Glu]-cysteine [Cys]-glycine) is a major endogenous antioxidant in both animal and plant cells. It also functions as a neurotransmitter mediating communication among neurons in the central nervous system of animals through modulating specific ionotropic Glu receptors (GLRs) in the membrane. Little is known about such signaling roles in plant cells. Here, we report that transient rises in cytosolic calcium triggered by exogenous GSH in Arabidopsis (*Arabidopsis thaliana*) leaves were sensitive to GLR antagonists and abolished in loss-of-function *atglr3.3* mutants. Like the GSH biosynthesis-defective mutant *PHYTOALEXIN DEFICIENT2*, *atglr3.3* showed enhanced susceptibility to the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000. Pathogen-induced defense marker gene expression was also decreased in *atglr3.3* mutants. Twenty-seven percent of genes that were rapidly responsive to GSH treatment of seedlings were defense genes, most of which were dependent on functional AtGLR3.3, while GSH suppressed pathogen propagation through the AtGLR3.3-dependent pathway. Eight previously identified putative AtGLR3.3 ligands, GSH, oxidized glutathione, alanine, asparagine, Cys, Glu, glycine, and serine, all elicited the AtGLR3.3-dependent cytosolic calcium transients, but only GSH and Cys induced the defense response, with the Glu-induced AtGLR3.3-dependent transcription response being much less apparent than that triggered by GSH. Together, these observations suggest that AtGLR3.3 is required for several signaling effects mediated by extracellular GSH, even though these effects may not be causally related.

Reduced glutathione (GSH; γ-Glu-Cys-Gly) is the most abundant short-chain peptide in cells and is enzymatically synthesized from Glu, Cys, and Gly. It is present in both intra- and extracellular compartments, although at least 90% is found inside the cell. Depending on the cell type, glutathione levels have been estimated to range from 1 to 10 mM, 90% of which is in reduced form, thus representing the major pool of endogenous nonprotein thiols within organisms (Noctor et al., 2011). Its Cys sulfur group can perform reversible redox reactions to form disulfides, notably with another glutathione Cys residue to produce oxidized glutathione (GSSG), which is continuously recycled at high rates to GSH by glutathione reductase existing in the cytosol and other cellular organelles. Therefore, glutathione serves a central role in free radical scavenging, maintaining the thiol status of proteins, and functions as a general antioxidant protecting cell membranes against oxidative stress and DNA against radiation (for review, see Franco and Cidlowski, 2009; Schmidt and Dringen, 2012).

In plants, GSH typically accumulates in cells to millimolar concentration. Intracellular GSH is essential for development and reactive oxygen species scavenging as well as oxidative signaling during stress responses (Foyer and Noctor, 2011; Noctor et al., 2012). Arabidopsis (*Arabidopsis thaliana*) knockout mutants completely deficit in GSH biosynthesis show either embryo- or seedling-lethal phenotypes (Cairns et al., 2006; Pasternak et al., 2008), demonstrating its indispensable role in plant development. Other Arabidopsis mutants that have various degrees of reduction in endogenous GSH content show altered expression of stress-related genes (Ball et al., 2004; Mhamdi et al., 2010; Han et al., 2013a, 2013b), enhancing sensitivity to excessive zinc (Shanmugam et al., 2012) and cadmium (Jozefczak et al., 2012) as well as pathogens (Parisy et al., 2007; Dubreuil-Maurizi and Poinssot, 2012).

In animal cells, GSH not only functions as an essential antioxidant as in plant cells, but GSH in the extracellular space of neurons in the central nervous system can act like a neurotransmitter or neuromodulator through specific interactions with Glu receptors (Levy et al., 1991; Juurlink, 1999; Dringen, 2000; Oja et al., 2000; Shaw et al., 2001; Aoyama et al., 2008). Glu receptors are either ionotropic (iGLRs) or metabotropic. iGLRs form ionic

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cation channels permeable to Ca^{2+}, Na^+, and K^+ and are named after their agonists *N*-methyl-D-Asp (NMDA), 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate, and kainite (Flores-Soto et al., 2012). Metabotropic Glu receptors are coupled to G proteins and functionally linked either to the formation of inositol phosphates and diacylglycerol or to the metabolism of cyclic nucleotides (Loane et al., 2012). The molecular structure of GSH tends to make it neuroactive, because all of its three individual amino acid residues can either function as neurotransmitters or interfere with glutamatergic neurotransmission, with Glu and Gly being excitatory and inhibitory neurotransmitters, respectively (Hnasko and Edwards, 2012). Furthermore, Cys is neurotoxic at high concentrations (Slivka and Cohen, 1993). GSH can stimulate NMDA receptor-dependent membrane depolarization and Ca^{2+} entry into rat neurons (Leslie et al., 1992; Janáky et al., 1993; Ogita et al., 1995; Varga et al., 1997), cultured hippocampal neurons (Chin et al., 2006), and pig cerebral cortical synaptic membranes (Janáky et al., 2000).

To the best of our knowledge, the first report of a potentially similar signaling role of extracellular GSH in plants to that found in the animal central nervous system was a study with tobacco (*Nicotiana tabacum*) expressing the Ca^{2+} reporter luminescence protein aequorin in the cytosol (Gomez et al., 2004). The authors reported that exogenous application of 5 mM GSH induces cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) transient elevation in the leaf (Gomez et al., 2004). In Arabidopsis roots, our previous study also demonstrated that 1 mM GSH can induce strong $[Ca^{2+}]_{cyt}$ elevation (Qi et al., 2006). Furthermore, GSH can induce AtGLR3.3-dependent membrane depolarization (Qi et al., 2006). AtGLR3.3 is one of 20 Glu receptor genes in Arabidopsis (Lacombe et al., 2001). The predicted protein structure of AtGLRs includes conserved transmembrane and extracellular ligand binding domains of iGLRs and G protein-coupled receptors (GPCRs; Chiu et al., 1999; Turano et al., 2001). Different plant GLR members are suggested to play roles in various physiological processes, including mineral nutrient homeostasis (Kim et al., 2001; Dubos et al., 2003; Kang et al., 2006; Aouini et al., 2012), carbon/nitrogen balance (Kang and Turano, 2003), root development (Li et al., 2006), stomata movement (Cho et al., 2009), abscisic acid sensing (Kang et al., 2004), gravitropism sensing (Miller et al., 2010), and pollen tube elongation (Michard et al., 2011). Recently, pharmacological approaches produced evidence that AtGLRs could be involved in innate immunity response in Arabidopsis seedlings (Kwaaitaal et al., 2011, 2012). In light of the established roles of GSH in plant defense responses (Ferrari et al., 2003; Parisy et al., 2007; Schlaeppi et al., 2008) and the requirement of AtGLR3.3 for GSH-induced signaling events in the roots (Qi et al., 2006), we hypothesized that GSH could mediate innate immunity responses in the leaf through AtGLR3.3-dependent pathways. This study reports evidence that strongly supports this notion and gives rise to several intriguing new questions about this novel signaling cascade in the defense response.

RESULTS

Exogenous GSH Induces AtGLR3.3-Dependent Heterogeneous $[Ca^{2+}]_{cyt}$ Rise

To investigate the signaling role of extracellular GSH in the leaf, we monitored how exogenous application of GSH ($[GSH]_{ext}$) affects $[Ca^{2+}]_{cyt}$ in detached Arabidopsis leaves, using cytosolic expression of the Ca^{2+} reporter protein aequorin. As observed previously (Qi et al., 2006), the background $[Ca^{2+}]_{cyt}$ concentration was around 0.1 μM (Fig. 1). Immediately after the delivery of a 10 μM GSH solution into the detached leaf, a rise in $[Ca^{2+}]_{cyt}$ was observed (Fig. 1). Increasing the $[GSH]_{ext}$ to 100 and 1,000 μM further promoted the response, which showed a strong concentration-dependent pattern (Fig. 1).

In our previous study with Arabidopsis roots, we demonstrated that 1 mM $[GSH]_{ext}$ induced membrane depolarization and that this effect largely depends on AtGLR3.3 (Qi et al., 2006), one of 20 Glu receptor-like genes in Arabidopsis. We hypothesized that the observed $[GSH]_{ext}$-induced $[Ca^{2+}]_{cyt}$ rise in this study was also mediated by AtGLR3.3. As an initial test of this hypothesis, we pretreated the leaf with the iGLRs antagonists (2*R*)-amino-5-phosphonopentanoate (AP5) and 6,7-dinitroquinoxaline-2,3-dione. Both of them,

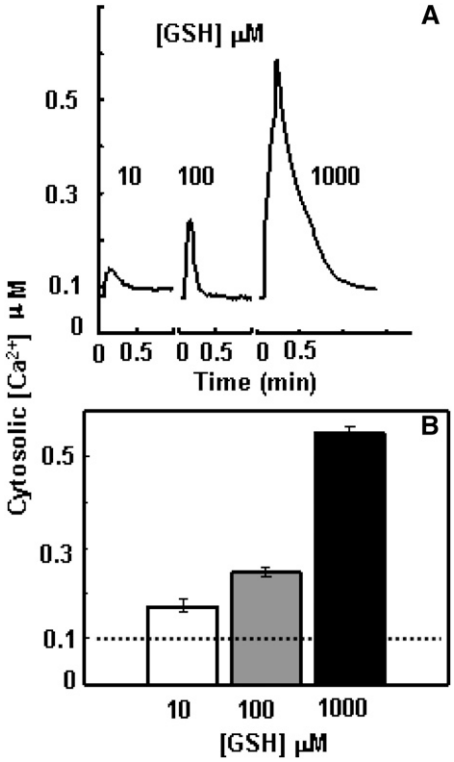

Figure 1. $[GSH]_{ext}$-induced concentration-dependent $[Ca^{2+}]_{cyt}$ rise in the leaf. A, Representative recording curve of 10, 100, and 1,000 μM GSH-induced $[Ca^{2+}]_{cyt}$ transient rise in a detached leaf. B, Averaged peak values with SE of the responses ($n = 5$), which are significantly different to each other at $P < 0.01$. The dash line indicates the background $[Ca^{2+}]_{cyt}$.

especially AP5, significantly suppressed the $[GSH]_{ext}$-induced responses (Fig. 2). We next introduced *aequorin* gene into the *atglr3.3-1* and *atglr3.3-2* knockout mutant backgrounds through cross pollination and monitored its response to $[GSH]_{ext}$. The GSH-induced $[Ca^{2+}]_{cyt}$ rise observed in the wild type was completely absent in the mutants (Fig. 3).

We next used pharmacological approaches to investigate which Ca^{2+} mobility pathways contribute to the $[GSH]_{ext}$-induced and AtGLR3.3-dependent $[Ca^{2+}]_{cyt}$ rise. Removal of extracellular Ca^{2+} and including 1 mM Ca^{2+} chelator EGTA in the recording buffer significantly, but not completely, suppressed 100 μM GSH-induced $[Ca^{2+}]_{cyt}$ rise (Fig. 2). This indicates that extracellular Ca^{2+} influx through certain plasma membrane Ca^{2+}-permeable channels partially, but not fully, contributes to the observed response. cAMP-gated calcium channels have been identified in the plasma membrane of Arabidopsis leaf protoplasts (Ali et al., 2007). Alloxan can interrupt the cAMP-dependent Ca^{2+} influx pathway by inhibiting adenylate cyclase activity and so blocking cAMP production. Pretreatment for 1 h with 1 mM alloxan significantly inhibited the rise in $[Ca^{2+}]_{cyt}$ (Fig. 2). Our previous study implicated Cyclic Nucleotide Gated Channel2 (CNGC2) in mediating the $[Ca^{2+}]_{cyt}$ rise associated with innate immunity (Ma et al., 2009). However, in this study, we found a normal response of the *cngc2* mutant expressing *aequorin* to the $[GSH]_{ext}$ (data not shown).

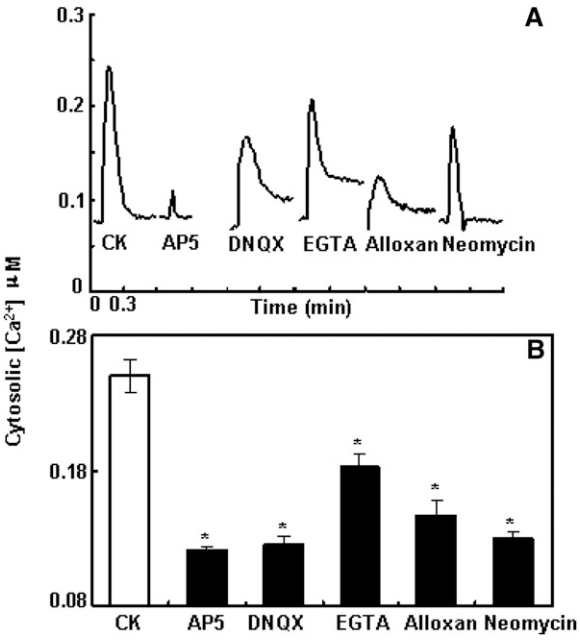

Figure 2. Pharmacological study of the $[GSH]_{ext}$-induced $[Ca^{2+}]_{cyt}$ transient response in the leaf. A, Representative recording curve of 100 μM $[GSH]_{ext}$-induced $[Ca^{2+}]_{cyt}$ rise in the CK and pretreated with 1 mM various Ca^{2+} mobility pathway blockers as indicated. B, Averaged peak values of these responses with SE ($n = 4$ for EGTA and $n = 5$ for the rest). The asterisk stands for the SD to the CK at $P < 0.01$. The average background $[Ca^{2+}]_{cyt}$ was around 0.08 μM.

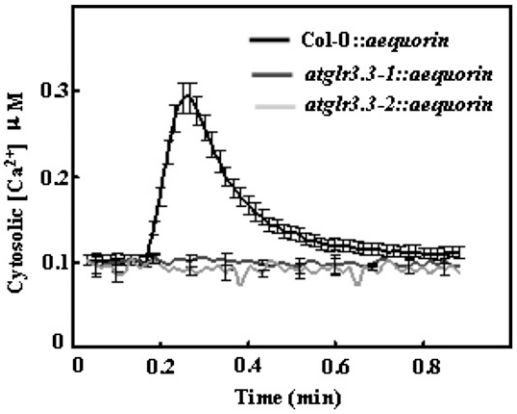

Figure 3. $[GSH]_{ext}$-induced $[Ca^{2+}]_{cyt}$ response was absent in the *atglr3.3* mutant. Averaged recording curves with SE of 100 μM $[GSH]_{ext}$-induced $[Ca^{2+}]_{cyt}$ rise in the leaf cells of the ecotype Columbia (Col-0) and *atglr3.3-1* and *atglr3.3-2* mutants expressing *aequorin* ($n = 10$ for each).

Chelating extracellular Ca^{2+} did not abolish the GSH-induced $[Ca^{2+}]_{cyt}$ response (Fig. 2), implicating Ca^{2+} release from certain internal Ca^{2+} storage pools in the response. 1,4,5-trisphosphate (IP3)-mediated Ca^{2+} release from internal Ca^{2+} pools has been demonstrated in Arabidopsis guard cells (Tang et al., 2007). Neomycin can block the IP_3-mediated Ca^{2+} mobility pathway by inhibiting the activity of phospholipase C, which catalyzes production of IP_3 from phosphatidylinositol 4,5 bisphosphate. One-hour 1 mM neomycin pretreatment also significantly suppressed the $[GSH]_{ext}$-induced Ca^{2+} response (Fig. 2). Taken together, these data demonstrated that the $[GSH]_{ext}$-induced Ca^{2+} response fully depended on AtGLR3.3 but was caused by increased Ca^{2+} mobility through various pathways.

AtGLR3.3 Mediates Part of the Early Transcriptional Response of the Leaf to $[GSH]_{ext}$

Ligand-induced $[Ca^{2+}]_{cyt}$ rise is often linked to downstream transcriptional responses (Dodd et al., 2010). To focus on the early transcriptional responses to $[GSH]_{ext}$ and to establish the importance of AtGLR3.3 for the GSH-mediated signaling cascades, we treated the wild-type and *atglr3.3-2* seedlings for 1 h with either the control (CK) recording buffer or the buffer containing 100 μM GSH. Two independent biological replicates were conducted for whole genome transcriptional profiling analysis with Agilent Arabidopsis microarrays. Under the CK condition, there were 35 genes showing at least 3-fold differential expression between the mutant and the wild type (Supplemental Table S1). As expected, *AtGLR3.3* was the lowest expressed transcript in the *atglr3.3* mutant compared with the wild type, with 47- and 50-fold changes in the two biological replicates, respectively (Supplemental Table S1).

GSH treatment of the wild type altered expression of 97 genes, with at least 3-fold transcript abundance change (Fig. 4A; Supplemental Tables S2 and S3). By

comparing the genes modulated by GSH in the *atglr3.3* mutant, we found that 70 (Supplemental Table S2) of the 97 genes showed no significant change in the mutant. The remaining 27 genes showed the same expression trends in response to GSH in both the wild type and the mutant (Fig. 4A; Supplemental Tables S3 and S4). Thus, this expression profiling analysis indicated that 72% (70/97) of the genes rapidly modulated by GSH in the wild type were dependent on the AtGLR3.3 pathway and 28% (27/97) independent of it.

To validate the transcriptional profiling data set, independent biological samples were produced, and eight genes selected from the list of genes regulated by GSH in the wild type were chosen for quantitative reverse transcript (qRT)-PCR analysis (Supplemental Tables S2 and S3). All eight genes showed significantly increased expression in response to GSH, an effect that was significantly attenuated in the *atglr3.3-2* mutant background (Supplemental Fig. S1). The trends of gene expression in Supplemental Figure S1 were consistent with those revealed by the microarray (Supplemental Table S2). These observations provide further evidence that early transcriptional responses induced by GSH are at least partially acting through AtGLR3.3.

GSH-Regulated AtGLR3.3-Dependent Defense and Signaling-Related Genes

To explore which cellular pathways were regulated by GSH in an AtGLR3.3-dependent manner, we performed categorization of these genes by Gene Ontology annotation analysis combined with manual checking of available literature associated with each of the 70 genes in Supplemental Table S2 through searching The Arabidopsis Information Resource (http://www.arabidopsis.org). This analysis showed that 27%, 20%, 20%, 20%, and 16% of these genes were functionally categorized as Defense, Signaling, Unknown, Transcription Factor, and Others, respectively (Fig. 4B; Supplemental Table S2). There were eight genes that were common to the categories Defense and Transcription Factor. All of the genes in the Defense category have been demonstrated using corresponding mutants to play roles during defense responses or found to be induced by various pathogens at transcriptional level (Table I; Supplemental Table S2). These genes notably include the essential salicylic acid (SA) biosynthesis gene *ISOCHORISMATE SYNTHASE1* (*ICS1*; Wildermuth et al., 2001) and its key regulator *SYSTEMIC ACQUIRED RESISTANCE DEFICIENT1* (*SARD1*; Zhang et al., 2010) as well as the transcription factors *CALMODULIN BINDING PROTEIN60-LIKEG* (*CBP60G*) (Wang et al., 2009), *WRKY33*, and *WRKY46* (Birkenbihl et al., 2012; Hu et al., 2012; Moreau et a., 2012), all of which are essential genes for mediating defense response of Arabidopsis.

Genes in the Signaling category include a kinase (At1g67000), receptor-like proteins *RLP7* and *RLP20*, calcium homeostasis-related proteins such as Glu receptors *AtGLR1.2*, *AtGLR2.8*, and *AtGLR2.9*, and *CALMODULIN LIKE37*. These proteins could be involved in relaying the GSH-induced AtGLR3.3-dependent rise in $[Ca^{2+}]_{cyt}$ transient to downstream signaling cascades (Fig. 3). In addition, this category includes At3g28580, an AAA-ATPase, which was up-regulated 6-fold by GSH in an AtGLR3.3-dependent manner (Supplemental Table S2). This component has recently been reported to play a central role in mediating plant responses to reactive oxygen species in conditions of environmental stress (Simková et al., 2012). Taken together, this analysis demonstrated that the AtGLR3.3-dependent early transcriptional responses of Arabidopsis to $[GSH]_{ext}$ primarily involved cellular defense and signaling, functional categories that account for 47% of the modulated genes (Supplemental Table S2).

AtGLR3.3 Is Required for GSH-Mediated Innate Immunity Response in Arabidopsis Leaf

Arabidopsis mutants defective in GSH biosynthesis show altered defense responses to various pathogens

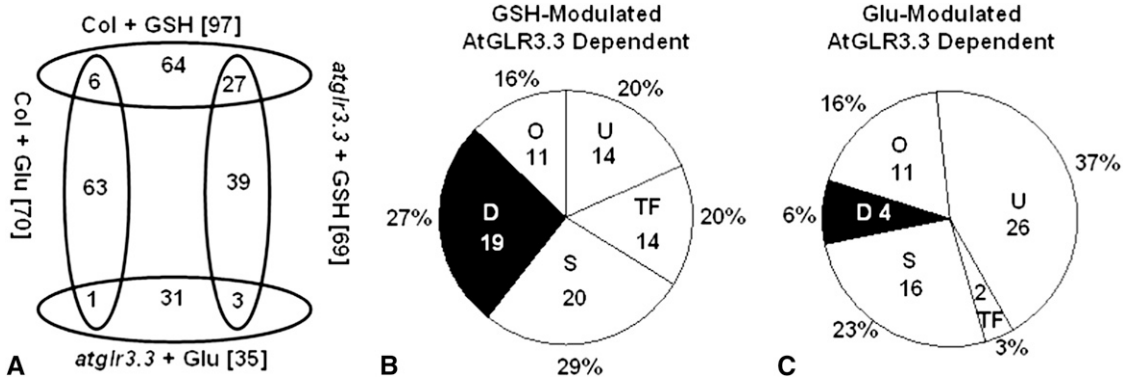

Figure 4. Overview of the GSH- and Glu-mediated AtGLR3.3-dependent gene expression. A, Number of overlapping genes modulated by GSH and Glu in the ecotype Columbia (Col-0) and *atglr3.3* mutant. B, Functional category of the genes modulated by GSH with AtGLR3.3 dependence. C, Functional category of the genes modulated by Glu with AtGLR3.3 dependence. The numbers in and the percentage symbol outside the pie chart indicate the number and percentage of the genes in the category, respectively. U, Unknown; TF, Transcription Factor; S, Signaling; O, Others; D, Defense.

Table I. *Defense genes induced by GSH in ecotype Columbia but not glr3.3-2*

Gene ID	FC01[a]	FC02[a]	Average	Annotation	Reference
Having confirmative role in defense					
AT5G13320	17.9	6.1	12	GRETCHEN HAGEN 3.12; SA biosynthesis	Okrent et al., 2009
AT2G46400	15.7	4.9	10.3	WRKY46 transcription factor	Hu et al., 2012
AT1G33960	12.5	3	7.75	*AVIRULENCE-INDUCED GENE1*	Reuber and Ausubel, 1996
AT1G74710	5.8	4.8	5.3	Isochorismate Synthase1; SA biosynthesis	Wildermuth et al., 2001
AT1G72520	3.3	6.6	4.95	Lipoxygenase4; jasmonic acid biosynthesis	Acosta and Farmer, 2010
AT2G35980	5.1	3.9	4.5	Yellow-leaf-specific gene9	Po-Wen et al., 2013
AT5G26920	4.4	3.4	3.9	CBP60G	Wang et al., 2009
AT1G73805	3.6	3.5	3.55	SARD1; SA biosynthesis	Zhang et al., 2010
AT2G38470	3.4	3.4	3.4	WRKY33 transcription factor	Birkenbihl et al., 2012
Having putative role in defense					
AT5G36970	14.8	23.1	18.95	HAIRPIN-INDUCED1 LIKE25; induced by pathogens	Varet et al., 2002
AT5G47850	7	13.2	10.1	CRINKLY44 related4; protein kinase; response to insect	Little et al., 2007
AT1G21240	7.5	3.3	5.4	Wall associated kinase3; induced by SA	He et al., 1999
AT2G18060	3.5	6.3	4.9	NAC (NAM, ATAF1/2 and CUC2) domain containing protein37; induced by chitin	Libault et al., 2007
AT3G50930	3.7	5.7	4.7	ATPases associated with diverse cellular activities type; induced by bacterial pathogen	Navarro et al., 2004
AT3G23230	3	6.3	4.65	Ethylene response factor98; induced by pathogen and jasmonic acid	McGrath et al., 2005
AT2G43000	3.7	5.1	4.4	NAC domain containing protein42; camalexin biosynthesis	Saga et al., 2012
AT1G14540	4	3.8	3.9	Peroxidase4; induced by oxidative stress and fungus infection	Rasul et al., 2012
AT1G18570	3.2	4.1	3.65	MYB51 transcription factor; induced by pathogen	Maekawa et al., 2012
AT4G33050	4	3.1	3.55	Embryo sac development arrest39; response to chitin and virus	Libault et al., 2007

[a]Fold changes of two replicates.

(Glazebrook and Ausubel, 1994; Roetschi et al., 2001; van Wees et al., 2003; Bohman et al., 2004; Parisy et al., 2007; Dubreuil-Maurizi and Poinssot, 2012). Innate immunity responses involve complicated signaling networks. We hypothesized that the *atglr3.3* mutants may have overlapping defense phenotypes with Arabidopsis mutants, such *PHYTOALEXIN DEFICIENT2-1* (*pad2-1*), defective in GSH biosynthesis. To test its pathogen phenotype, we inoculated mature leaves of the wild type and two allelic knockout mutants (*atglr3.3-1* and *atglr3.3-2*) with the hemibiotrophic model bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000). Pathogen growth was monitored at 0, 1, 2, and 3 d post inoculation (DPI). Figure 5 showed that the two *atglr3.3* mutants had significantly higher susceptibility to the pathogen infection than the wild type, indicating that AtGLR3.3 has a role in innate immunity of Arabidopsis. To validate our experimental system, we reexamined the pathogen phenotype of *pad2-1* along with the wild type. Supplemental Figure S3 showed that the pathogen population per unit in the mutant at 2 DPI was significantly higher than that of the wild type, which was consistent with the previous report (Glazebrook and Ausubel, 1994).

To investigate the role of AtGLR3.3 in the response, we monitored expression of two defense marker genes, *PATHOGENESIS-RELATED GENE1* (*PR1*) and *WRKY33*, in response to pathogen infection in both the wild type and the mutants at 0, 12, 24, and 48 h post inoculation. The pathogen-induced expression of the defense marker genes was significantly attenuated in both allelic *atglr3.3* loss-of-function mutants (Fig. 6). To investigate the physiological significance of the GSH-mediated AtGLR3.3-dependent defense gene expression (Fig. 4; Table I), we next examined the effect of $[GSH]_{ext}$ on pathogen propagation inside the leaves of the wild type and the mutants. One day prior to the pathogen infiltration, the leaves were infiltrated with the CK buffer or the CK buffer containing100 μM GSH. On the day after, all leaves were infiltrated with the pathogen suspension at 5×10^5 colony-forming units (cfu) mL^{-1}. The GSH pretreatment significantly suppressed pathogen propagation in the wild-type leaf. However, suppression of propagation by $[GSH]_{ext}$ was not evident in the *atglr3.3* mutants (Fig. 5). Thus, $[GSH]_{ext}$ promotes innate immunity responses in the leaf through AtGLR3.3-dependent pathways.

Ligand Specificity of AtGLR3.3 in the Innate Immune Response

Our previous study with Arabidopsis root and hypocotyl cells suggests that there are up to eight putative ligands of AtGLR3.3: GSH, GSSG, Ala, Asn, Cys, Gly, Glu, and Ser (Qi et al., 2006; Stephens et al., 2008). Among these, Glu plays a dominant role (Stephens et al., 2008). Given the results shown in Figure 5, we hypothesized that if any of the eight putative ligands are true biological ligands of AtGLR3.3 in vivo, they should be able to evoke AtGLR3.3-dependent innate immunity response in the leaf by exogenous application, in a similar fashion to GSH (Figs. 3–5). To test this hypothesis, we infiltrated the leaf of the wild type and two alleles of mutants with CK or CK solution containing 100 μM GSSG, Ala, Asn, Cys, Gly, and Glu

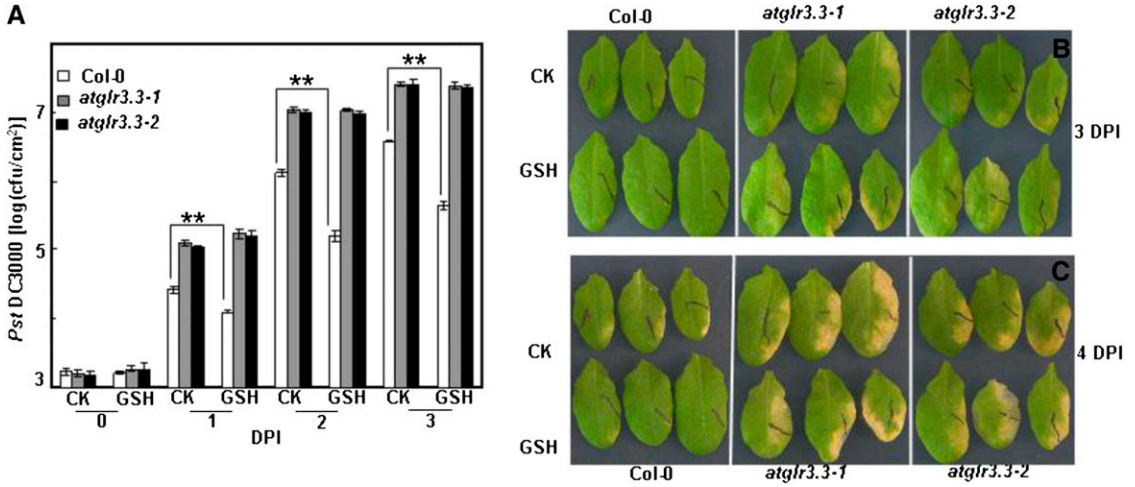

Figure 5. Role of *AtGLR3.3* in the innate immunity and GSH-triggered defense response in the leaf. A, Leaves of ecotype Columbia (Col-0) and *atglr3.3-1* and *atglr3.3-2* mutants were infiltrated with either CK solution or the solution containing 100 μM GSH 1 d before the inoculation of *Pst* DC3000 at 5×10^5 cfu mL^{-1}. The pathogen proliferation was monitored at 0, 1, and 2 DPI. Values presented are average with SE from at least four leaves from different plants. At 1, 2, and 3 DPI, there is SD between the Col-0 and two mutants at $P < 0.01$. Two asterisks indicated significance at $P < 0.01$. Five independent experiments were conducted and showed comparable results. B, Leaf symptoms of the Col-0, *atglr3.3-1*, and *atglr3.3-2* plants preinfiltrated with CK solution or the solution containing 100 μM GSH 3 DPI with *Pst* DC3000. C, Same leaves in B were kept 1 d longer, e.g. 4 DPI, in a sealed agar plate.

1 d before the pathogen inoculation. We also included Asp, a nonexciting amino acid, as a control. At 2 DPI, consistent with the previous results (Fig. 5), the *atglr3.3* mutants showed enhanced bacterial growth (Fig. 7). Among the compounds tested, only Cys and Asp were able to act similarly to GSH to significantly suppress pathogen propagation in the wild-type leaf (Fig. 7). The suppressing effects of Cys, but not Asp, were annulled in the *atglr3.3* loss-of-function mutants (Fig. 7). Thus, of the two amino acids, only the suppressive effect of Cys acts through an AtGLR3.3-dependent pathway, similar to GSH.

The Eight Putative Ligands, but Not Asp, Induce an AtGLR3.3-Dependent $[Ca^{2+}]_{cyt}$ Rise

It was unexpected that the eight ligands and Asp would have distinct effects on the plant's innate immunity responses (Figs. 5 and 7). We hypothesized that the difference could be related to (1) varying ability to initiate the transient rise in $[Ca^{2+}]_{cyt}$ and (2) different dependence on AtGLR3.3 in the leaves. We therefore examined effects of seven ligands and Asp on the $[Ca^{2+}]_{cyt}$ in the leaf cells of the wild type and *atglr3.3* mutants. All the tested ligands, except Asp but including Cys, evoked significant $[Ca^{2+}]_{cyt}$ responses (Fig. 8). These were of varying amplitudes, but all were dependent on AtGLR3.3 (Fig. 8). This result demonstrated that innate immunity responses triggered by the ligands GSH and Cys are not simply correlated with their ability to induce $[Ca^{2+}]_{cyt}$ transients.

The Glu-Mediated Early Transcriptional Response Is Distinct to That Induced by GSH

Glu is the principle exciting amino acid that activates GLRs in the central nervous system (Flores-Soto et al., 2012). It also evokes strong $[Ca^{2+}]_{cyt}$ responses in the leaf (Fig. 8A), which are at least comparable with, if not stronger than, those induced by GSH (Figs. 2 and 3). However, Glu and GSH had essentially distinct effects on the plant defense responses. Most notably, Glu had no suppressive effect on the pathogen propagation (Figs. 5 and 7).

To further explore the influence of the two compounds, we compared Glu-elicited transcriptomic responses with those induced by GSH (Fig. 4B; Table I; Supplemental Table S2). In total, treatment of wild-type leaves with 100 μM Glu modulated expression of 70 genes, 69 of which showed no significant changes in response to Glu in the *atglr3.3* mutant and can therefore be categorized as Glu-modulated AtGLR3.3-dependent genes (Fig. 4A; Supplemental Tables S5, S6, and S7). Among the 69 genes, only six genes were common to the GSH-modulated AtGLR3.3-dependent group (Fig. 4A; Supplemental Tables S2 and S5). Functional categorization of the 69 Glu-modulated AtGLR3.3-dependent genes showed that 37%, 16%, 23%, 3%, and 6% of these genes were classified as Unknown, Others, Signaling, Transcription Factor, and Defense, respectively (Fig. 4C). The Defense section only included four genes: *PR1*, *FLAVIN-DEPENDENT MONOOXYGENASE1* (Mishina and Zeier, 2006), *CELL WALL-ASSOCIATED KINASE1*, and *CELL WALL-ASSOCIATED KINASE3* (Brutus et al., 2010; De Lorenzo et al., 2011). Hence, it was strikingly

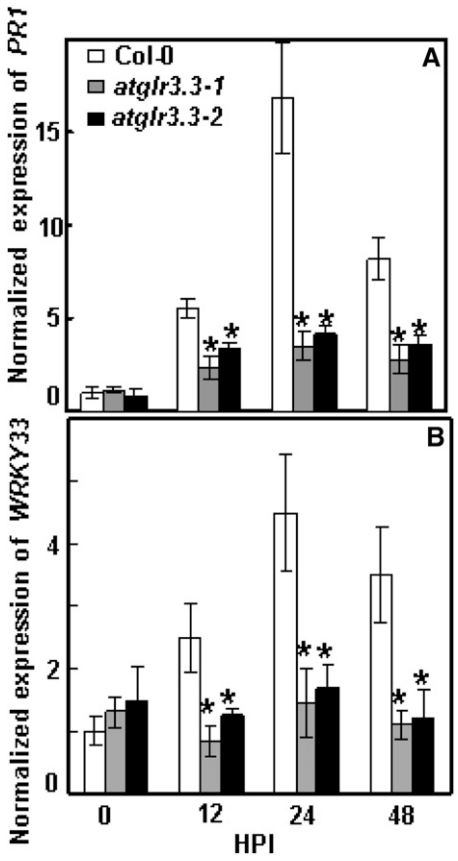

Figure 6. *Pst* DC3000-induced defense market gene expression in ecotype Columbia (Col-0) and *atglr3.3* mutants. *Pst* DC3000 at 5×10^5 cfu mL^{-1} was infiltrated into the leaves of Col-0, *atglr3.3-1*, and *atglr3.3-2*. The leaves were harvested at 0, 12, 24, and 48 h post infection (HPI) for *PR1* (A) and *WRKY33* (B) gene expression analysis with qRT-PCR. The transcript abundance was normalized with reference gene *Tubulin2* in Col-0 at 0 h post infection. Values presented are average with SE from three independent experiments. The asterisk indicated the SD between the mutants and Col-0 at same time point at $P < 0.05$.

different from the transcriptomic patterns elicited by GSH in an AtGLR3.3-dependent manner (Fig. 4B). Taken together, these results demonstrate that although GSH and Glu induce comparable AtGLR3.3-dependent $[Ca^{2+}]_{cyt}$ responses, they induce distinct downstream early transcription responses. Crucially, only GSH was able to induce expression of a significant proportion of defense-related genes, which could explain the specific capacity of GSH to suppress pathogen propagation in the leaves (Fig. 5).

DISCUSSION

Since the discovery of AtGLRs in Arabidopsis genome (Lam et al., 1998), there has been considerable interest in deciphering their physiological functions (Kudla et al., 2010). Previously, in root cells, we demonstrated that AtGLR3.3 is required for the membrane depolarization induced by eight putative ligands, including GSH, GSSG, Ala, Asn, Cys, Glu, Gly, and Ser (Qi et al., 2006). It is possible that different AtGLRs expressed in different tissue and under various conditions show different ligand specificity. The first evidence to support this notion was the finding that D-Ser, but not Glu and Gly, activates AtGLR1.2-dependent Ca^{2+} conductance in the plasma membrane of the pollen tube (Michard et al., 2011). Among the eight

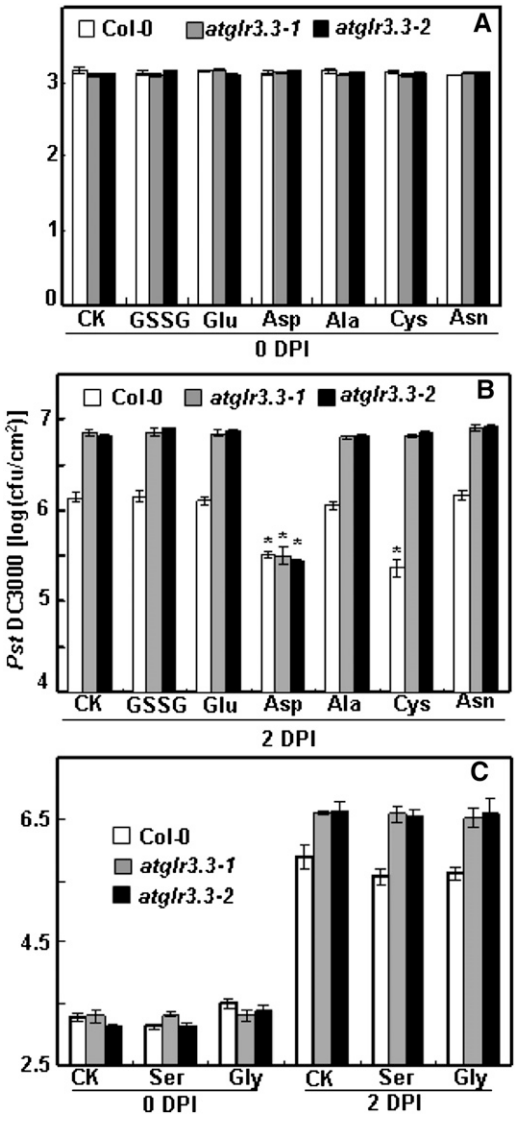

Figure 7. *Pst* DC3000 infection assay of ecotype Columbia (Col-0) and *atglr3.3* with putative ligands and Asp. The experiment procedure and analysis methods are same as those in Figure 5. *Pst* DC3000 proliferation in these leaves was monitored at 0 (A) and 2 DPI (B) preinfiltrated with either CK solution or the solution containing 100 μM GSSG, Glu, Asp, Ala, Cys, and Asn. C, The same assay was conducted separately for Ser and Gly. At 2 DPI in B and C, except for Asp, the values of *atglr3.3-1* and *atglr3.3-2* were significantly higher than that of Col-0 at $P < 0.01$. The asterisk in B indicated there was SD between the treatment and the CK for the corresponding genotype at $P < 0.01$.

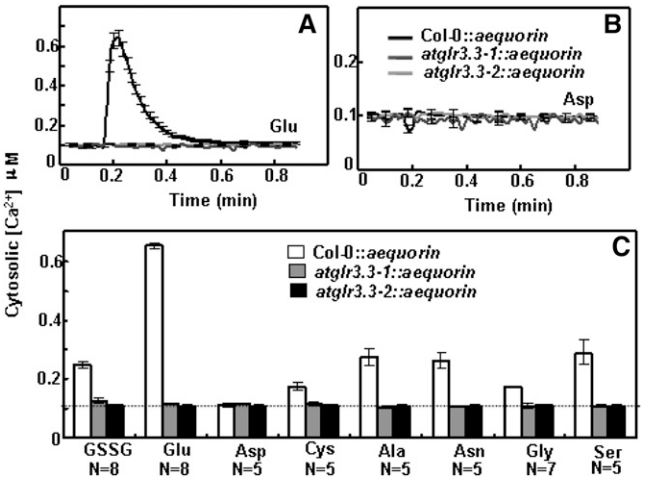

Figure 8. Effects of the seven putative ligands of AtGLR3.3 and Asp on the $[Ca^{2+}]_{cyt}$ in the leaf cells of ecotype Columbia (Col-0) and *atglr3.3* expressing *aequorin*. Averaged recording curves with SE of 100 μM Glu (A) and Asp (B) induced $[Ca^{2+}]_{cyt}$ rise in the leaf cells of Col-0 and *atglr3.3-1* and *atglr3.3-2* mutants expressing *aequorin* ($n = 8$ for Glu and $n = 5$ for Asp). C, the average peak values with SE of the response to the indicated ligands ($n = 8$ for GSSG and Glu, $n = 5$ for Asp, Cys, Ala, Asn, and Ser, and $n = 7$ for Gly). The asterisk indicated SD at $P < 0.01$ between the mutants and Col-0 for the same treatment. The dash line indicated the background $[Ca^{2+}]_{cyt}$.

putative ligands of AtGLR3.3, GSH has established physiological function as an essential antioxidant and signaling molecule involved in plant defense responses (Noctor et al., 2012). It led us to form hypotheses that AtGLR3.3 may play a role in innate immunity and in mediating GSH-induced defense responses. Results presented in this study support this hypothesis and open up several intriguing avenues of research for further exploration.

The GSH-Induced AtGLR3.3-Dependent $[Ca^{2+}]_{cyt}$ Transient Involves Several Calcium Stores

It was previously observed that exogenous application of GSH at millimolar concentrations induced a $[Ca^{2+}]_{cyt}$ rise in tobacco leaf and Arabidopsis root cells (Gomez et al., 2004; Qi et al., 2006). However, the identity of the proteins involved in this response was not known. In this study, we first demonstrated that as little as 10 μM GSH could evoke significant elevation of the $[Ca^{2+}]_{cyt}$ (Fig. 1), suggesting that the cell has sensitive mechanisms to detect extracellular GSH at physiological concentrations. Furthermore, using a pharmacological approach and a transfer DNA (T-DNA) insertion knockout mutant expressing the Ca^{2+} reporter protein aequorin, we demonstrated that the response fully depended on AtGLR3.3 in Arabidopsis leaf cells (Fig. 3). The result is consistent with the previous observation that AtGLR3.3 is required for GSH-induced membrane depolarization in the roots (Qi et al., 2006). In animal cells, iGLRs form cation channels permeable to Ca^{2+}, K^+, and Na^+ (Flores-Soto et al., 2012). Recently, AtGLR3.4, the closest homolog of AtGLR3.3, has been demonstrated to form an amino acid-gated Ca^{2+} channel when heterologously expressed in the plasma membrane of human embryonic kidney cells (Vincill et al., 2012).

It is possible that AtGLR3.3 forms similar Ca^{2+} channels to AtGLR3.4 in the plasma membrane of the leaf cells and is gated by extracellular GSH to conduct Ca^{2+} influx into the cytosol. However, chelating extracellular Ca^{2+} didn't completely abolish the GSH-mediated $[Ca^{2+}]_{cyt}$ response (Fig. 2). This implies that mobilization of internal Ca^{2+} pools also contributes to the response. AtGLRs are predicted to contain conserved domains of both iGLRs and GPCRs (Turano et al., 2001). It is possible that the AtGLR3.3, similar to GPCRs, is coupled to certain internal Ca^{2+} mobility-signaling pathways, such as IP_3-mediated Ca^{2+} release from internal Ca^{2+} pools (Loane et al.,2012). Support for this notion comes from the observation that pharmacologically interfering with IP_3 production significantly suppressed the GSH-mediated response (Fig. 2). In summary, the observed $[Ca^{2+}]_{cyt}$ elevation could be explained by (1) direct influx of extracellular Ca^{2+} through the AtGLR3.3 channel into the cytosol and (2) indirect activation of internal Ca^{2+}-releasing pathways through unknown intermediate signal transducers.

Role of AtGLR3.3 in Innate Immunity and GSH-Induced Defense Responses

Two alleles of *atglr3.3* loss-of-function mutants showed consistently enhanced susceptibility to *Pst* DC3000 (Fig. 5) and decreased expression of defense-related marker genes in response to the pathogen (Fig. 6). This implicates AtGLR3.3 as a novel genetic component of the innate immunity system in the leaf. Theoretically, any of the previously identified eight ligands could be biologically significant ligands of the receptor. By testing the effects of the exogenous application of the ligands individually on pathogen propagation, we found that both GSH and Cys were able to significantly suppress pathogen growth in an AtGLR3.3-dependent manner (Fig. 7). The common feature that GSH and Cys share, and which is absent from the other potential ligands, is a thiol group. It is possible that it is these groups that interact with AtGLR3.3, which in turn evokes specific downstream signaling cascades that ultimately lead to the defense responses. It is well documented that cytosolic GSH can move into the extracellular space during cell death in animal cells (Hammond et al., 2004; Schmidt and Dringen, 2012). In plants, pathogen infection increases GSH production (Vanacker et al., 2000; Parisy et al., 2007). Possibly, cytosolic GSH can be exported to the extracellular space through oligopeptide transporters (Koh et al., 2002) during the interaction between the plant cells and pathogens.

GSH serves as reservoir for Cys, and the two flanking amino acids Glu and Gly protect the sulfhydryl group of Cys against oxidation (Meyer, 2008; Hell and Wirtz, 2011). It is known that externally supplied Cys, after being transported into the cytosol, is transiently formed

into GSH (Hell and Wirtz, 2011). Therefore, it is possible that in addition to its direct binding to the AtGLR3.3, Cys can reinforce the GSH production and indirectly induce the defense response. Conversion of Cys to GSH may have contributed to the induced resistance to bacteria (preinfiltration 1 d prior to the pathogen inoculation), but the much shorter incubations used for the luminescence studies (minutes) mean that effects of Cys on $[Ca^{2+}]_{cyt}$ are likely to be direct. It is a very intriguing question how perception of extracellular GSH and Cys by the membrane-anchored AtGLR3.3 relays into downstream signaling cascade.

The suppressing effect of Asp on the pathogen propagation through the AtGLR3.3-independent pathway was unexpected (Fig. 7). Asp is closely linked to several metabolites that play essential roles in primary metabolic pathways such as the tricarboxylic acid cycle that contributes to cellular energy metabolism (Kirma et al., 2012). It was possible that excess Asp could interrupt the energy production process and in turn decrease the energy in the plant cells available for the pathogen consumption. It is also possible that excess Asp drives production of certain defense compounds to either directly limit the pathogen propagation or trigger the plant cells' innate immunity responses. However, Glu, another amino acid that is closely linked to the tricarboxylic acid cycle, did not suppress pathogen propagation.

Our study gives rise to questions about the physiological ligands of AtGLR3.3. In animal cells, $[GSH]_{ext}$ can induce release of other compounds, for example dopamine from mouse striatal slices (Janáky et al., 2007) and taurine from developing mouse hippocampus (Janáky et al., 2008). We cannot exclude the possibility that $[GSH]_{ext}$ induces release of other compounds from the plant cells to activate the AtGLR3.3-dependent innate immunity response.

GSH-Mediated AtGLR3.3-Dependent Early Transcription Response

Several studies report that $[GSH]_{ext}$ induces stress-associated gene expression, and partial and whole genome data have been presented for GSH- and glutathione reductase-deficient mutants (Ball et al., 2004; Mhamdi et al., 2010; Han et al., 2013b). In this study, we analyzed the early transcription responses of the plant cells to $[GSH]_{ext}$ with the aim of studying gene regulation effects of GSH acting as a signaling molecule rather than in its well-known role as an antioxidant. Because we only had two biological replicates, we set very strict standards as detailed in "Materials and Methods" to process the raw data. Its validity was supported by two findings: first, the AtGLR3.3 transcript was the most down-regulated in the *atglr3.3* mutant compared with that in the wild type (Supplemental Table S1); second, real-time qRT-PCR with independent biological samples were largely consistent with the microarray data set (Supplemental Fig. S1). The GSH treatment modulated expression of 97 genes in the wild type, 70 of which were not significantly changed in *atglr.3.3*, accounting for 72% of the total (Fig. 4A; Supplemental Table S2). It clearly indicated that AtGLR3.3 plays an essential role in the early transcriptional responses of the leaf to GSH. The genetic mechanism of the GSH-mediated and AtGLR3.3-independent gene expression is unknown. In animal cells, in addition to the GLRs, GSH has been shown to have profound regulative effects on the ryanodine receptor (Zable et al., 1997), death receptor (Morito et al., 2003), and GPCRs (Wang et al., 2006). It is possible that certain unknown receptors in Arabidopsis mediate the AtGLR3.3-independent GSH signaling cascade.

For the GSH-modulated AtGLR3.3-dependent genes, we did standard Gene Ontology annotation analysis combined with manual literature checking. It revealed that 27% of the genes have either confirmative roles in defense or are induced by biotic stresses (Table I; Supplemental Table S2), which accounted for the largest subgroup in the early transcription response. It makes strong sense in context of the defense function of GSH observed in this study (Fig. 5). Particularly interesting is the finding that GSH induced expression of *ICS1* and *SARD1* in an AtGLR3.3-dependent manner, two essential genes for SA biosynthesis (Wildermuth et al., 2001; Zhang et al., 2010). A regulatory role for SA in GSH production in plants has been described (Srivastava and Dwivedi, 1998; Yoshida et al., 2009). Our finding that GSH can strongly induce *ICS1* expression (Table I) implies that there is a positive AtGLR3.3-dependent feedback mechanism for GSH to enhance SA biosynthesis under stress conditions. Hydrogen peroxide induces production of SA in tobacco (Leon et al., 1995), while the GSH-deficient mutant *pad2-1* lost pathogen *Phytophthora brassicae*-triggered expression of *ICS1* (Dubreuil-Maurizi and Poinssot, 2012). Recently, it has been reported that hydrogen peroxide-triggered accumulation of SA in a catalase-deficient mutant can be largely blocked by blocking glutathione synthesis, and that is associated with the failure to up-regulate *ICS1* (Han et al., 2013a). It will be very interesting to investigate the role of AtGLR3.3 in the interaction between SA and GSH homeostasis.

Correlation of the $[Ca^{2+}]_{cyt}$ Transient Rise with the Defense Response

A large number of environmental and internal signals are able to evoke $[Ca^{2+}]_{cyt}$ transients in plant cells (Kudla et al., 2010). In most of these cases, two fundamental questions remain: (1) the molecular component underlying the transient $[Ca^{2+}]_{cyt}$ rise and (2) how the Ca^{2+} responses are linked to the downstream physiological function mediated by the signaling. In this study, we provided genetic evidence that the transient $[Ca^{2+}]_{cyt}$ rise induced by the eight putative ligands were fully dependent on AtGLR3.3 (Figs. 3 and 8). However, the link between the GSH-induced transient $[Ca^{2+}]_{cyt}$ rise and the defense functions of GSH still remain elusive.

It is possible that the two effects of GSH are not related. Evidence to support this argument is that Glu-induced AtGLR3.3-dependent $[Ca^{2+}]_{cyt}$ transient

rise was stronger than that of GSH (Fig. 8), even though unlike GSH, Glu did not induce significant amount of defense gene expression and defense responses (Figs. 4C and 7). Key points are the still unknown structure of the Glu receptor complex and the mechanism through which it specifically recognizes different ligands. In animal cells, functioning iGLRs are composed of different subunits encoded by individual iGLR genes and activated by different ligands. A typical case in this context is the NMDA iGLR, which is composed of four subunits, two from NMDA Receptor A (NRA), NRB, NRC, and NRD and two NR1s. NRA to NRD is activated by Glu and NR1 by Gly (Flores-Soto et al., 2012).

It is possible that the AtGLRs are also formed by different subunits that recognize different ligands. GSH and Cys could activate a unique subunit through their specific thiol group and induce additional signaling cascades that occur alongside, rather than downstream, of the transient $[Ca^{2+}]_{cyt}$ rise. Our previous study with the cross-desensitization technique indirectly reveals that the AtGLRs are composed by AtGLR3.3 and other subunits specifically activated by different putative ligands (Stephens et al., 2008).

The situation could be even more complex. It has been proposed for some time that transient $[Ca^{2+}]_{cyt}$ rises induced by different signals could be different in strength, frequency, and spatial distribution (Kudla et al., 2010). A recent study provides evidence in support of this and shows that transient $[Ca^{2+}]_{cyt}$ rises with different strength and spatial distribution are linked to different transcription responses (Wheeler et al., 2012). Such concepts could explain why GSH and Glu induce comparable changes in $[Ca^{2+}]_{cyt}$ but distinct gene expression responses. To completely elucidate this mechanism, studies will require more precise measurement and subcellular imaging of dynamic changes in $[Ca^{2+}]_{cyt}$. Whether the two GSH-mediated AtGLR3.3-dependent effects are related or not, our data suggest that the GSH-related functions of AtGLR3.3 are involved in signaling. The *atglr3.3* mutant showed wild-type growth on cadmium (Supplemental Fig. S2), a condition in which GSH plays an important but nonsignaling role as a precursor molecule of phytochelatins that sequester cadmium (Cobbett et al., 1998; Jozefczak et al., 2012).

CONCLUSION

Glu receptors play vital roles in mediating information exchange among neurons in the central nervous system. Their complex functions are determined by their versatile structures, subunit composition, various ligand types, and binging sites as well as binding affinity in different tissues and under different physiological conditions (Flores-Soto et al., 2012; Popescu, 2012). In this study, we provided evidence to support a novel role of AtGLR3.3 in the innate immunity and GSH-mediated defense response in Arabidopsis leaf. Our findings promote understanding of the AtGLR gene family and the related signaling roles of extracellular GSH and raise intriguing questions for further study.

MATERIALS AND METHODS

Arabidopsis AtGLR3.3 Mutants Expressing Aequorin

The Arabidopsis (*Arabidopsis thaliana*) *atglr3.3-1* and *atglr3.3-2* (At1g42540) homozygous knockout mutants used in this study were described previously (Qi et al., 2006) and were SALK_040458 and SALK_066009, respectively (Alonso et al., 2003), obtained from The Arabidopsis Biological Resource Center. To introduce the *aequorin* gene into the mutant background, mutants were crossed with the *aequorin* transgenic Arabidopsis line (Knight et al., 1991). In the F2 segregation population, homozygous T-DNA insertions were detected with gene-specific primers (SALK_040458, 5′-TTGTTTGTTGCAGCATTGAG-3′, 5′-GCCAATCTTGAGTTCTTTCCC-3′ and SALK_066009, 5′-AAGCACCAGACATCTTACGC-3′, 5′-TCAGATTGGTCCAATTTTAGC-3′) and the T-DNA left border-specific primer LBb1.3 (5′-ATTTTGCCGATTTCGGAAC-3′). The presence of active aequorin was validated by measuring luminescence intensity in the leaf, as described below.

$[Ca^{2+}]_{cyt}$ Measurement in Arabidopsis Leaf Expressing Aequorin

To measure dynamic changes in $[Ca^{2+}]_{cyt}$ in the leaf, Arabidopsis seeds were surface sterilized with 100% ethanol (v/v) for 15 min and dried on sterilized filter paper in a fume hood. Sterilized seeds were sown on 0.7% (w/v) agar medium containing one-half-strength Murashige and Skoog basic salts with 1% (w/v) Suc and 5 mM MES, pH 5.7. After 3-d treatment at 4°C, the plates were placed in a growth chamber at 22°C to 24°C , 12-h light/12-h dark, and a light intensity of 100 to 120 μmol E m^{-2} s^{-1}. One leaf per 12-d-old seedlings was cut and placed in a 1.5-mL centrifuge tube with 100 μL control buffer (CK buffer) composed of 1 mM KCl, 1 mM $CaCl_2$, and 5 mM MES, pH 5.7. We observed that leaves from precut seedlings had a very weak $[Ca^{2+}]_{cyt}$ response. To ensure consistent results, therefore, care was taken to sample only one leaf per seedling. Under dim light, *coelenterazine hcp* (Promega) was added into the buffer to a final concentration of 1 μM. After 12- to 14-h incubation in the dark, the tube with the leaf was put into a single tube luminometer with a detection limit of 10^{-21} mol luciferase (Turner Biosystem, 20/20) in a room with dim light. The tube was left to stand for 3 to 5 min to allow the leaf to recover from potential disturbances of $[Ca^{2+}]_{cyt}$ triggered by the handling. For the treatment, 100 μL control buffer with 2 times the final concentration of GSH was quickly delivered into the tube by positioning the pipette tip on the internal walls to enable the treatment solution to diffuse into the solution with the leaf. This procedure avoided directly dropping the treatment solution into the solution with the leaf and so aimed to minimize disturbance of the leaf $[Ca^{2+}]_{cyt}$ background.

Luminescence intensity was measured at 1-Hz frequency. At the end of each recording, 500 μL ice-cold 1 M $CaCl_2$ in 20% ethanol (v/v) was delivered into the tube to saturate the Ca^{2+} and aequorin protein-dependent luminescence. The absolute $[Ca^{2+}]_{cyt}$ concentration was calculated based on the method described previously (Rentel and Knight, 2004). To explore the contribution of external Ca^{2+} to the observed $[Ca^{2+}]_{cyt}$ rise, we incubated the leaf in Ca^{2+}-free CK buffer with 1 mM EGTA, a Ca^{2+} chelator. The GSH treatment was also performed in the same buffer. To address effects of AP5, alloxan, neomycin, and 6,7-dinitroquinoxaline-2,3-dione on the $[Ca^{2+}]_{cyt}$ responses, these chemicals were added into the recording buffer at the indicated concentration 1 h before the GSH treatments. All chemicals were purchased from Sigma.

Arabidopsis Seedling Treatment and RNA Extraction for Complementary DNA Microarray

Arabidopsis seedlings were cultured on 0.7% (w/v) agar medium containing one-half-strength Murashige and Skoog basic salt with 1% Suc (w/v) and 5 mM MES, pH 5.7. Ten-day-old seedlings were equilibrated in the CK buffer for 24 h. Then, these seedlings were either treated with the CK buffer or the CK buffer containing100 μM GSH and 100 μM Glu for 1 h, respectively. Total RNA was extracted from each sample using EasyPure Plant RNA kit (Transgene). Further RNA purification, probe preparation, hybridization, and microarray scanning were done at the National GeneChip Engineering Center (http://www.ebioserve.com). Labeled samples were hybridized with one Agilent Arabidopsis 4×44k array by following the manufacturer's instructions. Four biologically independent sets of Arabidopsis seedlings were prepared, two for the microarray experiment and two for real-time qRT-PCR analysis.

The raw data were normalized by quantile algorithm using Gene Spring Software 11.0. The following criteria were used in the National GeneChip Engineering Center for defining significantly differentially expressed genes: (1) genes that are statistically significant at the level of $P < 0.05$ after false

discovery rate correction and labeled "Present" call by the GeneChip Operation System output and (2) genes showing a change in the expression level between treatment and control at least 3-fold in the two replicates. The Gene Ontology annotation analysis was conducted using the online program at http://www.Arabidopsis.org/tools/bulk/go/index.jsp. The raw data of the microarray experiment was deposited at http://www.ebi.ac.uk/miamexpress/ with access number E–MEXP–3885.

qRT-PCR Analysis

Tissue preparation and RNA extraction were as for the microarray analyses. Total RNA was treated with RNAse-free DNAse to remove all DNA contamination (Takara). One microgram of RNA samples were used for complementary DNA synthesis with oligo(dT) primer and PrimeScript II reverse transcriptase (Takara) following a standard protocol. DNA contamination of the complementary DNA was further tested by PCR with a pair of primers spanning an intron. Before the qRT-PCR analysis, all primers were tested for their specificity and primer dimer formation by conducting semiquantitative reverse transcript PCR. qRT-PCR was conducted on a Biorad Chromo 4 real-time PCR system using SYBR Green Premix Ex Taq II kit (Takara). The Arabidopsis *Tubulin2* gene (At5g62690) was used as a constitutive reference to normalize the gene expression. Relative expression to the wild type under CK condition was presented in Figure 6 and Supplemental Figure S1. Primer sequences for the PCR are listed in Supplemental Table S8.

Bacterial Growth Assay

To prepare Arabidopsis plants for the assay, we grew two wild-type and two mutant plants in the same pot to minimize side effects of growth environment difference on the pathogen propagation results. The pots were maintained in a growth chamber with 50 to 70% humidity at 22°C to 24°C, 12-h light/12-h dark, and a light intensity of 100 to 120 μmol E m^{-2} s^{-1}. Four-week-old leaves were used in this study. For each plant, we chose two leaves for syringe-based infiltration. One day before the pathogen inoculation, we infiltrated the leaf with either the CK or CK containing100 μM GSH, GSSG, Ala, Asn, Cys, Gly, Glu, Ser, and Asp.

The bacterial strain used in this study was *Pst* DC3000. The –80°C stock of the pathogen was first streaked onto a low-salt Luria-Bertani plate (tryptone 10 g L^{-1}, yeast [*Saccharomyces cerevisiae*] extract 5 g L^{-1}, and NaCl 5 g L^{-1}) with 50 μg mL^{-1} rifampin. Plates with bacteria were cultured at 28°C for 38 to 42 h, then subcultured in liquid Luria-Bertani solution growing up to an optical density at 600 nm of 0.8 to 1.0. After collection by centrifuging, the pathogen was resuspended in sterile water to an optical density at 600 nm of 0.001, corresponding to 5×10^5 cfu mL^{-1} bacteria, and infiltrated into the leaf with a needless 1-mL syringe. At 0, 1, 2, and 3 DPI, the inoculated leaf was taken and briefly rinsed in 75% ethanol (v/v) and sterile water. A leaf disc with 0.5-cm diameter was sampled from each of four to eight different leaves from separate plants for each treatments of each genotype and ground in sterile water. A standard colony-forming unit dilution assay was conducted (Katagiri et al., 2002). Five independent biological replicates were performed.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. qRT-PCR analysis of GSH-induced AtGLR3.3-dependent gene expression.

Supplemental Figure S2. Root elongation responses of ecotype Columbia and *atglr3.3* to Cd^{2+}.

Supplemental Figure S3. *Pst* DC3000 infection assay of Arabidopsis *pad2-1* mutants.

Supplemental Table S1. Differential expressed gene in the *atglr3.3-2* mutant compared with that in ecotype Columbia.

Supplemental Table S2. Genes modulated by GSH in ecotype Columbia but not *atglr3.3-2* mutant.

Supplemental Table S3. Genes modulated by GSH in ecotype Columbia and *atglr3.3-2* mutant.

Supplemental Table S4. Genes modulated by GSH in *atglr3.3-2* but not ecotype Columbia.

Supplemental Table S5. Genes modulated by Glu in ecotype Columbia but not *atglr3.3-2* mutant.

Supplemental Table S6. Genes modulated by Glu in ecotype Columbia and *atglr3.3-2* mutant.

Supplemental Table S7. Genes modulated by Glu in *atglr3.3-2* but not ecotype Columbia.

Supplemental Table S8. Primers used for the quantitative PCR.

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LITERATURE CITED

Acosta IF, Farmer EE (2010) Jasmonates. The Arabidopsis Book **8:** e0129, doi/10.1199/tab.0129

Ali R, Ma W, Lemtiri-Chlieh F, Tsaltas D, Leng Q, von Bodman S, Berkowitz GA (2007) Death don't have no mercy and neither does calcium: *Arabidopsis* CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. Plant Cell **19:** 1081–1095

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653–657

Aouini A, Hernould M, Ariizumi T, Matsukura C, Ezura H, Asamizu E (2012) Overexpression of the tomato *glutamate receptor-like* genes *SlGLR1.1* and *SlGLR3.5* hinders Ca^{2+} utilization and promotes hypersensitivity to Na^+ and K^+ stresses. Plant Biotechnol **29:** 229–235

Aoyama K, Watabe M, Nakaki T (2008) Regulation of neuronal glutathione synthesis. J Pharmacol Sci **108:** 227–238

Ball L, Accotto GP, Bechtold U, Creissen G, Funck D, Jimenez A, Kular B, Leyland N, Mejia-Carranza J, Reynolds H, et al (2004) Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in *Arabidopsis*. Plant Cell **16:** 2448–2462

Birkenbihl RP, Diezel C, Somssich IE (2012) Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection. Plant Physiol **159:** 266–285

Bohman S, Staal J, Thomma BP, Wang M, Dixelius C (2004) Characterisation of an *Arabidopsis-Leptosphaeria maculans* pathosystem: resistance partially requires camalexin biosynthesis and is independent of salicylic acid, ethylene and jasmonic acid signalling. Plant J **37:** 9–20

Brutus A, Sicilia F, Macone A, Cervone F, De Lorenzo G (2010) A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. Proc Natl Acad Sci USA **107:** 9452–9457

Cairns NG, Pasternak M, Wachter A, Cobbett CS, Meyer AJ (2006) Maturation of Arabidopsis seeds is dependent on glutathione biosynthesis within the embryo. Plant Physiol **141:** 446–455

Chin TY, Chueh SH, Tao PL (2006) *S*-Nitrosoglutathione and glutathione act as NMDA receptor agonists in cultured hippocampal neurons. Acta Pharmacol Sin **27:** 853–860

Chiu J, DeSalle R, Lam HM, Meisel L, Coruzzi G (1999) Molecular evolution of glutamate receptors: a primitive signaling mechanism that existed before plants and animals diverged. Mol Biol Evol **16:** 826–838

Cho D, Kim SA, Murata Y, Lee S, Jae SK, Nam HG, Kwak JM (2009) Deregulated expression of the plant glutamate receptor homolog *AtGLR3.1* impairs long-term Ca^{2+}-programmed stomatal closure. Plant J **58:** 437–449

Cobbett CS, May MJ, Howden R, Rolls B (1998) The glutathione-deficient, cadmium-sensitive mutant, *cad2-1*, of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. Plant J **16:** 73–78

De Lorenzo G, Brutus A, Savatin DV, Sicilia F, Cervone F (2011) Engineering plant resistance by constructing chimeric receptors that recognize damage-associated molecular patterns (DAMPs). FEBS Lett **585:** 1521–1528

Dodd AN, Kudla J, Sanders D, Merchant S, Briggs W, Ort D (2010) The language of calcium signaling. Annu Rev Plant Biol **61:** 593–620

Dringen R (2000) Metabolism and functions of glutathione in brain. Prog Neurobiol **62:** 649–671

Dubos C, Huggins D, Grant GH, Knight MR, Campbell MM (2003) A role for glycine in the gating of plant NMDA-like receptors. Plant J **35:** 800–810

Dubreuil-Maurizi C, Poinssot B (2012) Role of glutathione in plant signaling under biotic stress. Plant Signal Behav **7:** 210–212

Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM (2003) *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2*, but not *SID2*, *EDS5* or *PAD4*. Plant J **35:** 193–205

Flores-Soto ME, Chaparro-Huerta V, Escoto-Delgadillo M, Vazquez-Valls E, González-Castañeda RE, Beas-Zarate C (2012) Structure and function of NMDA-type glutamate receptor subunits. Neurologia **27:** 301–310

Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol **155:** 2–18

Franco R, Cidlowski JA (2009) Apoptosis and glutathione: beyond an antioxidant. Cell Death Differ **16:** 1303–1314

Glazebrook J, Ausubel FM (1994) Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc Natl Acad Sci USA **91:** 8955–8959

Gomez LD, Noctor G, Knight MR, Foyer CH (2004) Regulation of calcium signalling and gene expression by glutathione. J Exp Bot **55:** 1851–1859

Hammond CL, Madejczyk MS, Ballatori N (2004) Activation of plasma membrane reduced glutathione transport in death receptor apoptosis of HepG2 cells. Toxicol Appl Pharmacol **195:** 12–22

Han Y, Chaouch S, Mhamdi A, Queval G, Zechmann B, Noctor G (2013a) Functional analysis of *Arabidopsis* mutants points to novel roles for glutathione in coupling H_2O_2 to activation of salicylic acid accumulation and signaling. Antioxid Redox Signal **18:** 2106–2121

Han Y, Mhamdi A, Chaouch S, Noctor G (2013b) Regulation of basal and oxidative stress-triggered jasmonic acid-related gene expression by glutathione. Plant Cell Environ **36:** 1135–1146

He ZH, Cheeseman I, He D, Kohorn BD (1999) A cluster of five cell wall-associated receptor kinase genes, *Wak1-5*, are expressed in specific organs of *Arabidopsis*. Plant Mol Biol **39:** 1189–1196

Hell R, Wirtz M (2011) Molecular biology, biochemistry and cellular physiology of cysteine metabolism. The Arabidopsis Book **9:** e0154, doi/10.1199/tab.0154

Hnasko TS, Edwards RH (2012) Neurotransmitter corelease: mechanism and physiological role. Annu Rev Physiol **74:** 225–243

Hu Y, Dong Q, Yu D (2012) *Arabidopsis* WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen *Pseudomonas syringae*. Plant Sci **185-186:** 288–297

Janáky R, Dohovics R, Saransaari P, Oja SS (2007) Modulation of [^{3}H] dopamine release by glutathione in mouse striatal slices. Neurochem Res **32:** 1357–1364

Janáky R, Shaw CA, Oja SS, Saransaari P (2008) Taurine release in developing mouse hippocampus is modulated by glutathione and glutathione derivatives. Amino Acids **34:** 75–80

Janáky R, Shaw CA, Varga V, Hermann A, Dohovics R, Saransaari P, Oja SS (2000) Specific glutathione binding sites in pig cerebral cortical synaptic membranes. Neuroscience **95:** 617–624

Janáky R, Varga V, Saransaari P, Oja SS (1993) Glutathione modulates the *N*-methyl-D-aspartate receptor-activated calcium influx into cultured rat cerebellar granule cells. Neurosci Lett **156:** 153–157

Jozefczak M, Remans T, Vangronsveld J, Cuypers A (2012) Glutathione is a key player in metal-induced oxidative stress defenses. Int J Mol Sci **13:** 3145–3175

Juurlink BH (1999) Management of oxidative stress in the CNS: the many roles of glutathione. Neurotox Res **1:** 119–140

Kang J, Mehta S, Turano FJ (2004) The putative glutamate *receptor 1.1* (*AtGLR1.1*) in *Arabidopsis thaliana* regulates abscisic acid biosynthesis and signaling to control development and water loss. Plant Cell Physiol **45:** 1380–1389

Kang J, Turano FJ (2003) The putative glutamate *receptor 1.1* (*AtGLR1.1*) functions as a regulator of carbon and nitrogen metabolism in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **100:** 6872–6877

Kang S, Kim HB, Lee H, Choi JY, Heu S, Oh CJ, Kwon SI, An CS (2006) Overexpression in *Arabidopsis* of a plasma membrane-targeting glutamate receptor from small radish increases glutamate-mediated Ca^{2+} influx and delays fungal infection. Mol Cells **21:** 418–427

Katagiri F, Thilmony R, He SY (2002) The Arabidopsis thaliana-Pseudomonas syringae interaction. The Arabidopsis Book **1:** 0039, doi/10.1199/tab.0039

Kim SA, Kwak JM, Jae SK, Wang MH, Nam HG (2001) Overexpression of the *AtGluR2* gene encoding an *Arabidopsis* homolog of mammalian glutamate receptors impairs calcium utilization and sensitivity to ionic stress in transgenic plants. Plant Cell Physiol **42:** 74–84

Kirma M, Araújo WL, Fernie AR, Galili G (2012) The multifaceted role of aspartate-family amino acids in plant metabolism. J Exp Bot **63:** 4995–5001

Knight MR, Campbell AK, Smith SM, Trewavas AJ (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature **352:** 524–526

Koh S, Wiles AM, Sharp JS, Naider FR, Becker JM, Stacey G (2002) An oligopeptide transporter gene family in Arabidopsis. Plant Physiol **128:** 21–29

Kudla J, Batistic O, Hashimoto K (2010) Calcium signals: the lead currency of plant information processing. Plant Cell **22:** 541–563

Kwaaitaal M, Huisman R, Maintz J, Reinstädler A, Panstruga R (2011) Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP-induced calcium influx in *Arabidopsis thaliana*. Biochem J **440:** 355–365

Kwaaitaal M, Maintz J, Cavdar M, Panstruga R (2012) On the ligand binding profile and desensitization of plant ionotropic glutamate receptor (iGluR)-like channels functioning in MAMP-triggered Ca^{2+} influx. Plant Signal Behav **7:** 1373–1377

Lacombe B, Becker D, Hedrich R, DeSalle R, Hollmann M, Kwak JM, Schroeder JI, Le Novère N, Nam HG, Spalding EP, et al (2001) The identity of plant glutamate receptors. Science **292:** 1486–1487

Lam HM, Chiu J, Hsieh MH, Meisel L, Oliveira IC, Shin M, Coruzzi G (1998) Glutamate-receptor genes in plants. Nature **396:** 125–126

Leon J, Lawton MA, Raskin I (1995) Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. Plant Physiol **108:** 1673–1678

Leslie SW, Brown LM, Trent RD, Lee YH, Morris JL, Jones TW, Randall PK, Lau SS, Monks TJ (1992) Stimulation of *N*-methyl-D-aspartate receptor-mediated calcium entry into dissociated neurons by reduced and oxidized glutathione. Mol Pharmacol **41:** 308–314

Levy DI, Sucher NJ, Lipton SA (1991) Glutathione prevents *N*-methyl-D-aspartate receptor-mediated neurotoxicity. Neuroreport **2:** 345–347

Li J, Zhu S, Song X, Shen Y, Chen H, Yu J, Yi K, Liu Y, Karplus VJ, Wu P, et al (2006) A rice glutamate receptor-like gene is critical for the division and survival of individual cells in the root apical meristem. Plant Cell **18:** 340–349

Libault M, Wan J, Czechowski T, Udvardi M, Stacey G (2007) Identification of 118 *Arabidopsis* transcription factor and 30 ubiquitin-ligase genes responding to chitin, a plant-defense elicitor. Mol Plant Microbe Interact **20:** 900–911

Little D, Gouhier-Darimont C, Bruessow F, Reymond P (2007) Oviposition by pierid butterflies triggers defense responses in Arabidopsis. Plant Physiol **143:** 784–800

Loane DJ, Stoica BA, Faden AI (2012) Metabotropic glutamate receptor-mediated signaling in neuroglia. Wiley Interdiscip Rev Membr Transp Signal **1:** 136–150

Ma W, Qi Z, Smigel A, Walker RK, Verma R, Berkowitz GA (2009) Ca^{2+}, cAMP, and transduction of non-self perception during plant immune responses. Proc Natl Acad Sci USA **106:** 20995–21000

Maekawa S, Sato T, Asada Y, Yasuda S, Yoshida M, Chiba Y, Yamaguchi J (2012) The Arabidopsis ubiquitin ligases ATL31 and ATL6 control the defense response as well as the carbon/nitrogen response. Plant Mol Biol **79:** 217–227

McGrath KC, Dombrecht B, Manners JM, Schenk PM, Edgar CI, Maclean DJ, Scheible WR, Udvardi MK, Kazan K (2005) Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. Plant Physiol **139:** 949–959

Meyer AJ (2008) The integration of glutathione homeostasis and redox signaling. J Plant Physiol **165:** 1390–1403

Mhamdi A, Hager J, Chaouch S, Queval G, Han Y, Taconnat L, Saindrenan P, Gouia H, Issakidis-Bourguet E, Renou JP, et al (2010) Arabidopsis GLUTATHIONE REDUCTASE1 plays a crucial role in leaf responses to intracellular hydrogen peroxide and in ensuring appropriate gene expression through both salicylic acid and jasmonic acid signaling pathways. Plant Physiol **153:** 1144–1160

Michard E, Lima PT, Borges F, Silva AC, Portes MT, Carvalho JE, Gilliham M, Liu LH, Obermeyer G, Feijó JA (2011) Glutamate

receptor-like genes form Ca^{2+} channels in pollen tubes and are regulated by pistil D-serine. Science **332:** 434–437

Miller ND, Durham Brooks TL, Assadi AH, Spalding EP (2010) Detection of a gravitropism phenotype in *glutamate receptor-like 3.3* mutants of *Arabidopsis thaliana* using machine vision and computation. Genetics **186:** 585–593

Mishina TE, Zeier J (2006) The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. Plant Physiol **141:** 1666–1675

Moreau M, Degrave A, Vedel R, Bitton F, Patrit O, Renou JP, Barny MA, Fagard M (2012) EDS1 contributes to nonhost resistance of *Arabidopsis thaliana* against *Erwinia amylovora*. Mol Plant Microbe Interact **25:** 421–430

Morito N, Yoh K, Itoh K, Hirayama A, Koyama A, Yamamoto M, Takahashi S (2003) Nrf2 regulates the sensitivity of death receptor signals by affecting intracellular glutathione levels. Oncogene **22:** 9275–9281

Navarro L, Zipfel C, Rowland O, Keller I, Robatzek S, Boller T, Jones JD (2004) The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. Plant Physiol **135:** 1113–1128

Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Garcia B, Queval G, Foyer CH (2012) Glutathione in plants: an integrated overview. Plant Cell Environ **35:** 454–484

Noctor G, Queval G, Mhamdi A, Chaouch S, Foyer CH (2011) Glutathione. The Arabidopsis Book **9:** e0142, doi/10.1199/tab.0142

Ogita K, Enomoto R, Nakahara F, Ishitsubo N, Yoneda Y (1995) A possible role of glutathione as an endogenous agonist at the *N*-methyl-D-aspartate recognition domain in rat brain. J Neurochem **64:** 1088–1096

Oja SS, Janáky R, Varga V, Saransaari P (2000) Modulation of glutamate receptor functions by glutathione. Neurochem Int **37:** 299–306

Okrent RA, Brooks MD, Wildermuth MC (2009) *Arabidopsis* GH3.12 (PBS3) conjugates amino acids to 4-substituted benzoates and is inhibited by salicylate. J Biol Chem **284:** 9742–9754

Parisy V, Poinssot B, Owsianowski L, Buchala A, Glazebrook J, Mauch F (2007) Identification of *PAD2* as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of Arabidopsis. Plant J **49:** 159–172

Pasternak M, Lim B, Wirtz M, Hell R, Cobbett CS, Meyer AJ (2008) Restricting glutathione biosynthesis to the cytosol is sufficient for normal plant development. Plant J **53:** 999–1012

Popescu GK (2012) Modes of glutamate receptor gating. J Physiol **590:** 73–91

Po-Wen C, Singh P, Zimmerli L (2013) Priming of the *Arabidopsis* pattern-triggered immunity response upon infection by necrotrophic *Pectobacterium carotovorum* bacteria. Mol Plant Pathol **14:** 58–70

Qi Z, Stephens NR, Spalding EP (2006) Calcium entry mediated by GLR3.3, an Arabidopsis glutamate receptor with a broad agonist profile. Plant Physiol **142:** 963–971

Rasul S, Dubreuil-Maurizi C, Lamotte O, Koen E, Poinssot B, Alcaraz G, Wendehenne D, Jeandroz S (2012) Nitric oxide production mediates oligogalacturonide-triggered immunity and resistance to *Botrytis cinerea* in *Arabidopsis thaliana*. Plant Cell Environ **35:** 1483–1499

Rentel MC, Knight MR (2004) Oxidative stress-induced calcium signaling in Arabidopsis. Plant Physiol **135:** 1471–1479

Reuber TL, Ausubel FM (1996) Isolation of *Arabidopsis* genes that differentiate between resistance responses mediated by the *RPS2* and *RPM1* disease resistance genes. Plant Cell **8:** 241–249

Roetschi A, Si-Ammour A, Belbahri L, Mauch F, Mauch-Mani B (2001) Characterization of an *Arabidopsis-Phytophthora* pathosystem: resistance requires a functional PAD2 gene and is independent of salicylic acid, ethylene and jasmonic acid signalling. Plant J **28:** 293–305

Saga H, Ogawa T, Kai K, Suzuki H, Ogata Y, Sakurai N, Shibata D, Ohta D (2012) Identification and characterization of *ANAC042*, a transcription factor family gene involved in the regulation of camalexin biosynthesis in *Arabidopsis*. Mol Plant Microbe Interact **25:** 684–696

Schlaeppi K, Bodenhausen N, Buchala A, Mauch F, Reymond P (2008) The glutathione-deficient mutant *pad2-1* accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore *Spodoptera littoralis*. Plant J **55:** 774–786

Schmidt MM, Dringen R (2012) GSH synthesis and metabolism. *In* **Gruetter R, Choi I**, eds, Advance in Neurobiology, **Vol 4**. Springer, New York, pp 1029–1050.

Shanmugam V, Tsednee M, Yeh KC (2012) *ZINC TOLERANCE INDUCED BY IRON 1* reveals the importance of glutathione in the cross-homeostasis between zinc and iron in *Arabidopsis thaliana*. Plant J **69:** 1006–1017

Shaw C, Janaky R, Ogita K (2001) Glutathione-mediated signal transduction in mammalian CNS. J Neurochem **78:** 120

Simková K, Moreau F, Pawlak P, Vriet C, Baruah A, Alexandre C, Hennig L, Apel K, Laloi C (2012) Integration of stress-related and reactive oxygen species-mediated signals by Topoisomerase VI in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **109:** 16360–16365

Slivka A, Cohen G (1993) Brain ischemia markedly elevates levels of the neurotoxic amino acid, cysteine. Brain Res **608:** 33–37

Srivastava MK, Dwivedi UN (1998) Salicylic acid modulates glutathione metabolism in pea seedlings. J Plant Physiol **153:** 409–414

Stephens NR, Qi Z, Spalding EP (2008) Glutamate receptor subtypes evidenced by differences in desensitization and dependence on the *GLR3.3* and *GLR3.4* genes. Plant Physiol **146:** 529–538

Tang RH, Han S, Zheng H, Cook CW, Choi CS, Woerner TE, Jackson RB, Pei ZM (2007) Coupling diurnal cytosolic Ca^{2+} oscillations to the CAS-IP_3 pathway in *Arabidopsis*. Science **315:** 1423–1426

Turano FJ, Panta GR, Allard MW, van Berkum P (2001) The putative glutamate receptors from plants are related to two superfamilies of animal neurotransmitter receptors via distinct evolutionary mechanisms. Mol Biol Evol **18:** 1417–1420

van Wees SC, Chang HS, Zhu T, Glazebrook J (2003) Characterization of the early response of Arabidopsis to *Alternaria brassicicola* infection using expression profiling. Plant Physiol **132:** 606–617

Vanacker H, Carver TL, Foyer CH (2000) Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hypersensitive response in the barley-powdery mildew interaction. Plant Physiol **123:** 1289–1300

Varet A, Parker J, Tornero P, Nass N, Nürnberger T, Dangl JL, Scheel D, Lee J (2002) *NHL25* and *NHL3*, two *NDR1/HIN1-1ike* genes in *Arabidopsis thaliana* with potential role(s) in plant defense. Mol Plant Microbe Interact **15:** 608–616

Varga V, Jenei Z, Janáky R, Saransaari P, Oja SS (1997) Glutathione is an endogenous ligand of rat brain *N*-methyl-D-aspartate (NMDA) and 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. Neurochem Res **22:** 1165–1171

Vincill ED, Bieck AM, Spalding EP (2012) Ca^{2+} conduction by an amino acid-gated ion channel related to glutamate receptors. Plant Physiol **159:** 40–46

Wang L, Tsuda K, Sato M, Cohen JD, Katagiri F, Glazebrook J (2009) *Arabidopsis* CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. PLoS Pathog **5:** e1000301

Wang M, Yao Y, Kuang D, Hampson DR (2006) Activation of family C G-protein-coupled receptors by the tripeptide glutathione. J Biol Chem **281:** 8864–8870

Wheeler DG, Groth RD, Ma H, Barrett CF, Owen SF, Safa P, Tsien RW (2012) Ca_V1 and Ca_V2 channels engage distinct modes of Ca^{2+} signaling to control CREB-dependent gene expression. Cell **149:** 1112–1124

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414:** 562–565

Yoshida S, Tamaoki M, Ioki M, Ogawa D, Sato Y, Aono M, Kubo A, Saji S, Saji H, Satoh S, et al (2009) Ethylene and salicylic acid control glutathione biosynthesis in ozone-exposed *Arabidopsis thaliana*. Physiol Plant **136:** 284–298

Zable AC, Favero TG, Abramson JJ (1997) Glutathione modulates ryanodine receptor from skeletal muscle sarcoplasmic reticulum. Evidence for redox regulation of the Ca^{2+} release mechanism. J Biol Chem **272:** 7069–7077

Zhang Y, Xu S, Ding P, Wang D, Cheng YT, He J, Gao M, Xu F, Li Y, Zhu Z, et al (2010) Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. Proc Natl Acad Sci USA **107:** 18220–18225