Arabidopsis Glutamate Receptor Homolog3.5 Modulates Cytosolic Ca^{2+} Level to Counteract Effect of Abscisic Acid in Seed Germination[1[OPEN]]

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Seed germination is a critical step in a plant's life cycle that allows successful propagation and is therefore strictly controlled by endogenous and environmental signals. However, the molecular mechanisms underlying germination control remain elusive. Here, we report that the Arabidopsis (*Arabidopsis thaliana*) glutamate receptor homolog3.5 (*AtGLR3.5*) is predominantly expressed in germinating seeds and increases cytosolic Ca^{2+} concentration that counteracts the effect of abscisic acid (ABA) to promote germination. Repression of *AtGLR3.5* impairs cytosolic Ca^{2+} concentration elevation, significantly delays germination, and enhances ABA sensitivity in seeds, whereas overexpression of *AtGLR3.5* results in earlier germination and reduced seed sensitivity to ABA. Furthermore, we show that Ca^{2+} suppresses the expression of *ABSCISIC ACID INSENSITIVE4* (*ABI4*), a key transcription factor involved in ABA response in seeds, and that ABI4 plays a fundamental role in modulation of Ca^{2+}-dependent germination. Taken together, our results provide molecular genetic evidence that AtGLR3.5-mediated Ca^{2+} influx stimulates seed germination by antagonizing the inhibitory effects of ABA through suppression of ABI4. These findings establish, to our knowledge, a new and pivotal role of the plant glutamate receptor homolog and Ca^{2+} signaling in germination control and uncover the orchestrated modulation of the AtGLR3.5-mediated Ca^{2+} signal and ABA signaling via ABI4 to fine-tune the crucial developmental process, germination, in Arabidopsis.

Germination converts a seed from a quiescent embryonic state to a highly active phase leading to seedling establishment, which is considered to be the most critical step in the life cycle of seed plants and represents the entry of the plant into the ecosystem (Weitbrecht et al., 2011; Rajjou et al., 2012). Germination begins with water uptake by dry nondormant seeds (i.e. imbibition) and terminates with two visible sequential events: testa (seed coat) rupture due to the expansion of the embryo and endosperm and radicle (embryonic root) protrusion through the endosperm (Nonogaki et al., 2010; Weitbrecht et al., 2011). This progression is controlled by various complex intrinsic signals, such as phytohormones, and environmental factors that include water, temperature, and light (Weitbrecht et al., 2011; Rajjou et al., 2012).

Plant hormones play crucial roles in germination regulation, among which abscisic acid (ABA) has been best investigated (Schopfer and Plachy, 1985; Finkelstein et al., 2002). ABA exerts its influence in germination by suppressing water uptake by the seed to reduce embryo growth potential at early stages (Schopfer and Plachy, 1985) and by inhibiting endosperm rupture to delay germination (Müller et al., 2006). Genetic and molecular studies seeking mutants with altered ABA sensitivity have revealed that transcriptional regulation is important in ABA responses in seeds (Finkelstein et al., 2002; Kucera et al., 2005; Holdsworth et al., 2008), which is mediated chiefly by three seed-expressed *ABSCISIC ACID-insensitive* (*ABI*) transcription factors, *ABI3*, *ABI4*, and *ABI5* (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Penfield et al., 2006). *ABI3* is essential for seed maturation and desiccation tolerance during embryogenesis (Nambara et al., 1995), and both *ABI4* and *ABI5* act in the ABA inhibition of seed germination (Finkelstein, 1994; Lopez-Molina et al., 2001; Penfield et al., 2006). In contrast to the negative regulation by ABA, another plant hormone GA promotes germination (Ogawa et al., 2003) through weakening the mechanical restraint of tissues that surround the embryo and through increasing the embryo growth potential (Ogawa et al., 2003). During the germination process, the endogenous ABA content of seeds declines, while bioactive GA levels accumulate prior to radicle

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emergence (Ogawa et al., 2003; Weitbrecht et al., 2011). This GA-to-ABA ratio in the seeds is the primary control of germination vigor (Kucera et al., 2005; Holdsworth et al., 2008; Rajjou et al., 2012). Emerging evidence shows that other signals are also involved in seed germination. For example, reactive oxygen species act as second messengers required for germination (Bailly et al., 2008) and interact with plant hormones to coordinate germination (Müller et al., 2009). Overall, germination control, a sophisticated process, is determined by concerted actions among various endogenous signals including some yet to be discovered.

Not only is calcium (Ca^{2+}) an indispensable nutrient for plants (White and Broadley, 2003), it also acts as a versatile second messenger through changes in free cytosolic Ca^{2+} level ($[Ca^{2+}]_{cyt}$) to participate in numerous developmental and adaption processes in plants, such as root hair elongation, pollen tube growth, stomatal movements, plant-microbe interactions, light signaling, and hormone responses (McAinsh and Pittman, 2009; Dodd et al., 2010; Kudla et al., 2010). The $[Ca^{2+}]_{cyt}$ elevations, relative to their low levels in the resting conditions (McAinsh and Pittman, 2009), are caused by extracellular Ca^{2+} influx or internal Ca^{2+} release from the stores, both of which may encode specific cellular Ca^{2+} signatures to further initiate downstream gene expressions and biological events (McAinsh and Pittman, 2009; Dodd et al., 2010; Kudla et al., 2010). The spatiotemporal changes of $[Ca^{2+}]_{cyt}$ in living cells are accurately controlled and sensed by an efficient Ca^{2+} signaling regulatory network, among which Ca^{2+} channels play a large role (Dodd et al., 2010; Kudla et al., 2010). In plants, several gene families are thought to encode Ca^{2+}-permeable channels (Ward et al., 2009; Hedrich, 2012), among them Arabidopsis (*Arabidopsis thaliana*) glutamate receptor homologs (AtGLRs) share high structural similarity to ionotropic Glu receptor Ca^{2+} channels in animals (Lam et al., 1998), and consist of 20 gene family members (Lacombe et al., 2001). Studies have shown that these plant GLRs participate in diverse physiological and developmental procedures, including pollen tube morphogenesis (Michard et al., 2011), root development (Kang et al., 2004; Li et al., 2006; Vincill et al., 2013), stomatal closure regulation (Cho et al., 2009), and various stress responses (Kim et al., 2001; Meyerhoff et al., 2005; Li et al., 2013; Mousavi et al., 2013), which are mostly dependent on Ca^{2+}. Despite the importance of AtGLR-related Ca^{2+} modulation in these processes, the function and molecular mechanism of Ca^{2+} signaling and AtGLRs in seed germination, the early plant developmental event that greatly impacts the plant's entire life and agricultural performance (Rajjou et al., 2012), is largely unknown.

Physiological studies have shown that Ca^{2+} plays a protective role in salt stress responses (Liu and Zhu, 1997; Bonilla et al., 2004) and that more Ca^{2+} can alleviate the effects of salinity toxicity on seed germination in agricultural crops (Bonilla et al., 2004). However, the modulation of germination by Ca^{2+} and the underlying molecular mechanism remain elusive. In this study, we investigated and characterized the importance of the cytosolic Ca^{2+} signal, mediated by Arabidopsis glutamate receptor homolog3.5 (*AtGLR3.5*), in germination. AtGLR3.5 regulates $[Ca^{2+}]_{cyt}$, its expression levels correlate with the onset and completion of the germination process, and it is required for normal germination. Furthermore, we show that the AtGLR3.5-mediated Ca^{2+} signal antagonizes the effect of ABA in seeds through inhibiting the expression of *ABI4* and that ABI4 plays a fundamental role in Ca^{2+} responses in germination. Our results demonstrate and uncover the mechanism by which AtGLR3.5 regulates Ca^{2+} signaling in germination, thus providing a molecular explanation for the observation that Ca^{2+} alleviates decreasing in germination caused by stress. Therefore, the results highlight the significance of Ca^{2+} in this developmental process, particularly under stress conditions.

RESULTS AND DISCUSSION

Extracellular Calcium Enhances Seed Germination

To gain insight into the role of Ca^{2+} in germination, we investigated whether and how Ca^{2+} modulates seed germination in Arabidopsis. As shown in Figure 1A, the application of $CaCl_2$-stimulated germination (enhanced radicle emergence and subsequent root growth) compared with the control, whereas chelating Ca^{2+} using EGTA severely inhibited these processes. External Ca^{2+} at physiological levels promoted germination in a dose-dependent manner (Fig. 1B; $P < 0.01$ at 0.1 mM, $P < 0.001$ at 1 and 5 mM), although high concentrations of external Ca^{2+} (20–80 mM) delayed germination (Supplemental Fig. S1, A–C), presumably due to a cytotoxic effect of too much Ca^{2+} (White and Broadley, 2003). Moreover, EGTA drastically reduced germination, to as low as 30% of the control at 10 mM (Fig. 1C; $P < 0.001$ at 5 and 10 mM). These data show that adequate external Ca^{2+} is required for seeds to germinate, which points to an important role of Ca^{2+} in the earliest phase of the plant life cycle.

Next, we carried out a time course analysis of seed germination at 0 and 5 mM external Ca^{2+} concentration and found a rapid increase in the germination between 30 and 40 h after the transfer of stratified seeds to a growth chamber, with 36 h being approximately one-half of the maximum germination (Fig. 1D). This observation is comparable with previous reports showing that germination is completed between 31 and 45 h after sowing (Dekkers et al., 2013) or 36 h after imbibition under optimal conditions (Holdsworth et al., 2008; Piskurewicz et al., 2008). This supports the appropriateness of our experimental conditions and reliability of our analysis. The rapid germination increase was dramatically diminished by the removal of the Ca^{2+} using EGTA (Fig. 1D), indicating that Ca^{2+} is required for this developmental process. It is noteworthy that at approximately 60 h, when most seeds had germinated even without a Ca^{2+} supply, EGTA continued to inhibit germination (Fig. 1D). As the concentration of EGTA used in the experiment was

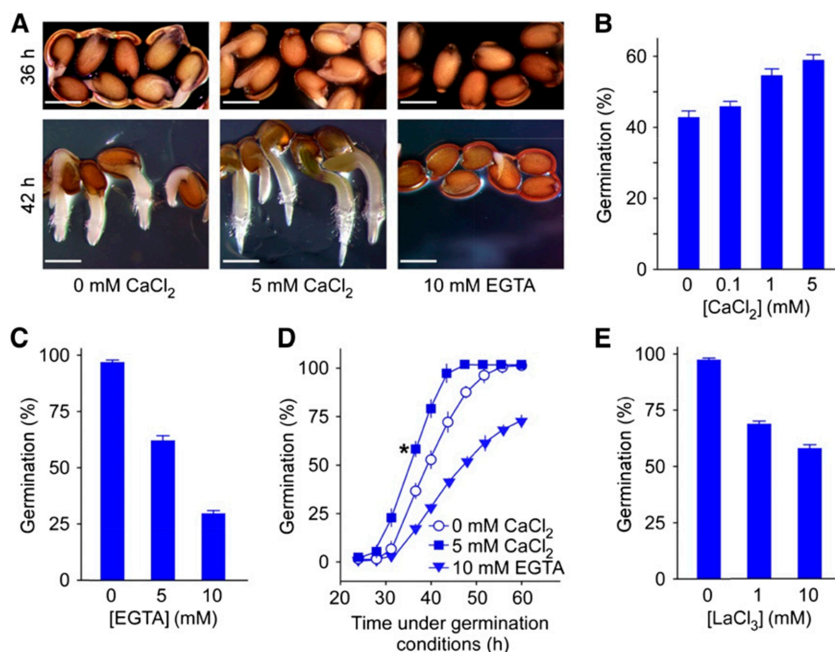

Figure 1. Seed germination is enhanced by external calcium. A, Effect of calcium on germination. Arabidopsis wild-type seeds sown on modified MS medium containing 0 or 5 mM $CaCl_2$ or 0 mM $CaCl_2$ supplemented with 10 mM EGTA were incubated at 4°C (in the dark) for 3 d and transferred to the growth chamber (22°C) for 36 (top) or 42 h (bottom) prior to analysis. Three biological replicates were performed, and representative images are shown. B, Quantification of germination percentage at various concentrations of external Ca^{2+}. Seeds were sown on modified MS medium containing indicated amounts of $CaCl_2$ and scored for germination 36 h after incubation under the germination conditions. C, Effect of EGTA on germination. Seeds were sown on modified MS medium containing no $CaCl_2$ supplemented with 5 or 10 mM EGTA and scored for germination 42 h after incubation under the germination conditions. D, Time course quantification of germination at various amounts of external Ca^{2+}. Seeds were incubated on the same medium as in A at 22°C for 24 to 60 h and scored for germination. Asterisk indicates the germination percentage at 36 h after incubation of the seeds under the germination conditions. E, Effect of Ca^{2+} channel blocker $LaCl_3$ on germination. Seeds were incubated on MS medium supplemented with indicated concentrations of $LaCl_3$ and scored for germination 42 h after incubation under the germination conditions. For the germination analysis in B to E, data from three independent replicates are shown; error bars indicate SE of the mean. Bar = 0.5 mm.

high enough to chelate all the Ca^{2+} ions in the media, it appears likely that Ca^{2+}, other than that in the medium, also contributes to germination. Because high amounts of Ca^{2+} exist in the seed coat (Punshon et al., 2012), cell wall, and apoplast (Clarkson, 1984; Hepler and Wayne, 1985) and Ca^{2+}-permeable channels function in Ca^{2+} influx to the cytoplasm (Dodd et al., 2010; Kudla et al., 2010), we hypothesized that extracellular Ca^{2+} enters the cytosol through plasma membrane Ca^{2+}-permeable channels to positively regulate germination. Our hypothesis was supported by pharmacological assays that showed $LaCl_3$, a commonly used external Ca^{2+} channel blocker (Knight et al., 1996), significantly reduced germination (Fig. 1E; $P < 0.001$ at 1 and 10 mM). Together, these results suggest that extracellular Ca^{2+} influx by Ca^{2+}-permeable channels in the plasma membrane plays a large role in germination.

AtGLR3.5 Mediates $[Ca^{2+}]_{cyt}$ Elevation and Is Required for Seed Germination

Because our results indicate the involvement of Ca^{2+} channels in germination (Fig. 1), we searched publicly available gene expression databases for candidate Arabidopsis GLR Ca^{2+} channels that modulate seed germination. Using the eFP Browser that presents gene expression data throughout Arabidopsis development (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al., 2007), we found that among the 20 members of the AtGLR family, *AtGLR3.5* (At2g32390) and *AtGLR3.7* (At2g32400) show clear transcriptional increases during the transition from dry seeds to imbibed seeds. We focused on *AtGLR3.5* in this study because its expression increases much more markedly during the germination transition than *AtGLR3.7*, and *AtGLR3.5* is most highly expressed in imbibed seeds but much less in dry seeds and vegetative tissues compared with the ubiquitous, high expression of *AtGLR3.7* in various tissues. *AtGLR3.7* knockout seeds showed a weak germination phenotype (Supplemental Fig. S2; $P < 0.05$).

To verify the connection of *AtGLR3.5* with germination, we performed quantitative reverse transcription (qRT)-PCR analysis that showed clear up-regulation of *AtGLR3.5* mRNA transcript in 24-h-imbibed seeds (Supplemental Fig. S3A). Then, to obtain a more detailed understanding of the *AtGLR3.5* expression pattern, we analyzed *AtGLR3.5* transcript levels during the entire

germination process. This revealed an immediate steep increase in the transcript level at the outset and a gradual decrease upon completion of germination, peaking at 24 h (approximately 12-fold; Fig. 2A). These data show that *AtGLR3.5* is predominantly expressed in germinating seeds but not in dry or fully germinated seeds. To determine the site of *AtGLR3.5* expression in germinating seeds, we created transgenic plants expressing the *GUS* reporter gene under a 2.1-kb promoter sequence of *AtGLR3.5* and found that *AtGLR3.5* was mainly expressed in embryonic cotyledons (Fig. 2B). At the seedling stage, *AtGLR3.5* transcript was detected in both roots and shoots (Supplemental Fig. S3B). Compared with no detectable GUS signal at 0 h, *AtGLR3.5* expression was highly up-regulated at 24 h and then decreased to a lower level at 48 h after the onset of germination (Fig. 2B), consistent with the qRT-PCR results (Fig. 2A). Intriguingly, the dynamic changes (increase and decrease) in *AtGLR3.5* expression level in seeds (Fig. 2A) correlate with the time points of the onset of the rapid germination (30–40 h; Fig. 1D) and the completion of germination (60 h; Fig. 1D). The spatiotemporal expression pattern of *AtGLR3.5* strongly implies a possible role for the gene in this critical developmental process.

To assess the function of AtGLR3.5 in germination, we produced transgenic plants with altered *AtGLR3.5* expressions and tested their germination phenotypes. A *35S*::*AtGLR3.5* RNA interference (RNAi) construct and a *35S*::*AtGLR3.5* overexpression (OE) construct were introduced into wild-type Arabidopsis, and a large reduction or increase in the *AtGLR3.5* transcript level was found in the resulting *AtGLR3.5* RNAi or *AtGLR3.5* OE lines, respectively (Supplemental Fig. S4, A and B). Significantly, the transcript levels of *AtGLR3.4*, the closest homolog to *AtGLR3.5* (Chiu et al., 2002), and *AtGLR3.1* were unaffected in the two *AtGLR3.5* RNAi lines (Supplemental Fig. S5). The *AtGLR3.5* RNAi seeds and *AtGLR3.5* OE seeds displayed opposing patterns in germination in Murashige and Skoog (MS) media, with the former germinating more slowly and the latter faster than the wild type (Fig. 2, C and D; $P < 0.01$), suggesting that AtGLR3.5 has an essential positive effect on germination.

We next checked the connection of AtGLR3.5 with calcium in seed germination. It was found that the *AtGLR3.5* RNAi seeds were much less sensitive to $LaCl_3$ than the wild type (Fig. 3A), implying the Ca^{2+} channel function of AtGLR3.5 in germination. As external Ca^{2+} concentration increases, wild-type germination increased, an effect that was largely abolished in the *AtGLR3.5* RNAi seeds (Fig. 3B; $P < 0.001$ at all Ca^{2+} concentrations examined), indicating the importance of AtGLR3.5 in mediating Ca^{2+} influx in germination. External Ca^{2+} treatment had no clear effect on the expression of *AtGLR3.5* gene under the germination conditions (Supplemental Fig. S6), suggesting a posttranscriptional and/or posttranslational regulation of AtGLR3.5 by Ca^{2+}. These results together indicate that the essential role of AtGLR3.5 in germination is presumably achieved by mediating Ca^{2+} influx.

We further examined whether AtGLR3.5 directly modulates $[Ca^{2+}]_{cyt}$ by using calcium indicator proteins aequorin (Knight et al., 1991) and yellow cameleon3.60 (YC3.60; Rincón-Zachary et al., 2010). Aequorin luminescence-based Ca^{2+} imaging analyses showed that knockdown of *AtGLR3.5* lowered the steady-state $[Ca^{2+}]_{cyt}$ in young seedlings (Fig. 3, C and D). Importantly, the

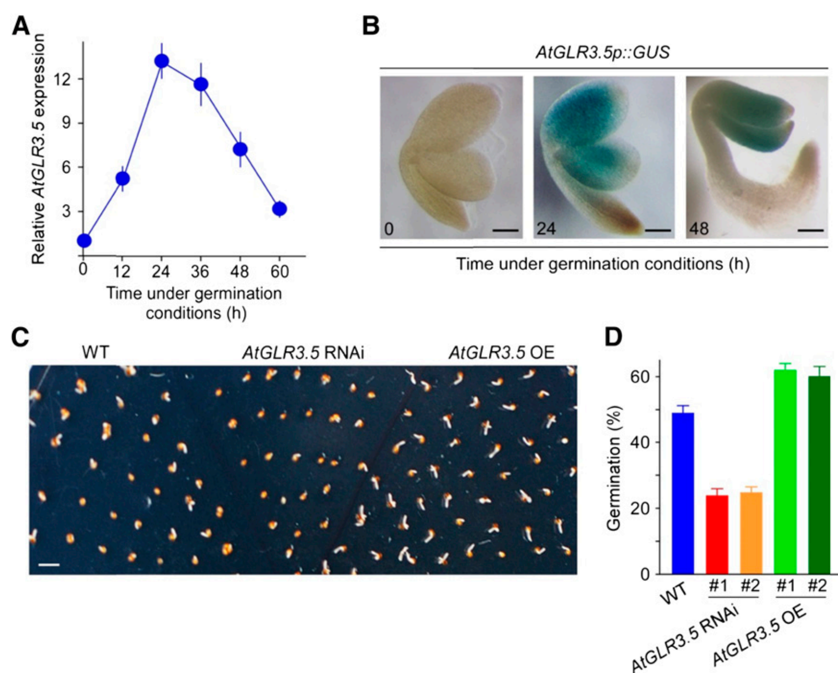

Figure 2. *AtGLR3.5* is required for seed germination. A, Time course analysis of *AtGLR3.5* expression during germination by qRT-PCR. Wild-type (WT) seeds incubated in water were stratified and collected at the indicated time points after incubation under the germination conditions. Data shown indicate means ± SE of the mean (n = 3). B, *AtGLR3.5p*::*GUS* reporter analysis in seed embryo during germination. Seeds were stratified and collected for staining at the indicated time points after incubation under the germination conditions. Images were taken after removal of the seed coat. C, Germination phenotype of wild-type, *AtGLR3.5* RNAi, and *AtGLR3.5* OE seeds on MS medium 40 h after incubation under the germination conditions. Three biological replicates were performed, and a representative image is shown. D, Germination analyses of wild-type, *AtGLR3.5* RNAi, and *AtGLR3.5* OE seeds 36 h after incubation under germination conditions. Bars = 100 μM (B) and 2 mm (D).

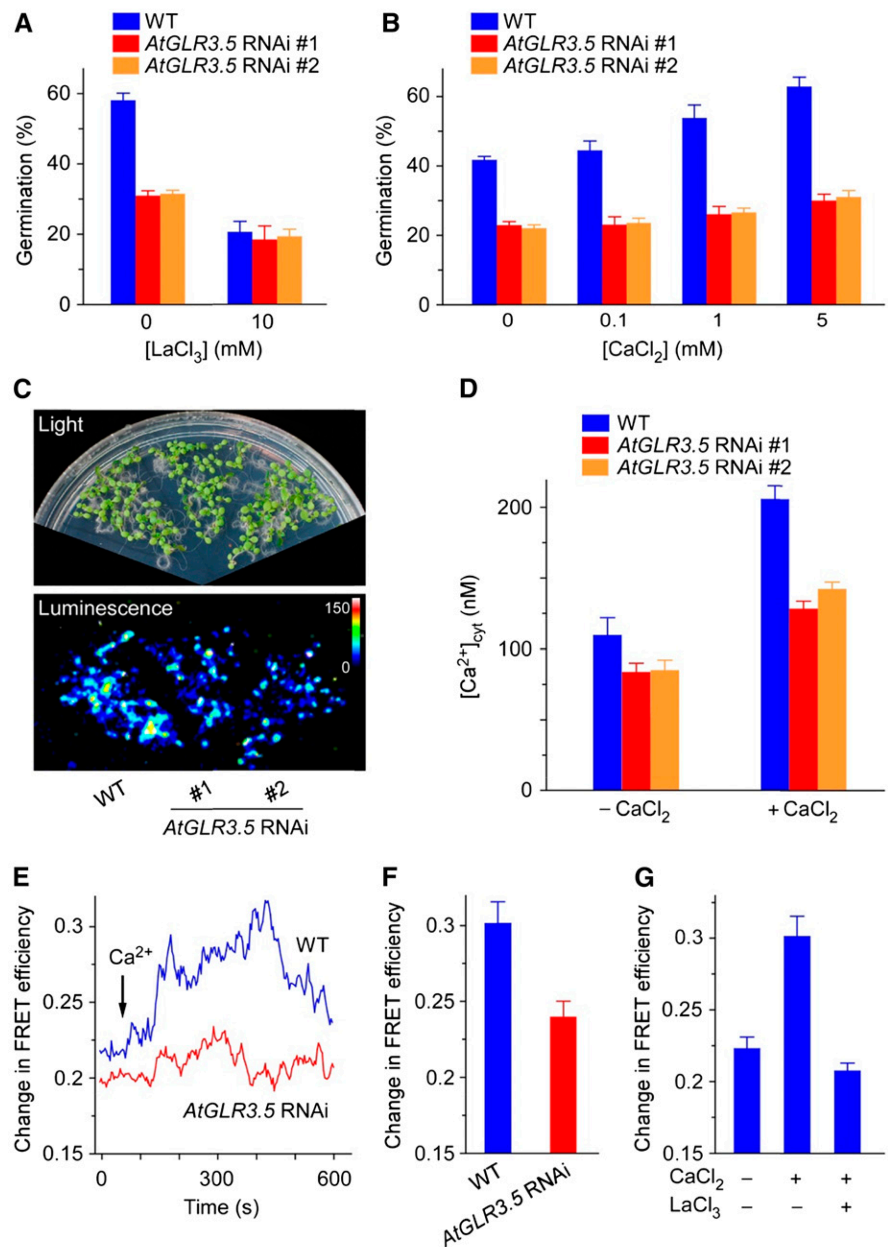

Figure 3. AtGLR3.5 modulates $[Ca^{2+}]_{cyt}$ fluctuation. A, Germination analysis of *AtGLR3.5* RNAi seeds in the absence and presence of $LaCl_3$. Seeds sown on MS medium supplemented with indicated concentrations of $LaCl_3$ were scored for germination 36 h after incubation under the germination conditions. B, Germination analysis of *AtGLR3.5* RNAi seeds at various concentrations of $CaCl_2$. Seeds sown on modified MS medium containing indicated amounts of $CaCl_2$ were scored for germination 36 h after incubation under the germination conditions. C, Ca^{2+}-sensitive photoprotein aequorin imaging analyses of $[Ca^{2+}]_{cyt}$ in 8-d-old wild-type (WT) and *AtGLR3.5* RNAi seedlings. D, Aequorin luminescence-based $[Ca^{2+}]_{cyt}$ quantification in 8-d-old seedlings before and after 10 mM $CaCl_2$ treatment. Data shown are means ± SE of the mean (n = 30 seedlings). E, $[Ca^{2+}]_{cyt}$-dependent FRET efficiency changes in wild-type and *AtGLR3.5* RNAi seedlings in response to $CaCl_2$ (10 mM). The primary roots of 4-d-old Arabidopsis plants expressing YC3.60 were used for the analysis. Arrows indicate the time point of $CaCl_2$ addition. F, Quantification of $[Ca^{2+}]_{cyt}$ changes in the wild type and *AtGLR3.5* RNAi mutant 300 s after $CaCl_2$ (10 mM) application, based on FRET efficiency changes as in E. Data shown are means ± SE of the mean (n = 10 seedlings). G, Effects of $CaCl_2$ (10 mM) and $LaCl_3$ (10 mM) on FRET efficiency changes in wild-type plants. Data shown are means ± SE of the mean (n = 16 seedlings). In A and B, data shown indicate means ± SE of the mean (n = 3).

reduction in $[Ca^{2+}]_{cyt}$ in the *AtGLR3.5* RNAi seedlings was more pronounced upon external Ca^{2+} treatment by which Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase (CICI) occurred (Fig. 3D; $P < 0.01$). These results suggest that AtGLR3.5 controls the resting $[Ca^{2+}]_{cyt}$ as well as CICI response. We further conducted fluorescence resonance energy transfer (FRET)-sensitized emission imaging analysis of Arabidopsis plants expressing YC3.60, as previously described (Rincón-Zachary et al., 2010). We observed the expected CICI in the wild type and strongly impaired response in *AtGLR3.5* RNAi plants (Fig. 3, E and F; $P < 0.01$). By contrast, the hydrogen peroxide (H_2O_2)-induced $[Ca^{2+}]_{cyt}$ increase (Demidchik et al., 2007) appeared normal in both wild-type and *AtGLR3.5* RNAi plants (Supplemental Fig. S7). Moreover, preincubation of wild-type seedlings with $LaCl_3$ largely abolished the CICI (Fig. 3G), giving further evidence that the CICI is mediated primarily by Ca^{2+}-permeable channels on the plasma membrane. Thus, the findings that *AtGLR3.5* RNAi lines had lower $[Ca^{2+}]_{cyt}$ than the wild type (Fig. 3, C and D) as well as impaired CICI response (Fig. 3, D and E) are in agreement with and also explain our prior observations that *AtGLR3.5* RNAi seeds geminated more slowly than wild-type controls (Fig. 2, C and D) and were less sensitive to various amounts of external Ca^{2+} in germination (Fig. 3B). It indicates that external Ca^{2+} may activate AtGLR3.5 at the protein level, rather than up-regulation of the gene expression to promote seed germination (Supplemental Fig. S6). In addition, the dynamic expression pattern of *AtGLR3.5* during

seed germination (Fig. 2A) may indicate a transient induction of *AtGLR3.5* and an increase in $[Ca^{2+}]_{cyt}$ in germinating seeds. Collectively, these results imply that AtGLR3.5 modulates Ca^{2+} influx, and thus $[Ca^{2+}]_{cyt}$ increases during the germination process, to ensure proper seed germination.

AtGLR3.5-Mediated Ca^{2+} Signal Alleviates the Inhibitory Effects of ABA on Germination

The phytohormone ABA has a prominent role in germination inhibition (Schopfer and Plachy, 1985; Finkelstein et al., 2002). Because the effect of Ca^{2+} in promoting radicle emergence and germination was observed (Fig. 1), we were curious as to whether the effects of Ca^{2+} and ABA, two signaling molecules that have opposite influences in germination regulation, are correlated. To test this idea, we scored seed germination at various concentrations of Ca^{2+} and ABA and found that with an increase in external Ca^{2+} amount, the inhibitory effect of exogenous ABA on germination was mitigated (Fig. 4A). Supporting this finding, *AtGLR3.5* RNAi lines, which had lower $[Ca^{2+}]_{cyt}$ and were less responsive to external Ca^{2+} than the wild type (Fig. 3, C–F), were hypersensitive to ABA in seed germination compared with the wild type (Fig. 4, B and C; $P < 0.001$). Also consistent, *AtGLR3.5* OE seeds were more resistant to ABA than the wild type (Fig. 4, D and E; $P < 0.001$). qRT-PCR analysis shows that ABA had no clear effect on the *AtGLR3.5* expression level under germination conditions (Supplemental Fig. S8). Taken together, these results indicate that the Ca^{2+} signal, mediated by AtGLR3.5, antagonizes the effect of ABA in suppressing seed germination.

Cytosolic Ca^{2+} Signal Mediated by AtGLR3.5 Acts against ABI4 to Foster Seed Germination

To understand the molecular mechanism by which Ca^{2+} counteracts the effect of ABA on seed germination, we examined the expression of some key ABA regulators involved in germination, including *ABI3*, *ABI4*, and *ABI5* from the ABA signaling network, *9-CIS-EPOXYCAROTENOID DIOXYGENASE6* (*NCED6*) and *ABSCISIC ALDEHYDE OXIDASE3* (*AAO3*) from the ABA biosynthesis pathway, and *CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE2* (*CYP7072A2*) in ABA catabolism, in wild-type and *AtGLR3.5* RNAi seeds. As shown in Figure 5A, a drastic up-regulation (approximately 15-fold) of *ABI4* expression was observed in stratified *AtGLR3.5* RNAi seeds compared with the wild type. This elevation began to decrease and gradually dropped to the wild-type level at approximately 48 h after incubation of the seeds under germination conditions. A 3-fold increase in the *ABI3* transcript level was found in the stratified *AtGLR3.5* RNAi seeds compared with the wild type, but there was no difference in the *ABI3* transcript level between wild-type and *AtGLR3.5* RNAi seeds after 24-h incubation under the germination conditions (Fig. 5B). In addition, a previous study has shown that *ABI3* expression does not regulate seed germination (Bassel et al., 2006). No apparent difference was found in the expression level of *ABI5* during the entire germination period (Fig. 5C). In contrast to the higher expression of *ABI4* in the germinating *AtGLR3.5* RNAi seeds (Fig. 5A), the stratified *AtGLR3.5* OE seeds had lower *ABI4* expression than the wild type (Supplemental Fig. S9; $P < 0.01$).

A recent finding has shown that ABI4 positively regulates ABA biogenesis during seed germination

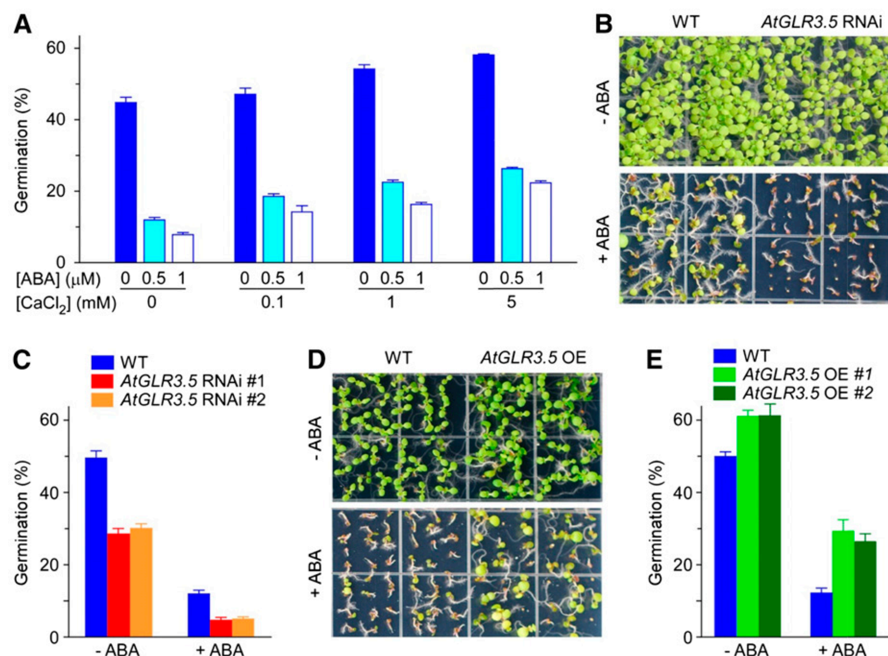

Figure 4. *AtGLR3.5* and calcium affect ABA sensitivity in seed germination. A, Calcium alleviates the inhibitory effect of ABA on germination. B, Phenotypes of 15-d-old wild-type (WT) and AtGLR3.5 RNAi seedlings treated with or without ABA (1 μM). C, Germination analysis of wild-type and *AtGLR3.5* RNAi seeds treated with or without ABA (1 μM). D, Phenotypes of 9-d-old wild-type and *AtGLR3.5* OE seedlings in the absence and presence of ABA (1 μM). E, Germination analysis of wild-type and *AtGLR3.5* OE seeds in the presence and absence of ABA (1 μM). For the germination analysis in A, C, and E, seeds were scored for germination 36 h after incubation under the germination conditions, and data shown are means ± SE of the mean ($n = 3$). In B and D, three independent experiments were performed and representative images are shown.

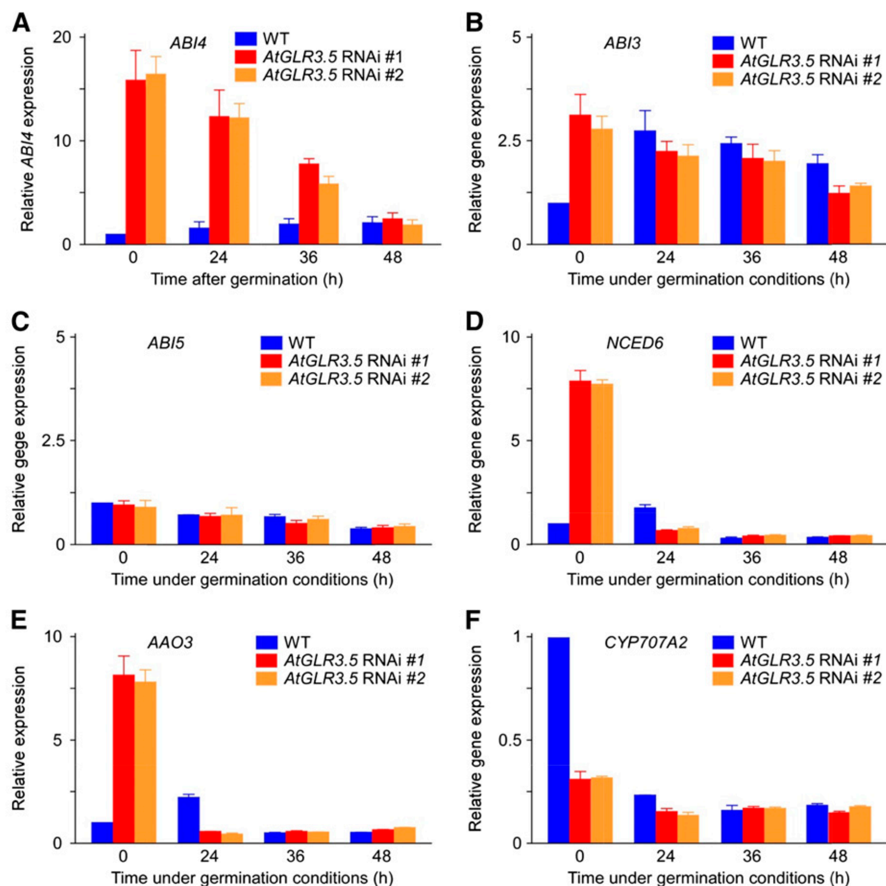

Figure 5. *AtGLR3.5* influences the expression of key ABA regulators in seed germination. Expression analyses of genes involved in ABA signaling (A–C) and metabolic pathways (D–F) in wild-type (WT) and *AtGLR3.5* RNAi seeds at indicated time points after incubation under germination conditions. Seeds were stratified at 4°C for 3 d, transferred to a growth chamber for indicated time periods, and collected for analysis. Data shown are means ± SE of the mean ($n = 3$).

and directly represses the expression of the ABA catabolic gene *CYP707A2* (Shu et al., 2013). In line with this report, our results showed that the expression levels of two crucial ABA biosynthesis genes *NCED6* and *AAO3* were significantly higher in stratified *AtGLR3.5* RNAi seeds and then rapidly decreased to levels lower than the wild type 24 h after incubation under germination conditions (Fig. 5, D and E). We also found that the transcript of *CYP707A2* was down-regulated in the *AtGLR3.5* RNAi seeds prior to germination and recovered to the wild-type level 24 h after incubation under germination conditions (Fig. 5F). Consistent with the expression patterns of these ABA biogenesis and catabolic genes, the ABA content in the stratified *AtGLR3.5* RNAi seeds was higher than the wild type, and this difference did not persist 24 h after incubation of the seeds (Supplemental Fig. S10; $P < 0.01$ at 0-h time point). The expression levels of *NCED6* and *AAO3* were lower in the stratified *AtGLR3.5* OE seeds (Supplemental Fig. S9; $P < 0.01$), and the ABA content in the stratified *AtGLR3.5* OE seeds was clearly lower than that in the wild type (Supplemental Fig. S10; $P < 0.05$ at 0 h). Thus, we conclude that the expression level of *AtGLR3.5* affects both ABA biogenesis and ABA signaling in seed germination and that the expression of *ABI4* is significantly increased at early germination stages in the *AtGLR3.5* RNAi seeds compared with the wild type.

To further determine whether the increase in *ABI4* expression level in the *AtGLR3.5* RNAi seeds is relevant to the impaired elevation in $[Ca^{2+}]_{cyt}$ (Fig. 3, C–F), we next analyzed the effect of external Ca^{2+} or EGTA on *ABI4* expression in wild-type seeds. As shown in Figure 6A, the *ABI4* expression level was clearly suppressed by $CaCl_2$ but greatly induced by EGTA after 24-h incubation under germination conditions. In addition, histochemical analysis of GUS activity in *ABI4p::GUS* transgenic seeds displayed a very similar pattern. Compared with the control, the addition of Ca^{2+} caused a decrease in *ABI4* expression, while adding EGTA had the opposite effect (Fig. 6B). It is worth noting that the suppression of *ABI4* expression by Ca^{2+} occurred preferentially in the embryonic cotyledons (Fig. 6B), the same region in seeds where *AtGLR3.5* is predominantly expressed (Fig. 2B). Together, these results provide strong evidence that AtGLR3.5-mediated $[Ca^{2+}]_{cyt}$ increase causes a decrease in *ABI4* expression in germinating seeds.

ABI4 regulates the ABA response in seeds (Finkelstein et al., 2002; Penfield et al., 2006), and its expression is induced by ABA (Penfield et al., 2006). Because Ca^{2+} represses *ABI4* expression during germination (Fig. 6, A and B) and antagonizes the effects of ABA (Fig. 4A), we examined whether the influence of Ca^{2+} on *ABI4* expression is effective in the presence of ABA. As shown

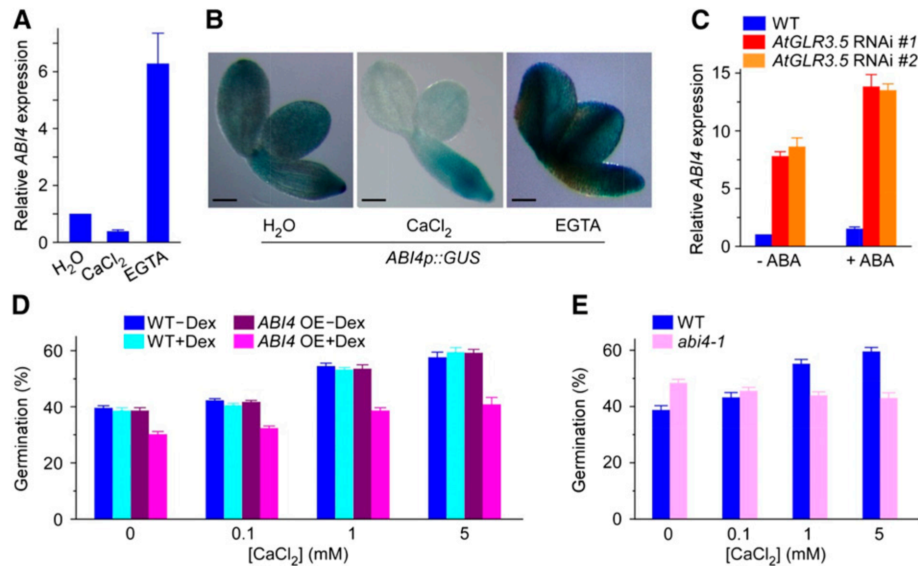

Figure 6. Interplay between calcium and ABA converges at ABI4. A, Relative expression of *ABI4* in response to $CaCl_2$ (1 mM) or EGTA (5 mM) in wild-type (WT) seeds. B, Histochemical staining of the GUS reporter driven by the *ABI4* promoter (*ABI4p::GUS*) in response to $CaCl_2$ (1 mM) or EGTA (5 mM) treatment. Images were taken after removal of the seed coat. C, Expression analysis of *ABI4* in wild-type or *AtGLR3.5* RNAi lines in response to ABA (20 μM). D, Germination analysis of wild-type and Dex-inducible *ABI4* OE seeds at various concentrations of $CaCl_2$ in the absence or presence of Dex (2 μM). E, Germination analysis of *abi4-1* mutant at various concentrations of $CaCl_2$. In A to C, seeds were stratified in indicated solution and transferred to the growth chamber for 24 h before analysis. In D and E, seeds were scored for germination 36 h after incubation under the germination conditions. In A and C, data shown are means ± SE of the mean (n = 3). Bar = 100 μM.

in Figure 6C, compared with the control (no ABA treatment), the increase in the level of *ABI4* transcript was much larger in *AtGLR3*.5 RNAi seeds than the wild type in the presence of ABA, indicating that *AtGLR3.5* plays a crucial role in repression of ABA-induced *ABI4* expression during germination. Together, these results provide unequivocal molecular evidence illustrating the importance of the change in the $[Ca^{2+}]_{cyt}$ mediated by AtGLR3.5 in ABA response during seed germination, which is consistent with our former observations that AtGLR3.5 and Ca^{2+} affect ABA sensitivity in seeds (Fig. 4, A, C, and E). Furthermore, our results also suggest that AtGLR3.5 may impede ABA signaling, at least in part, through modulating *ABI4* expression to promote seed germination.

Our results show that the effects of the AtGLR3.5-mediated Ca^{2+} signal on germination may be largely determined by changes in *ABI4* expression (Fig. 6, A–C). Thus, we further tested the Ca^{2+} responses in *ABI4* OE and *abi4-1* mutant plants. The germination of *ABI4* OE seeds in which *ABI4* expression is driven by the dexamethasone (Dex)-inducible promoter (Shkolnik-Inbar and Bar-Zvi, 2010) was indistinguishable from the wild type when Dex was absent (Fig. 6D). In the presence of Dex, however, the germination of *ABI4* OE seeds was significantly lower than the wild type, irrespective of external Ca^{2+} concentrations (Fig. 6D; $P < 0.001$), indicating the inhibitory effect of *ABI4* on seed germination. Thus, the phenotype of *AtGLR3.5* RNAi seeds, which show highly up-regulated *ABI4* expression, late germination, and less responsiveness to external Ca^{2+} compared with the wild type (Figs. 2D, 3B, and 5A), bears a strong resemblance to that of the *ABI4* OE plants (Fig. 6D; Shkolnik-Inbar and Bar-Zvi, 2010). By contrast, the *abi4-1* mutants that contain no functional ABI4 (Finkelstein, 1994) showed unimpaired or slightly better germination on medium containing 0 or 0.1 mM Ca^{2+} and reduced germination at 1 and 5 mM Ca^{2+} compared with wild-type seeds (Fig. 6E). These results suggest that ABI4 plays not only negative but also positive roles in germination when Ca^{2+} is present. Interestingly, a previous report has shown both positive and negative roles of ABI4 in root growth (Cui et al., 2012). Together, these results indicate a fundamental role of ABI4 in modulation of Ca^{2+}-dependent germination and further support that the elevated expression of *ABI4* is a major cause of the delayed germination of *AtGLR3.5* RNAi seeds.

The ability of the seed embryo to resume its biochemical and molecular activities determines germination vigor (Rajjou et al., 2012). ABI4 (Finkelstein et al., 1998), an APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (AP2/ERF) transcription factor involved in ABA responses in seeds, is specifically transcribed in the embryo, where it represses the breakdown of seed lipids (Penfield et al., 2006), thereby contributing to ABA sensitivity in the seed embryo (Penfield et al., 2006; Weitbrecht et al., 2011). Studies have shown that transcriptional regulation plays a critical role in the ABA response in germinating seeds (Finkelstein et al., 2002; Kucera et al., 2005; Holdsworth

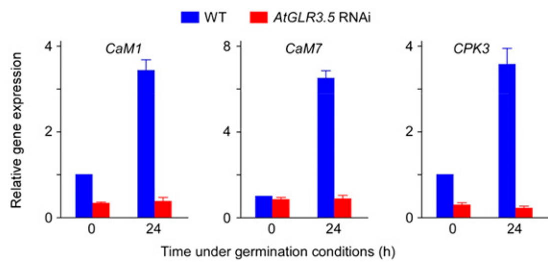

Figure 7. AtGLR3.5 affects the expression of cellular Ca^{2+}-sensing molecules. Expression analyses of *CaM1*, *CaM7*, and *CPK3* in wild-type (WT) and *AtGLR3.5* RNAi seeds at indicated time points after incubation under the germination conditions. Seeds were stratified and collected at 0 or 24 h after transfer to a growth chamber. Data shown are means ± SE of the mean (n = 3).

et al., 2008). Thus, the AtGLR3.5-mediated Ca^{2+} repression of *ABI4* expression (Figs. 5A and 6, A–C) may indicate more lipid breakdown and better embryonic growth potential, which is supported by our observations that *AtGLR3.5* OE seeds germinated faster than the wild type (Fig. 2, C and D) and that Ca^{2+} stimulates enhanced germination both in the presence and absence of ABA (Figs. 1 and 4A).

The completion of seed germination requires coordination between the embryo and the surrounding endosperm and testa layers (Penfield et al., 2006). In Arabidopsis, ABA represses embryo growth potential in seeds (Schopfer and Plachy, 1985) and delays endosperm rupture but not testa rupture (Müller et al., 2006). The *ABI4* transcript is confined to the embryo (Penfield et al., 2006), while *ABI5* is expressed in both embryo and micropylar endosperm, a barrier for the radicle protrusion (Lopez-Molina et al., 2001; Penfield et al., 2006). These two *ABI* transcriptional factors play a synergistic role in regulating downstream gene expression and physiological responses (Reeves et al., 2011). Our results demonstrate that the $[Ca^{2+}]_{cyt}$ signal generated by AtGLR3.5 specifically affects *ABI4* but not *ABI5* expression in germinating seeds (Fig. 5, A and C), and the *ABI4* regulation by Ca^{2+} also occurs when ABA is present (Fig. 6C), suggesting a specific role of Ca^{2+} in the embryo tissue during germination. Studies have shown that an early signal originating from the embryo is required to induce endosperm weakening to facilitate germination (Müller et al., 2006). Our results suggest that Ca^{2+} regulation of *ABI4* expression occurs at early germination stages, 24 h before germination onset (Fig. 6, A and B). Thus, it will be interesting to investigate whether Ca^{2+} modulation of *ABI4* in the embryo tissue also plays a role in the early embryo-endosperm interaction and endosperm rupture during seed germination.

Beyond ABA, germination is largely modulated by plant hormone GA (Ogawa et al., 2003; Kucera et al., 2005). A recent report shows that ABI4 positively regulates ABA biogenesis and negatively regulates GA biosynthesis during seed germination (Shu et al., 2013). Because we have found that AtGLR3.5-mediated Ca^{2+} signal represses *ABI4* expression and counteracts the ABA effect on germination, interesting future work might be to investigate whether Ca^{2+} is also a part of GA signaling system in germination control.

AtGLR3.5-Modulated Seed Germination May Involve Cellular Ca^{2+}-Sensing Molecules

As a crucial cellular signaling molecule, Ca^{2+} functions by eliciting characteristic transient $[Ca^{2+}]_{cyt}$ fluctuations at various developmental stages or upon specific stimuli (Dodd et al., 2010; Kudla et al., 2010). These Ca^{2+} signatures are detected and decoded by downstream cellular Ca^{2+}-sensing components that contain Ca^{2+}-binding domains, including calmodulin (CaM), Ca^{2+}-dependent protein kinase (CPK), and calcineurin B-like protein, which convert the information of the Ca^{2+} transients into biochemical events and physiological responses (Luan, 2009; Dodd et al., 2010; Kudla et al., 2010). Interestingly, studies have shown that disturbing the function of some Ca^{2+}-sensing proteins cause germination problems (Rivetta et al., 1997; Pandey et al., 2004; Zhao et al., 2011). The finding that AtGLR3.5 modulates $[Ca^{2+}]_{cyt}$ and germination prompted us to examine the expression of Ca^{2+}-sensing genes in *AtGLR3.5* RNAi seeds. It was found that consistent with the up-regulation of *AtGLR3.5* at early germination stages (Fig. 2A), the expression of *CaM1*, *CaM7*, and *CPK3* in wild-type seeds was significantly up-regulated as germination begins; however, these increases were largely abolished in germinating *AtGLR3.5* RNAi seeds (Fig. 7). Germinating *AtGLR3.5* OE seeds had a higher level of *CaM1*, *CaM7*, and *CPK3* transcripts compared with the wild type (Supplemental Fig. S11, $P < 0.01$ in A and C and $P < 0.05$ in B). These results indicate the involvement of the

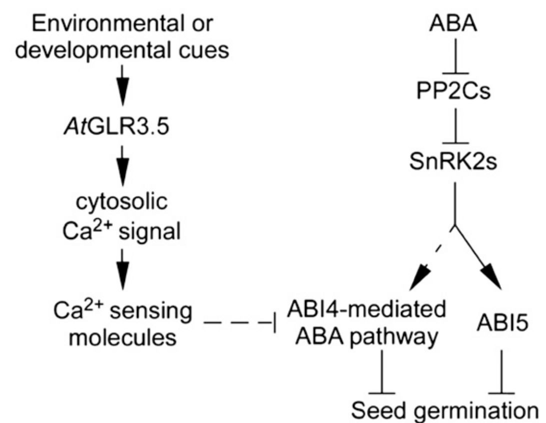

Figure 8. A working model for seed germination regulated by the AtGLR3.5-cytosolic Ca^{2+}-ABI4 network and Ca^{2+}-independent ABA signaling pathway. Arrows and t bars indicate activation and repression, respectively. Black lines represent direct effects, and dotted lines represent effects that have not yet been known to occur directly. PP2Cs, Type 2C protein phosphatases; SnRK2s, SNF1-related protein kinases.

Ca^{2+}-decoding system triggered by the AtGLR3.5-mediated $[Ca^{2+}]_{cyt}$ signal in germination control. Ca^{2+}-sensing proteins either combine the calcium-sensing function mediated by the Ca^{2+}-binding domains with an enzymatic activity (e.g. CPKs) or have calcium-binding abilities but lack other effector domains (e.g. CaMs), which therefore need to interact with target proteins to further transmit the Ca^{2+} signals (Luan, 2009; Dodd et al., 2010; Kudla et al., 2010). A recent report has shown that CPK5 directly phosphorylates NADPH/respiratory burst oxidase protein D, which is a prerequisite for the downstream signal propagation (Dubiella et al., 2013). While it has been established that CaM7 directly interacts with the promoters of several light-inducible genes and regulates their expression (Kushwaha et al., 2008), other CaMs appear to modulate gene expression through their interactions with Ca^{2+}/calmodulin-binding transcription activators that function as transcriptional (co)regulators (Finkler et al., 2007). Thus, our finding of the altered expression of *CaM1*, *CaM7*, and *CPK3* in germinating *AtGLR3.5* RNAi and OE seeds may indicate a complex Ca^{2+}-decoding network involving protein phosphorylation, protein-protein interaction, and even protein-DNA interaction, which acts downstream of the AtGLR3.5-mediated Ca^{2+} signal prior to ABI4. Future investigation into the Ca^{2+}-decoding network will more precisely elucidate the signaling mechanism as to how AtGLR3.5 and Ca^{2+} signal to ABI4 in germination control.

Ca^{2+} signals are core regulators of numerous physiological events and stress responses in plants (White and Broadley, 2003; McAinsh and Pittman, 2009; Dodd et al., 2010; Kudla et al., 2010). In this study, we investigated the role of Ca^{2+} signal in seed germination, the first step of plant life cycle. We show that Ca^{2+} signal, mediated by AtGLR3.5, positively regulates seed germination and antagonizes the inhibitory effect of ABA. AtGLR3.5 modulates steady-state $[Ca^{2+}]_{cyt}$ as well as transient $[Ca^{2+}]_{cyt}$ elevations upon Ca^{2+} stimuli. The AtGLR3.5-mediated Ca^{2+} signal represses ABA effects in seeds, largely through the transcription factor ABI4. Our data suggest that the signal transduction from the AtGLR3.5-mediated $[Ca^{2+}]_{cyt}$ elevation to ABI4 involves cellular Ca^{2+}-sensing molecules. A working model illustrating the AtGLR3.5-Ca^{2+}-ABI4 regulatory network in seed germination along with the known ABA pathway that has not been approved to be Ca^{2+} dependent is presented in Figure 8.

Since the discovery of GLR genes in plants (Lam et al., 1998), many studies have demonstrated that Ca^{2+} levels mediated by *AtGLR*s affect myriad cellular processes (Kim et al., 2001; Meyerhoff et al., 2005; Cho et al., 2009; Michard et al., 2011; Li et al., 2013; Mousavi et al., 2013; Vincill et al., 2013). To our knowledge, this is the first report demonstrating that cytoplasmic Ca^{2+} dynamics modulated by a plant GLR play a pivotal role in seed germination. Recently, amino acid activation of AtGLR Ca^{2+} channels has been reported (Qi et al., 2006; Michard et al., 2011; Vincill et al., 2012; Tapken et al., 2013). Identification of additional AtGLR members involved in the $[Ca^{2+}]_{cyt}$ modulation and germination regulation and discovering the amino acid(s) that may activate such AtGLRs would help to better describe the role of Ca^{2+} and *AtGLR*s in controlling plant physiology.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) genotypes used in this study are in the ecotype Columbia background. Seeds of the *abi4-1* mutant and transgenes expressing *ABI4p*::*GUS*, *35S*::*aequorin*, *35S*::*YC3.60*, or Dex-inducible *ABI4* OE plants were kindly provided by Drs. Ruth Finkelstein (University of California, Santa Barbara), Marc Knight (Durham University), Magaly Rincón-Zachary and Elison B. Blancaflor (Samuel Roberts Noble Foundation), and Dudy Bar-Zvi (Ben-Gurion University), respectively. The transfer DNA insertion mutant *atglr3.7* (SALK_103942) was obtained from the Arabidopsis Biological Resource Center. Arabidopsis plants were grown in soil or in petri dishes in a controlled growth chamber at 22°C under white fluorescent light (110 μmol m^{-2} s^{-1}) with a 16-h-light/8-h-dark photoperiod. For the germination assay, seeds were surface sterilized with 10% (v/v) bleach (Glorix) for 20 min and rinsed five times with sterile water. Seeds were then plated on MS-based medium containing MS major salts with indicated concentrations of $CaCl_2$, MS minor salts (Sigma), vitamins (Sigma), 1% (w/v) Suc (Sigma), and 0.8% (w/v) agar (Sigma type A) supplemented with EGTA (Sigma), $LaCl_3$ (Sigma), or ABA (Sigma) as needed. After stratification for 3 d at 4°C in darkness, the plates were transferred to the growth chamber (22°C) for indicated time periods. Germination was scored by the first sign of radicle tip appearance based on the criteria described previously (Bewley, 1997). For the germination frequency analysis, 60 to 80 seeds per sample in each treatment were tested, and three biological replicates were performed. Seeds tested in one experiment were harvested the same day.

Plasmid Construction and Generation of Arabidopsis Transgenic Plants

As no homozygous *AtGLR3.5* transfer DNA insertion lines (SALK_023880 and SALK_035264) were found, we generated *AtGLR3.5* knockdown lines using the pFGC5941 RNAi vector (http://www.chromdb.org). A 279-bp fragment of the *AtGLR3.5* DNA sequence was amplified by PCR using primers 5′-AATCTAGAGGCGCGCCCTCGATGTTTCTTCAGCCAAGGC-3′ (containing *Xba*I and *Asc*I restriction sites) and 5′-AAGGATCCATTTAAATCT-GAGAAACCTGTATGATTCGACC-3′ (containing *Bam*HI and *Swa*I restriction sites). The obtained fragment was cloned into the pFGC5941 vector first in sense orientation after cutting with *Asc*I and *Swa*I and then in the opposite orientation after digesting with *Xba*I and *Bam*HI, generating an *AtGLR3.5* RNAi construct under the control of the 35S promoter. The *AtGLR3.5 OE* and *AtGLR3.5p*::*GUS* constructs were created using the Gateway cloning system (Invitrogen) by cloning the full-length *AtGLR3.5* coding sequence or the 2.1-kb promoter region of *AtGLR3.5* first into the pENTR/D-TOPO cloning vector and then into the binary vector pMDC32 or pMDC163 (Karimi et al., 2002), respectively. Primers used for the PCR amplifications are as follows: *AtGLR3.5* full-length coding sequence, 5′-CACCATGATTCTTTCATTGGAAGAGC-3′ and 5′-TCACTGTGGAGTTTCGTGATC-3′; and *AtGLR3.5* promoter region, 5′-ATTCCACTCTATAGGAAGAAAATTGTT-3′ and 5′-AAGTAAACA-GAGCTCCAACGTTTACAG-3′. All constructs were verified by DNA sequencing. The resulting plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101, which were subsequently transformed into Arabidopsis wild-type plants by the floral dip method (Clough and Bent, 1998). Transformants were selected with Basta or hygromycin. Homozygous lines carrying a single insertion in the T3 generation were used for further analysis.

Gene Expression Analysis

Total RNA was extracted using Spectrum Plant Total RNA Kit (Sigma) and reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) after treatment with DNase I (Ambion). For the expression analyses of *AtGLR3.5*, *AtGLR3.4*, and *AtGLR3.1* in the *AtGLR3.5* RNAi lines, PCR amplifications were performed with 32 cycles for *AtGLR3.5* and *Ubiquitin21* (*UBQ21*) and 30 cycles for *AtGLR3.4* and *AtGLR3.1*, using the following gene-specific primers: *AtGLR3.5*, 5′-ATCTCATAAGTGAAGTTGCTGCAA-3′

and 5′-TTGCTATTAAAGTGTCCATTCCTT-3′; *AtGLR3.4*, 5′-CAAATAGCA-ACTGCAAATTCCGTA-3′ and 5′-TGATTGTGAAGTCCCAGCTGCACT-3′; *AtGLR3.1*, 5′-TCAGATTACTGTAAGTTTGCCAT-3′ and 5′-TCATATGGGTC-TTCTAGATGCAGT-3′; and *UBQ21*, 5′-TAGAGATGCAGGCATCAAGAGC-GCGACTG-3′ and 5′-GCGGCGAGGCGTGTATACATTTGTGCCATT-3′. Quantitative PCR was performed using SYBR Green PCR Master Mix (Bio-Rad) in a CFX96 Real-Time PCR System (Bio-Rad). The expression values were normalized to that of the housekeeping gene *ACTIN2*. Gene-specific primers used were designed by AtRTPrimer (Han and Kim, 2006) with the following sequences: *AtGLR3.5*, 5′-AGGAGGCTGGTTCTTCTAGAGG-3′ and 5′-TCTCACTGTGGAGTTTCGTG-3′; and *ACTIN2*, 5′-GGTAACATTGTGCTCA-CTCGTGG-3′ and 5′-AACGACCTTAATCTTCATGCTGC-3′. Primers used for expression analyses of genes involved in ABA or GA signaling and biosynthesis pathways including *ABI3*, *ABI4*, *ABI5*, *NCED6*, *AAO3*, and *CYP707A2* are as described (Nelson et al., 2009; Footitt et al., 2011). Primers used for the expression analyses of *CaM1*, *CaM7*, and *CPK3* are as described (Liu et al., 2005; Lario et al., 2013). For each expression analysis, three biological replicates were performed with similar results, and values shown are from one experimental repetition.

Measurement of $[Ca^{2+}]_{cyt}$ Level

Cytosolic free calcium concentrations were monitored using plants harboring a single functional copy of aequorin or YC3.60 as described (Knight et al., 1996; Tang et al., 2007; Rincón-Zachary et al., 2010). *AtGLR3.5* RNAi lines expressing aequorin or YC3.60 were obtained by genetic crosses. For aequorin bioluminescence-based Ca^{2+} imaging analysis, sterilized seeds were sowed on MS plates and incubated in a growth chamber for 8 d. Seedlings were then treated with 20 μM coelenterazine (Gold Bio) 18 h prior to Ca^{2+} imaging. Bright-field images were taken before aequorin luminescence recording. Aequorin images were taken after 20-min recording using a PIXIS CCD camera (Princeton Instruments), both before external $CaCl_2$ treatment to detect the resting $[Ca^{2+}]_{cyt}$ level and after 10 mM $CaCl_2$ treatment to monitor the CICI. WinView32 (Roper) software was used for the image analysis. The total amount of aequorin was estimated by treating the plants with discharging solution (0.9 M $CaCl_2$ in 10% [v/v] ethanol). The $[Ca^{2+}]_{cyt}$ was calibrated based on the aequorin luminescence intensity as described (Knight et al., 1996). Experiments were performed at room temperature.

For YC3.60-based FRET-sensitized emission imaging analysis, sterilized seeds were sowed on MS medium and incubated in a chamber for 4 d. The FRET-sensitized emission images were taken using a Leica TCS SP5 confocal laser-scanning microscope (Leica Microsystems) as described previously (Rincón-Zachary et al., 2010). Briefly, the primary roots of the seedlings were mounted with 0.1% (w/v) low-melting agarose on glass bottom microwell dishes (MatTek) and then treated with 10 mM $CaCl_2$ or 10 mM H_2O_2 to elicit CICI. Plants expressing cyan fluorescent protein or yellow fluorescent protein alone were used to obtain calibration images. $[Ca^{2+}]_{cyt}$-dependent FRET efficiency changes were calculated using the Leica software as described (Rincón-Zachary et al., 2010).

Measurement of ABA Levels in Seeds

Seeds (100 mg per sample) were stratified at 4°C for 3 d and collected at 0 and 24 h after incubation under germination conditions. ABA content was measured by liquid chromatography-tandem mass spectrometry at the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center. Three replicates were performed.

Histochemical GUS Activity Analysis

GUS activity was assayed as described (Jefferson et al., 1987). Briefly, transgenic seeds or seedlings were incubated in GUS staining buffer containing 2 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-GlcA, 100 mM sodium phosphate, pH 7.0, 1 mM EDTA, pH 8.0, 1% (v/v) Triton X-100, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide at 37°C in the dark overnight and cleaned with 70% (v/v) ethanol to remove chlorophyll. Samples in one experiment were stained for the same amount of time for comparison. Photographs were taken using a Zeiss Stemi SV6 dissection microscope equipped with a Zeiss Axio CamICc digital camera.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. High concentrations of external calcium delay seed germination.

Supplemental Figure S2. Germination analysis of *atglr3.7* mutant seeds.

Supplemental Figure S3. Expression of *AtGLR3.5* in seeds (dry seeds versus imbibed seeds) and seedlings.

Supplemental Figure S4. Relative *AtGLR3.5* expression in 2-week-old *AtGLR3.5* RNAi and *AtGLR3.5* OE transgenic lines.

Supplemental Figure S5. Reverse transcription-PCR analyses show that the *AtGLR3.5* RNAi construct specifically silenced *AtGLR3.5* transcript but not transcripts of *AtGLR.3.4* and *AtGLR3.1* in *AtGLR3.5* RNAi lines.

Supplemental Figure S6. Effect of $CaCl_2$ on the expression of *AtGLR3.5* in seeds.

Supplemental Figure S7. FRET-sensitized emission analysis showing $[Ca^{2+}]_{cyt}$ changes in wild-type and *AtGLR3.5* RNAi seedlings in response to 10 mM H_2O_2 treatment.

Supplemental Figure S8. Effect of ABA on the expression of *AtGLR3.5* in seeds.

Supplemental Figure S9. Relative expression of *ABI4*, *AAO3*, and *NCED6* in stratified wild-type and *AtGLR3.5* OE seeds.

Supplemental Figure S10. ABA contents in wild-type, *AtGLR3.5* RNAi, and *AtGLR3.5* OE seeds.

Supplemental Figure S11. Relative expression of *CaM1*, *CaM7*, and *CPK3* in germinating wild-type and *AtGLR3.5* OE seeds.

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