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Glutamate Receptor Homolog3.4 is Involved in Regulation of Seed Germination Under Salt Stress in Arabidopsis

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Seed germination is sensitive to salt stress. ABA and Ca^{2+} are involved in the regulation of seed germination under salt stress. Ca^{2+} influx mediated by glutamate receptors (GLRs) plays important roles in many physiological processes in plants. Here, we investigated the correlation of GLRs, Ca^{2+} and ABA during seed germination in response to salt stress by using *Arabidopsis thaliana* wild-type and T-DNA insertion knockout mutants of glutamate receptor homolog3.4. We demonstrated that *atglr3.4-1* and *atglr3.4-2* mutants were more sensitive to NaCl during seed germination and post-germination growth than wild-type plants. Treatments of wild-type seedlings with NaCl evoked a marked elevation in cytosolic Ca^{2+} activity ($[Ca^{2+}]_{cyt}$), and the elevation was inhibited by antagonists of GLRs, while the NaCl-induced elevation in $[Ca^{2+}]_{cyt}$ was impaired in *atglr3.4-1* and *atglr3.4-2* mutants. Moreover, the mutants exhibited a lower expression of *SOS3*, *SOS2* and *SOS1*, and greater accumulation of Na^+ than wild-type seeds in the presence of NaCl. Mutation of *AtGLR3.4* rendered the mutants more sensitive to ABA, while overexpression of *AtGLR3.4* made the transgenic lines more tolerant to ABA in terms of seed germination. However, there was no difference in ABA content between *atglr3.4* mutants and wild-type seeds, accompanied by lower expression of *ABI3* and *ABI4* in *atglr3.4* mutants when challenged with NaCl. These results demonstrate that AtGLR3.4-mediated Ca^{2+} influx may be involved in the regulation of seed germination under salt stress by modulating Na^+ accumulation through the SOS pathway.

Keywords: *Arabidopsis thaliana* • AtGLR3.4 • SOS pathway • Na accumulation • ABA • Salt stress • Seed germination.

Abbreviations: ABI, abscisic acid insensitive; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitriquinoxaline-2,3-dione; GLR, glutamate receptor; GUS, β-glucuronidase; MS, Murashige and Skoog; qRT–PCR, quantitative reverse transcription–PCR; SOS, salt overly sensitive.

Introduction

Seed germination is a critical stage in the plant life cycle that converts a quiescent seed to a highly active growth phase (Finch-Savage and Leubner-Metzger 2006, Rajjou et al. 2012). Seed germination starts with uptake of water by the quiescent dry seed and terminates with two sequential phases of testa and endosperm rupture through the expansion of the embryo and endosperm weakening (Bewley 1997, Finch-Savage and Leubner-Metzger 2006). As sessile organisms, most plant species cannot leave the places where their seeds germinate through their life spans (Park et al. 2011). Therefore, in order to ensure offspring propagation, timing of seed germination is accurately regulated by endogenous and environmental cues (Finkelstein et al. 2008, Park et al. 2011).

Salinity is one of the major abiotic stresses for seed germination and early seedling establishment for many plant species (Zhu 2001, Daszkowska-Golec 2011, Cheng et al. 2016). The high Na^+ concentrations in saline soils often reduce the soil water potential to inhibit water absorption of plants from soils, thus suppressing seed imbibition and embryo growth (Khajeh-Hosseini et al. 2003, Daszkowska-Golec 2011, Rajjou et al. 2012). Moreover, the excessive accumulation of Na^+ often leads to inhibition of cell survival, growth and division (Zehra et al. 2012). Therefore, maintaining a low concentration of Na^+ in the cytoplasm is essential to improve salt tolerance in plants. The SOS (SALT OVERLY SENSITIVE) signaling pathway, consisting of SOS3, SOS2 and SOS1, is a key regulator to maintain a low cytoplasmic Na^+ concentration in plant cells (Zhu 2002, Deinlein et al. 2014). The salt stress-induced calcium signal can be sensed by the calcium sensor SOS3, which interacts with and activates protein kinase SOS2. The SOS3–SOS2 protein kinase complex phosphorylates SOS1, a plasma membrane Na^+/H^+ antiporter, resulting in an efflux of excess Na^+ to maintain ion homeostasis (Zhu 2001, Zhu 2002).

The phytohormone ABA is regarded as the primary regulator to control the transition from dormancy to germination in seeds (Finch-Savage and Leubner-Metzger 2006, Holdsworth et al. 2008). Genetic and molecular studies have revealed that transcriptional regulation of ABA signaling plays an important role in seed development and germination (Finkelstein et al. 2002, Kucera et al. 2005, Holdsworth et al. 2008). For example, abscisic acid insensitive4 (ABI4) plays a unique role in embryonic lipid catabolism during seed germination (Penfield et al. 2006), while ABI5 can interact with ABI3 to execute an ABA-dependent growth arrest during germination, conferring osmotic tolerance to harsh environmental conditions (Lopez-Molina et al. 2001, Lopez-Molina et al. 2002). In the study of seed

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germination, Ca^{2+} has been demonstrated to be a critical signaling molecule for ABA-mediated seed germination. For example, several members of the Arabidopsis calcium-dependent protein kinase (CDPK) superfamily have been identified as positive ABA signaling regulators in seed germination (Choi et al. 2005, Zhu et al. 2007, Zhao et al. 2011). Pandey et al. (2008) found that the calcium sensor CBL9 and CBL-interacting protein kinase CIPK3 physically and functionally interact with each other to regulate the ABA-mediated seed germination. Kong et al. (2015) demonstrated that Arabidopsis glutamate receptor homolog3.5 modulates the cytosolic Ca^{2+} level to stimulate seed germination by antagozing the inhibitory effecct of ABA.

As a crucial cellular signaling molecule, Ca^{2+} signaling cascades participate in numerous processes associated with development and responses to environmental cues by eliciting a transient increase in cytosolic Ca^{2+} activity ($[Ca^{2+}]_{cyt}$) (Dodd et al. 2010, Kudla et al. 2010). Membrane transporters, including channels, pumps and carriers, are invovled in the generation of specific Ca^{2+} signals in response to specific stimuli (Dodd et al. 2010, Kudla et al. 2010). Several gene families are found to encode Ca^{2+}-permeable channels in plasma membranes, among which ionotropic glutamate receptor (iGLR) homologs are potential candidates to mediate Ca^{2+} influx (Jammes et al. 2011, Swarbreck et al. 2013). Twenty genes encoding homologs of animal iGluRs have been identified in Arabidopsis. The GLRs in Arabidopsis can be classfied into three clades based on their sequence similaries (Lam et al. 1998, Chiu et al. 1999, Lacombe et al. 2001). In mammalian cells, iGluRs are ligand-gated, cation-permeable channels to mediate the excitatory neurotransmission in the central nervous system (Dingledine et al. 1999). Similar to the iGluRs in mammalian cells, AtGLR1.4 and AtGLR3.4 have been shown to act as non-selective, Ca^{2+}-permeable channels through heterologous expression systems (Vincill et al. 2012, Tapken et al. 2013). In Arabidopsis, GLRs regulate many developmental and stress adaptation processes by mediating $[Ca^{2+}]_{cyt}$ fluctuation, including those of seed germination, pollen tube morphogenesis, root development and plant defense response (Michard et al. 2011, Li et al. 2013, Manzoor et al. 2013, Vincill et al. 2013, Kong et al. 2015, Singh et al. 2016).

Our previous studies have demonstrated that *atglr3.4* and *atglr3.7* T-DNA insertion mutants were hypersensitive to salt stress during seed germination probably by regulating Ca^{2+} influx (Cheng et al. 2016). However, whether knockout of *atglr3.4* would impair Na-induced $[Ca^{2+}]_{cyt}$ elevation in *atglr3.4* mutants and why *atglr3.4* mutants are more sensitive to salt stress than wild-type plants in terms of seed germination remain unclear. Here, we reported that loss of function of *AtGLR3.4* impaired the NaCl-induced increase in $[Ca^{2+}]_{cyt}$, which in turn may render the mutants more sensitive to salt stress due to greater accumulation of Na^+ in seeds. We further revealed that *atglr3.4* mutants exhibited lower expression levels of *SOS1*, *SOS2* and *SOS3*, in contrast to comparable ABA content and lower expression levels of *ABI3* and *ABI4* in *atglr3.4* mutants than those in wild-type under salt stress. Taken together, our results provide experimental evidence in support of involvement of AtGLR3.4-dependent Ca^{2+} signaling cascades in the inhibition of seed germination by salt stress.

Results

AtGLR3.4 regulates seed germination and early seedling establishment under salt stress

Our previous results showed that seed germination of *atglr3.4* mutants was more sensitive to NaCl treatment than that of the wild type (Cheng et al. 2016). To investigate further the role of AtGLR3.4 in plant response to salt stress, seeds of *atglr3.4-1* (SALK_079842), *atglr3.4-2* (SALK_016904) and the wild type were sown on 1/2 Murashige and Skoog (MS) medium supplemented with different concentrations of NaCl after 2 d stratification. Seed germination and subsequent growth of seedlings were monitored at the indicated time points. Consistent with the previous results (Cheng et al. 2016), germination rates of *atglr3.4-1* and *atglr3.4-2* were significantly lower than those of the wild type in the presence of 150 and 200 mM NaCl, while there was no difference in seed germination between wild-type and *atglr3.4* mutants when they were exposed to 100 mM NaCl (**Fig. 1A;** Supplementary Fig. S1). The subsequent growth of mutants was also lower compared with that of the wild-type plants. For instance, cotyledon greening rates of *atglr3.4* mutants were significantly lower than those of wild-type seeds in the presence of NaCl (**Fig. 1B**). In addition, root elongation in both the wild type and the *atglr3.4* mutants was inhibited in the presence of NaCl, with greater inhibitory effects in *atglr3.4* mutants than in wild-type seedlings.

We further analyzed the tissue expression patterns of *AtGLR3.4* by quantitative reverse transcription–PCR (qRT–PCR), and found that the transcripts of *AtGLR3.4* were detected across the tissues examined, with the highest and lowest transcript levels in leaves and roots, respectively (Supplementary Fig. S2A). By analyzing the transgenic plants expressing the β-glucuronidase (GUS) reporter gene under their own promoter sequence of *AtGLR3.4*, we observed that GUS staining was visible in roots, stems, leaves, siliques and germinating seeds (Supplementary Fig. S2B). Given the broad expression patterns of *AtGLR3.4*, we thus tested whether AtGLR3.4 is involved in salt stress during the seedling phase. The sensitivity of *atglr3.4-1*, *atglr3.4-2* and wild-type plants to salt stress was investigated by measuring survival rates of whole plants exposed to NaCl treatment. Similar to seed germination, wild-type plants displayed higher survival rates than *atglr3.4* T-DNA insertion mutants (Supplementary Fig. S3). However, the differences in survival rates between the wild type and *atglr3.4* mutants were less evident than those of germination rates and early seedling establishment. Therefore, AtGLR3.4 probably plays a more important role in seed germination and early seedling development than in adult seedlings under salt stress.

Both ion toxicity and osmotic stress account for hypersensitity of *atglr3.4* mutants to salt stress

Seeds exposed to NaCl suffer from both Na toxicity and osmotic stress (Wilson et al. 2014). To distinguish these effects, we examined seed germination in the presence of varying concentrations of mannitol. Similar to NaCl treatment, supplementation of the medium with mannitol delayed seed

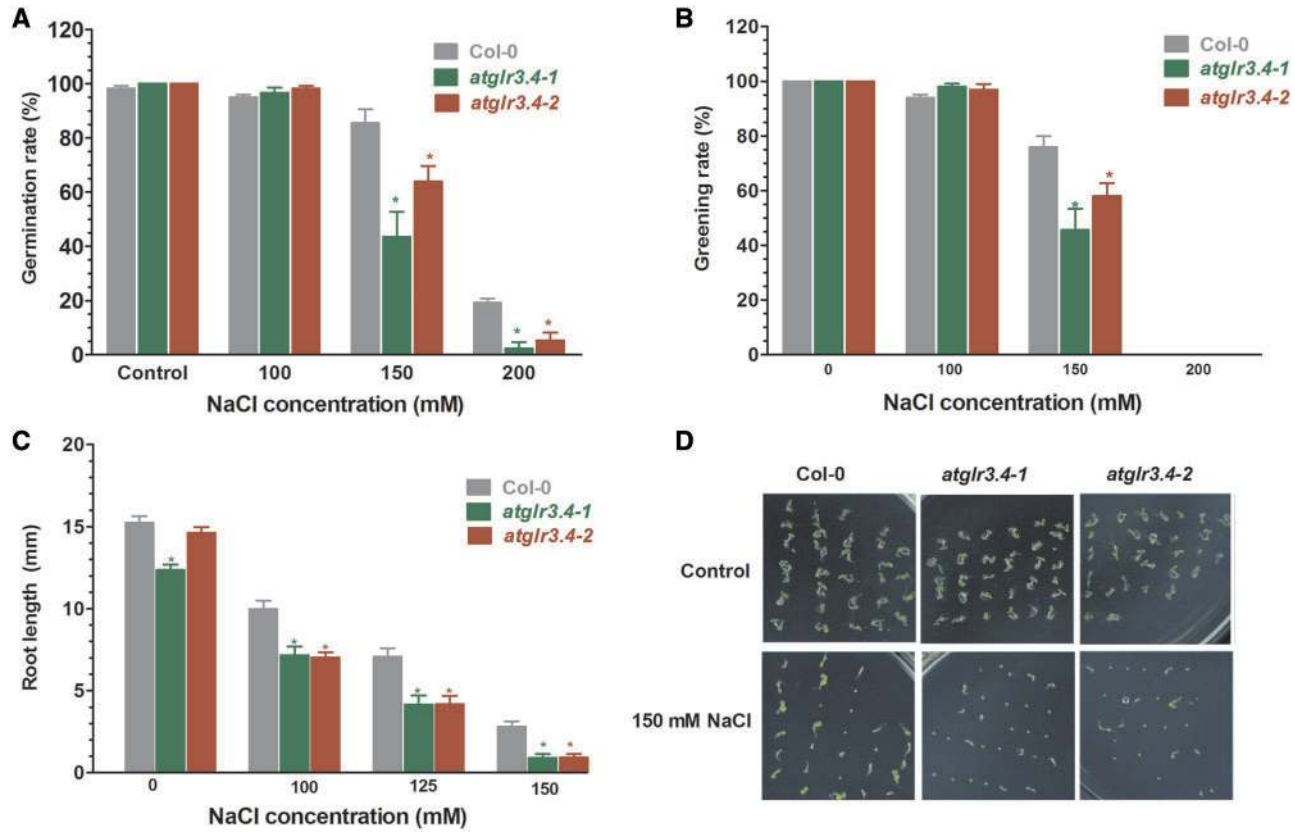

Fig. 1 AtGLR3.4 is involved in seed germination and early seedling establishment under salt stress. (A) Seed germination rates of the wild type (Col-0) and *atglr3.4* mutants were analyzed on the third day under varying concentrations of NaCl. Cotyledon greening rates (B) and early root growth (C) of the wild type, and *atglr3.4-1* and *atglr3.4-2* mutants were statistically analyzed after 7 d germination in different NaCl concentrations. (D) The representative photographs show seed germination of the wild type and *atglr3.4* mutants. Data are means ± SE with three replicates. Asterisks indicate significant differences ($P < 0.05$).

germination, and the inhibitory effect of mannitol on seed germination became more evident with increasing mannitol concentrations (**Fig. 2**). In addition, seed germination of *atglr3.4* mutants was more sensitive to mannitol treatment than the wild-type. Moreover, the inhibitory effect of NaCl on seed germination was consistently greater than that of mannitol, leading to a larger difference in seed germination between the wild type and the mutants. These results suggest that both ion toxicity and osmotic stress account for the difference in seed germination between the wild type and *atglr3.4* mutants under salt stress.

Loss of function of *AtGLR3.4* leads to impaired $[Ca^{2+}]_{cyt}$ induction in response to salt stress

Vincill et al. (2012) reported that AtGLR3.4 functions as an amino acid-gated, Ca^{2+}-permeable channel. To test whether AtGLR3.4 is involved in the regulation of $[Ca^{2+}]_{cyt}$ under salt stress, we monitored NaCl-induced $[Ca^{2+}]_{cyt}$ variation using wild-type Arabidopsis seedlings with cytosolic expression of the Ca^{2+} reporter protein aequorin. As shown in **Fig. 3**, exposure of Arabidopsis seedlings to NaCl led to a rapid rise in $[Ca^{2+}]_{cyt}$, and the magnitude of the elevation in $[Ca^{2+}]_{cyt}$ increased with increasing NaCl concentrations in the incubation medium. Chelation of extracellular Ca^{2+} by EDTA significantly suppressed this NaCl-induced elevation in $[Ca^{2+}]_{cyt}$. Moreover, treatment with the Ca channel blocker $LaCl_3$ resulted in a strong inhibition of NaCl-induced elevation in $[Ca^{2+}]_{cyt}$ (**Fig. 3C, D**).

Our previous studies revealed that salt stress-induced inhibition of seed germination can be alleviated by $CaCl_2$, and the alleviatory effects of $CaCl_2$ on seed germination rates of *atglr3.4* mutants were much less than those of the wild type (Cheng et al. 2016). In the present study, we found that germination rates of *atglr3.4* mutants were less sensitive to $LaCl_3$ under NaCl treatment (Supplementary Fig. S4) and supplementation of $CaCl_2$ in the growth medium had no effect on the abundance of *atglr3.4* transcripts in the presence of NaCl (Supplementary Fig. S5). More importantly, a pre-treatment with DNQX (6,7-dinitriquinoxaline-2, 3-dione) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), which are competitive GLR inhibitors, significantly suppressed the NaCl-induced elevation in $[Ca^{2+}]_{cyt}$ (**Fig. 3E, F**). In order to investigate further whether AtGLR3.4 modulates $[Ca^{2+}]_{cyt}$ variation when exposed to salt stress, the aequorin gene was introduced into the *atglr3.4-1* and *atglr3.4-2* mutants through cross-pollination, and their responses to exogenous NaCl were monitored. As shown in **Fig. 4**, an increase in $[Ca^{2+}]_{cyt}$ induced by NaCl was markedly suppressed in *atglr3.4-1* and *atglr3.4-2* mutants. Moreover, we analyzed the

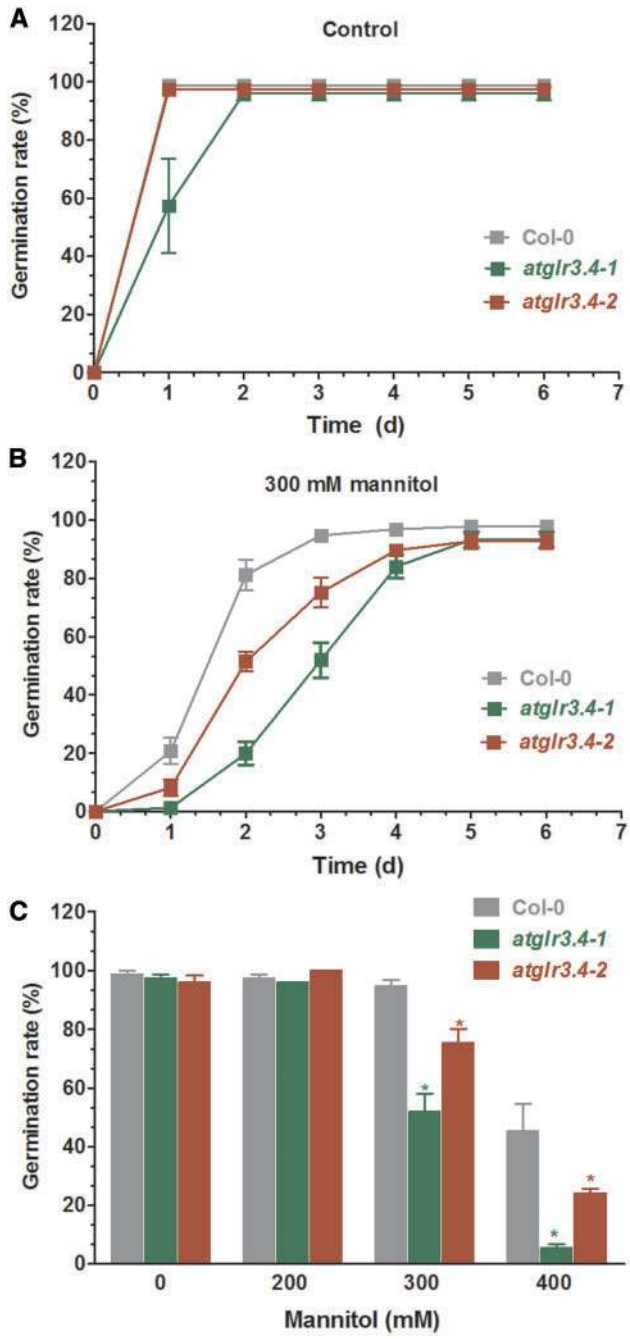

Fig. 2 Seed germination of *atglr3.4* knockouts was hypersensitive to osmotic stress. (A, B). Time-course quantification of germination of Col-0, *atglr3.4-1* and *atglr3.4-2* seeds grown on medium containing the indicated amounts of mannitol. (C). Seed germination rates of Col-0, *atglr3.4-1* and *atglr3.4-2* with the indicated amounts of mannitol. Data are means ± SE for three replicates, with each replicate containing 30 seeds. An asterisk indicates a significant difference ($P < 0.05$).

effect of NaCl on $[Ca^{2+}]_{cyt}$ by aequorin-based luminescence imaging in Arabidopsis seedlings. Consistent with the above results, the NaCl-induced elevations in $[Ca^{2+}]_{cyt}$ were annulled in *atglr3.4* mutants (**Fig. 4C**). These results provide direct evidence to support the involvement of AtGLR3.4 in mediating elevation in $[Ca^{2+}]_{cyt}$ in response to salt stress.

AtGLR3.4 mediates Na^+ accumulation in germinating seeds under salt stress

The SOS pathway has been reported to play an important role in maintaining low cytoplasmic Na^+ concentrations in plant cells (Zhu 2001, Julkowska and Testerink 2015). Given that loss of function of *AtGLR3.4* led to an impaired $[Ca^{2+}]_{cyt}$ elevation in response to salt stress (**Fig. 4**), we hypothesized that the salt stress-hypersensitive phenotype of the *atglr3.4* mutants may result from their aberrant SOS pathway in seed germination. To validate this hypothesis, Na^+ concentration, K^+ concentration and the Na^+/K^+ ratio in wild-type, *atglr3.4-1* and *atglr3.4-2* seeds were measured when they were sown on the growth medium after 24 h in the absence and presence of NaCl. As shown in **Fig. 5**, there were no differences in Na^+ concentrations in seeds of the wild type and *atglr3.4* mutants in the control medium. Exposure to NaCl medium led to a significant increase in Na^+ concentration of *atglr3.4* mutant and wild-type seeds. Further, Na^+ concentrations in *atglr3.4-1* and *atglr3.4-2* seeds were higher than those in wild-type seeds (**Fig. 5A**), while K^+ concentrations in *atglr3.4* mutants were comparable with those in wild-type seeds in the absence and presence of NaCl (**Fig. 5B**). Accordingly, the ratio of Na^+ to K^+ was much higher in *atglr3.4-1* than in wild-type seeds under salt stress (**Fig. 5C**). In contrast, there was no difference in the Na^+/K^+ ratio in seeds between the wild type and *atglr3.4-2* (**Fig. 5C**).

The expression patterns of *SOS3* in *atglr3.4-1*, *atglr3.4-2* and wild-type seeds were further monitored upon exposure of seeds to salt stress. Exposure to NaCl led to a marked up-regulation of *SOS3* expression across all genotypes examined. However, the abundance of *SOS3* transcripts in *atglr3.4-1* and *atglr3.4-2* was lower than that in wild-type seeds under saline conditions. We further investigated the expression profile of *SOS2*, and found that the abundance of *SOS2* transcripts in *atglr3.4-2* was lower than that in the wild type, while the abundance of *SOS2* transcripts in *atglr3.4-1* was higher than that in the wild type in control medium (**Fig. 5**). Exposure to NaCl led to a profound up-regulation of *SOS2* expression across the genotypes examined, with a greater abundance of *SOS2* transcripts in wild-type seeds than in *atglr3.4* mutants (**Fig. 5A**). A similar expression profile of *SOS1* in *atglr3.4-1*, *atglr3.4-2* and wild-type seeds was observed in control medium. There was a marked up-regulation of *SOS1* expression in all genotypes upon exposure to NaCl, and the abundance of *SOS1* transcripts in wild-type seeds was higher than in *atglr3.4* mutants (**Fig. 5D**). Taken together, these results reveal that the expression of *SOS1*, *SOS2* and *SOS3* was significantly lower in *atglr3.4* mutants than in wild-type seeds in the presence of NaCl.

AtGLR3.4 is involved in ABA-mediated seed germination

The phytohormone ABA plays a prominent role in seed germination under conditions of environmental stress (Nambara et al. 2010). Recently, Kong et al. (2015) reported that GLR3.5-mediated Ca^{2+} influx stimulates seed germination by antagonizing the inhibitory effect of ABA. To test whether ABA is involved in the AtGLR3.4-mediated seed germination under

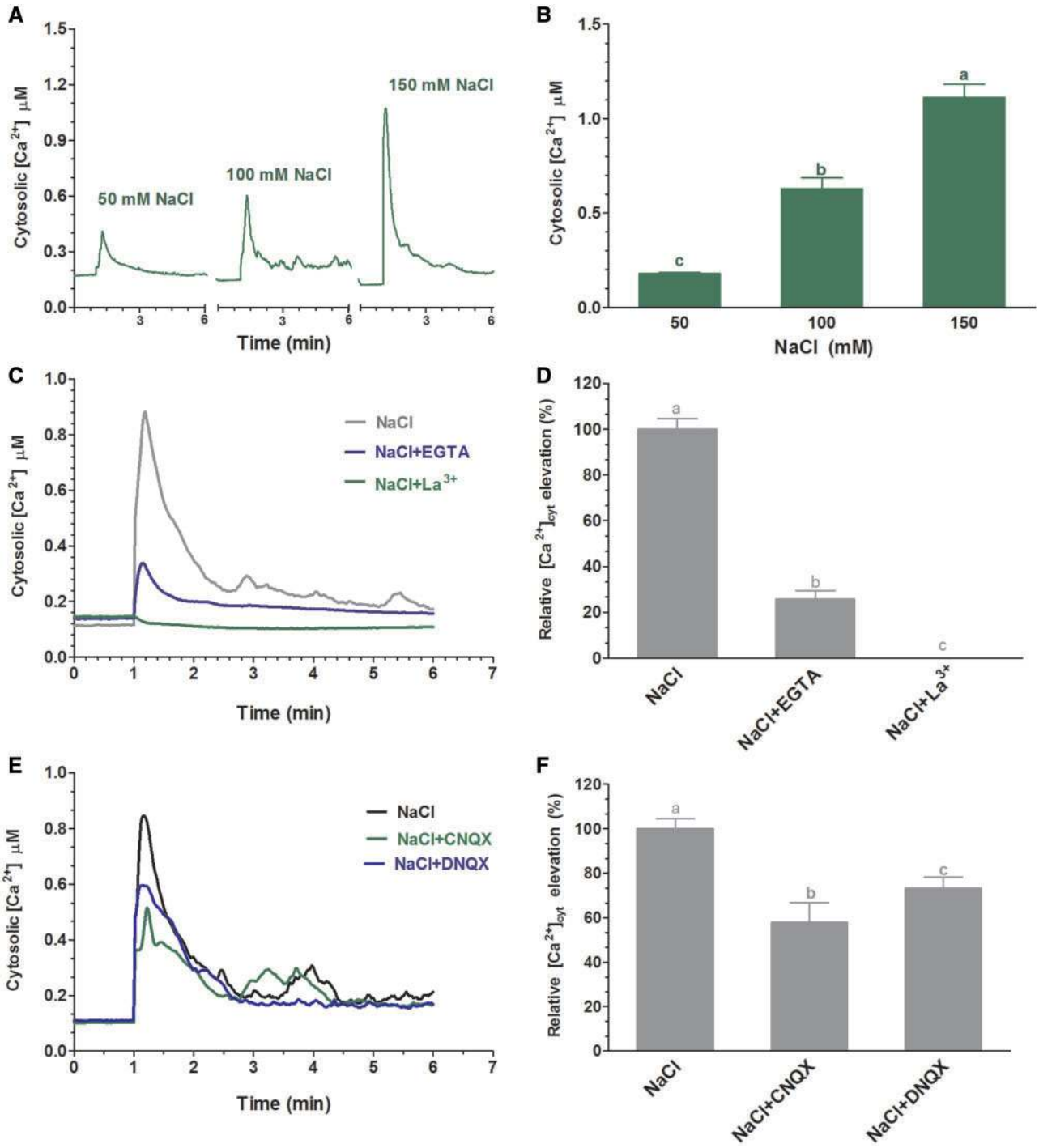

Fig. 3 Effect of NCl treatment on $[Ca^{2+}]_{cyt}$. (A) Representative curves of 50, 100 and 150 mM NaCl-induced $[Ca^{2+}]_{cyt}$ transients in 7 d seedlings. (B) Average peak values of $[Ca^{2+}]_{cyt}$ variation. Representative curves of 150 mM NaCl-induced $[Ca^{2+}]_{cyt}$ transients in 7-day-seedlings with or without application of 1 mM EGTA and 1 mM $LaCl_3$ (C) or CNQX and DNQX (E). Average peak values of $[Ca^{2+}]_{cyt}$ with or without EGTA or $LaCl_3$ (D), and CNQX and DNQX (F), Expressed as a percentage of the maximal peak response after subtracting the background luminescence of the corresponding controls. Data are means ± SE for at least five replicates. Inhibitor treatments were caried out 2 h prior to NaCl treatment. Different letters shown above the error bars indicate significant differences among treatments at $P < 0.05$.

conditions of salt stress, the effects of ABA on seed germination rates of *atglr3.4-1*, *atglr3.4-2* and the wild type were examined. No discernible differences in seed germination rates among *atglr3.4-1*, *atglr3.4-2* and the wild type were detected in the control medium after 2 d incubation (**Fig. 6A**). Seed germination of the wild type and mutants was delayed significantly upon exposure to ABA. However, seed germination in *atglr3.4-1* and *atglr3.4-2* mutants was more sensitive to ABA compared with the wild type (**Fig. 6A, B**). We further generated overexpression of *AtGLR3.4* transgenic plants. Two individual

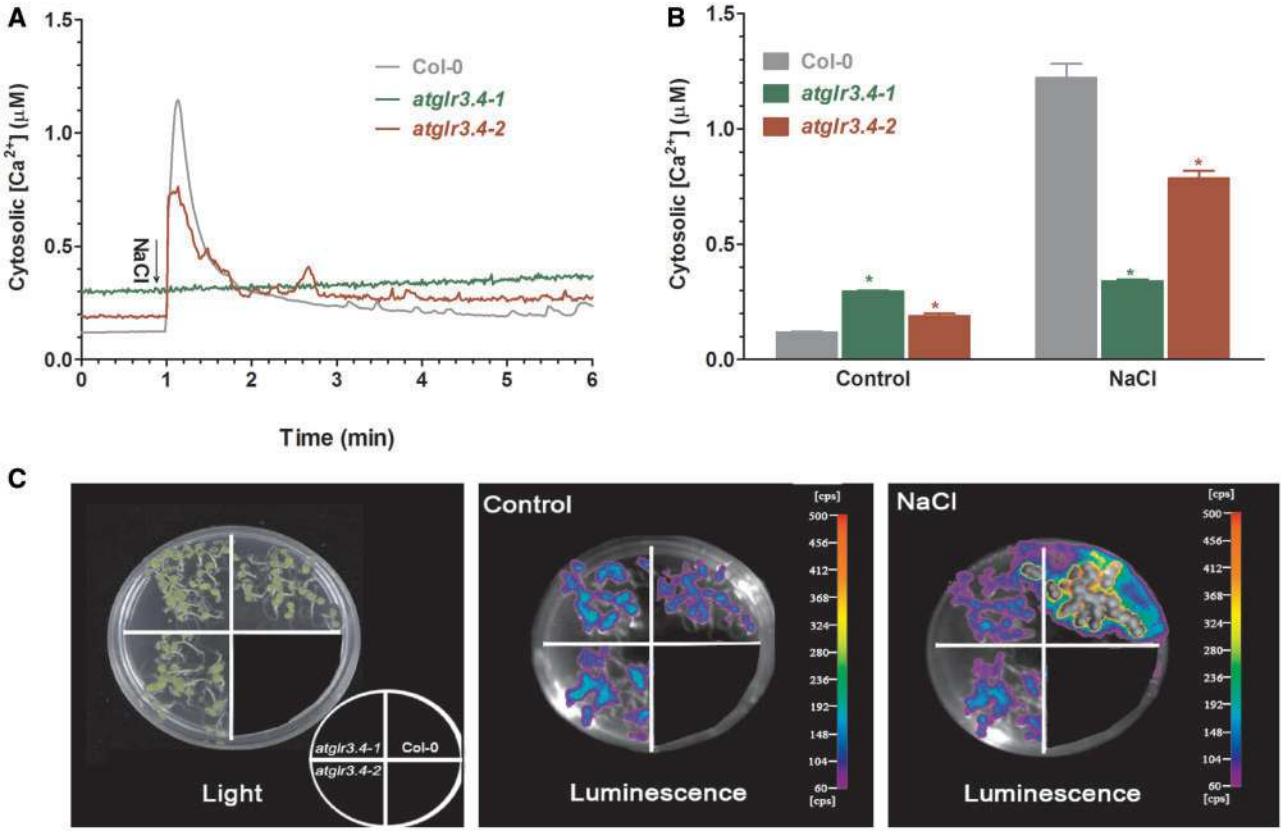

Fig. 4 AtGLR3.4 modulated $[Ca^{2+}]_{cyt}$ transient induction under salt stress. (A) Representative recording curves of 150 mM $[NaCl]_{ext}$-induced calcium transient rise in 7-day-old Col-0, *atglr3.4-1* and *atglr3.4-2* seedlings. (B) Averaged peak values with SE of the responses ($n \geq 5$). An asterisk indicates significant differences ($P < 0.05$) between Col-0 and glutamate mutants within a treatment. (C) Ca^{2+}-sensitive photoprotein aequorin imaging analysis of $[Ca^{2+}]_{cyt}$ in 7-day-old Col-0, *atglr3.4-1* and *atglr3.4-2* seedlings before and after 150 mM NaCl treatment.

overexpressing lines were selected to investigate the effect of ABA on seed germination (**Fig. 6C**). The seed germination rates of overexpression lines were more resistant to ABA than those of wild-type seeds (**Fig. 6D**). These results prompted us to hypothesize that the salt stress-sensitive phenotype of *atglr3.4* mutants is a consequence of altered ABA response in seed germination. To examine the role of ABA in AtGLR3.4-mediated seed germination under salt stress, we first investigated the effect of fluridone, an inhibitor of ABA biosynthesis, on seed germination in the presence and absence of NaCl. Exogenous application of fluridone significantly enhanced germination rates of all lines, with the magnitude of the stimulation of germination rates by fluridone in the *atglr3.4-1* and *atglr3.4-2* mutant seeds similar to that of wild-type seeds (Supplementary Fig. S6A). Accordingly, ABA contents of wild-type, *atglr3.4-1* and *atglr3.4-2* seeds treated with and without NaCl were measured. Surprisingly, endogenous ABA contents in wild-type and *atglr3.4* seeds were not altered by NaCl treatment. Moreover, ABA contents in *atglr3.4-1*and *atglr3.4-2* seeds were similar to those in wild-type seeds in the absence and presence of NaCl (Supplementary Fig. S6B). These results discount the possibility that the greater sensitivity of *atglr3.4* mutants to salt stress during seed germination results from the altered ABA contents in the mutants. In addition, we analyzed the expression patterns of several key transcription factors in ABA-mediated seed germination, such as *ABI3*, *ABI4* and *ABI5* (Supplementary Fig. S6C). The expression of *ABI3*, *ABI4* and *ABI5* was higher in *atglr3.4* mutants under control conditions, which is consistent with the lower germination rate of *atglr3.4* mutants in control medium (Supplementary Fig. S1C; **Fig. 2A**). When treated with NaCl, expression of *ABI3*, *ABI4* and *ABI5* was significantly up-regulated across all lines examined. However, expression levels of *ABI3* and *ABI4* were lower in *atglr3.4* mutants than those in wild-type seeds, and the expression level of *ABI5* in *atglr3.4* mutants was similar to that in wild-type seeds (Supplementary Fig. S6C).

Discussion

Many studies have revealed that GLRs are involved in the regulation of several plant physiological and developmental processes (Michard et al. 2011, Li et al. 2013, Manzoor et al. 2013, Vincill et al. 2013, Kong et al. 2015, Singh et al. 2016). In our previous studies, we demonstrated that mutation of *AtGLR3.4* rendered *atglr3.4* mutants more sensitive to salt stress than wild-type plants in terms of seed germination. However, the mechanisms underlying the AtGLR3.4-mediated seed germination under salt stress remain elusive. In the present study,

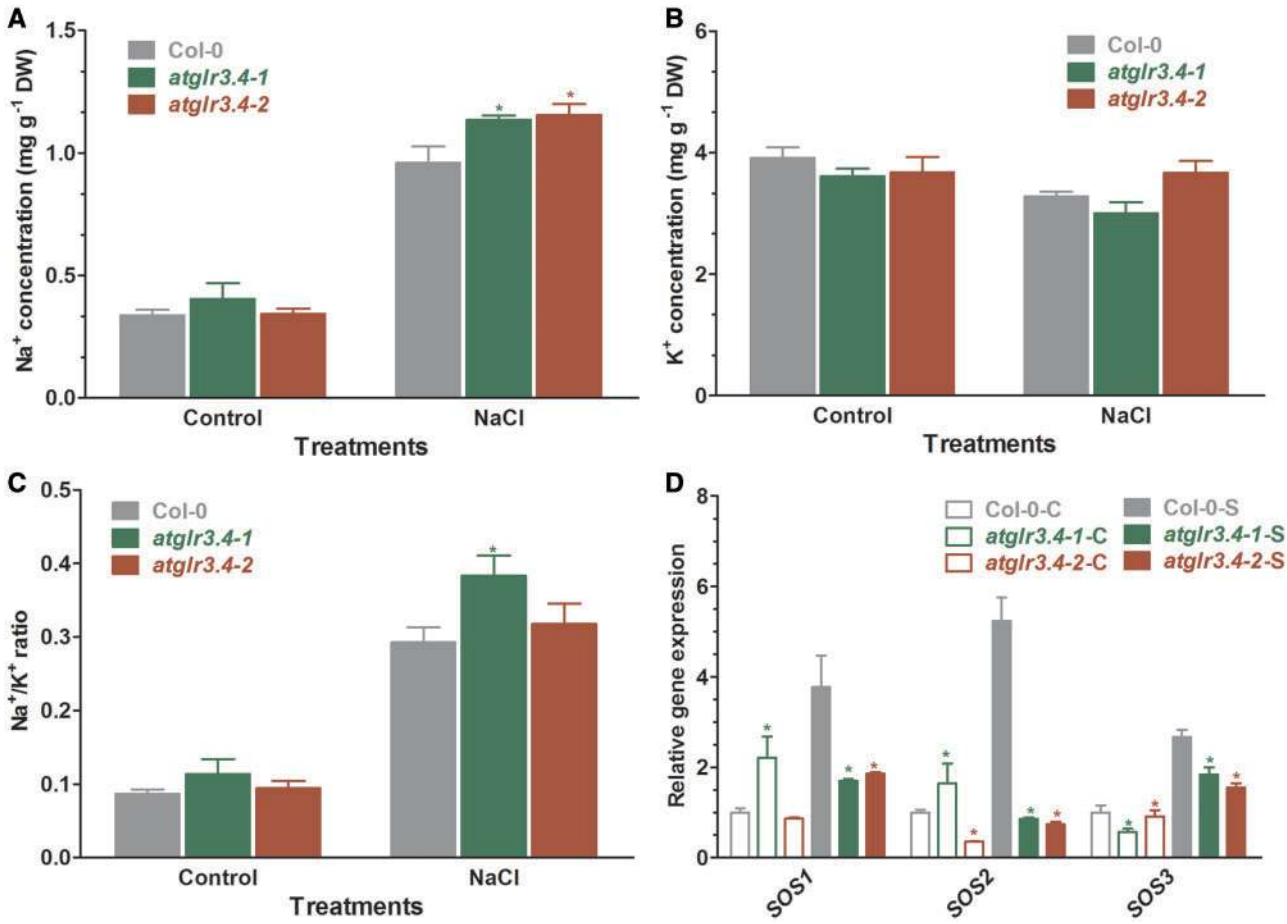

Fig. 5 AtGLR3.4 regulated Na^+ accumulation in germinating seeds under salt stress. (A–C) Concentrations of Na^+ (A), K^+ (B) and Na^+/K^+ (C) in wild-type, *atglr3.4-1* and *atglr3.4-2* seeds germinating for 24 h with or without NaCl. (D) Expression analysis of *SOS* genes in wild-type, *atglr3.4-1* and *atglr3.4-2* seeds germinating for 24 h. The seeds were sown on 1/2 MS medium with or without 150 mM NaCl after 2 d stratification, and germinating seeds were harvested at 24 h. Data are means ± SE with three replicates. Asterisks indicate significant differences ($P < 0.05$) from the wild type (Col-0) within a treatment.

we found that NaCl treatment induced a significant elevation in $[Ca^{2+}]_{cyt}$ in Arabidopsis seedlings (**Fig. 3A, B**), and the elevation was inhibited by GLR antagonists and mutation of *AtGLR3.4* (**Fig. 3C, F**). Moreover, we found that *atglr3.4* seeds accumulated greater amounts of Na^+ and exhibited lower expression levels of *SOS1*, *SOS2* and *SOS3* than the wild type under salt stress. Taken together, these results highlight that AtGLR3.4-mediated Ca^{2+} influx participates in the regulation of seed germination probably by modulating SOS-dependent Na^+ accumulation under salt stress.

Consistent with previous results (Meyerhoff et al. 2005), we found that *AtGLR3.4* showed a broad expression pattern, with the highest transcription level in leaves (Supplementary Fig. S2). To evaluate the role of AtGLR3.4 in response to salt stress, we compared several physiological processes associated with salt stress response between *atglr3.4* and wild-type plants on saline growth medium. One important finding is that *atglr3.4* mutants were more sensitive to salt stress than wild-type plants in terms of seed germination, cotyledon greening rates, early root elongation and survival rates (**Fig. 1**; Supplementary Fig. S3). Given that the differences in survival rate between the wild type and *atglr3.4* mutants were less evident than those in the seed germination rate and early seedling establishment, we conclude that AtGLR3.4 probably functions mainly in seed germination under saline conditions.

A rapid increase in $[Ca^{2+}]_{cyt}$ in response to NaCl treatment has been reported in plant cells (Knight *et al.*, 1997). However, the mechanism underlying this salt stress-induced increase in $[Ca^{2+}]_{cyt}$ is not clear. In our previous studies, we found that AtGRL3.4 was involved in alleviation of the effect of amino acids on NaCl-induced inhibition of seed germination in Arabidopsis, possibly by mediating Ca^{2+} influx (Cheng et al. 2016). Moreover, Vincill et al. (2012) demonstrated that AtGLR3.4 functions as an asparagine/serine, glycine-gated Ca^{2+}-permeable channel to mediate $[Ca^{2+}]_{cyt}$ variation. Here, we found that exposure of Arabidopsis seedlings to NaCl treatment evoked a rapid elevation in $[Ca^{2+}]_{cyt}$ (**Fig. 3A, B**), and the NaCl-induced $[Ca^{2+}]_{cyt}$ rise was inhibited by the chelating agent EDTA and antagonists of GLRs (**Fig. 3C–F**). In accordance with these results, seed germination of *atglr3.4* mutants was less sensitive to the Ca channel blocker $LaCl_3$ than wild-type plants under salt stress (Supplementary Fig. S4), and mutation of *AtGLR3.4* led to a drastic decline in NaCl-induced

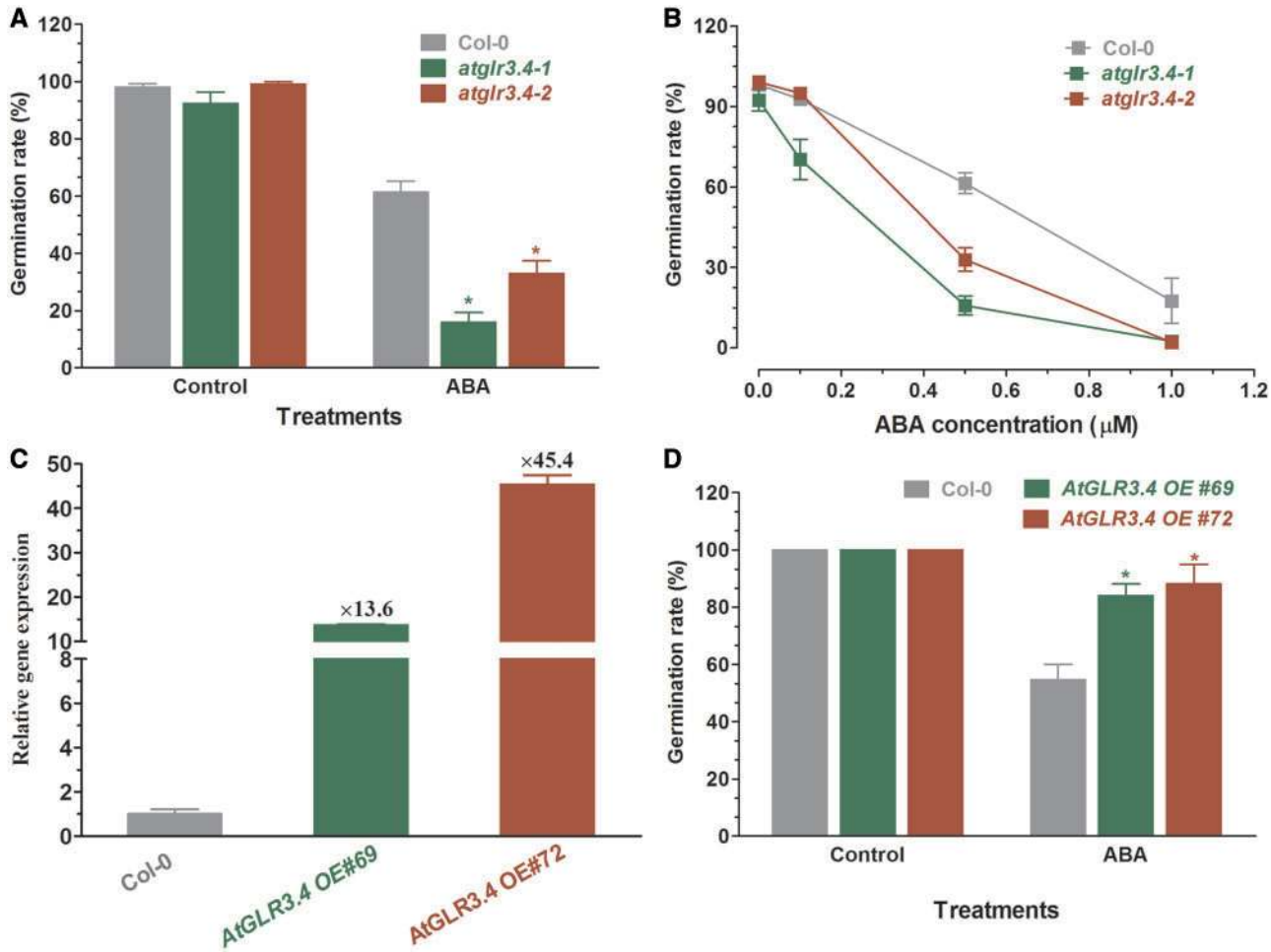

Fig. 6 *atglr3.4* mutants exhibited an ABA-hypersensitive phenotype in seed germination. (A) Germination rates of wild-type and *atglr3.4* mutant seeds treated with or without ABA (0.5 μM) after 2 d incubation following stratification. (B) Seed germination rates of the wild type (Col-0), *atglr3.4-1* and *atglr3.4-2* after 2 d incubation following stratification at the indicated concentrations of ABA. (C) Quantitative real-time PCR analysis of *AtGLR3.4* expression in Col-0 and its overexpression lines. Data are means ± SE for three replicates. (D) Germination analysis of wild-type and *AtGLR3.4*-overexpresing seeds treated with or without ABA (0.5 μM) after 2 d incubation. Data are means ± SE for three replicates, with each replicate containing 30 seeds. Asterisks indicate significant differences ($P < 0.05$) from the wild-type (Col 0) within a treatment.

$[Ca^{2+}]_{cyt}$ elevation (**Fig. 4**). Taken together, our results demonstrated that AtGLR3.4 is involved in mediating Ca^{2+} influx under salt stress, providing a mechanistic explanation for the salt stress-induced elevation in $[Ca^{2+}]_{cyt}$.

The ability to minimize net Na^+ influx into the cytoplasm is critical to improve salt tolerance for plants (Zhu 2001, Julkowska and Testerink 2015). The plasma membrane Na^+/H^+ antiporter SOS1 is the only Na^+ efflux protein so far identified in plant cells (Almeida et al. 2017). Our results revealed that mutation of *AtGLR3.4* caused a lower expression of *SOS1*, *SOS2* and *SOS3*, and higher Na^+ accumulation in mutants than in wild-type plants under salt stress (**Fig. 5**). These results suggest that AtGLR3.4-mediated Ca^{2+} influx is involved in the regulation of expression of *SOS* genes to mediate Na^+ accumulation in seeds when suffering from salt stress, highlighting the important role of the SOS pathway in seed germination under salt stress.

The phytohormone ABA plays a prominent role in seed germination and dormancy (Lopez-Molina et al. 2001, Lopez-Molina et al. 2002). When seeds suffer from osmotic stress and/or exogenous application of ABA, germination is often arrested or delayed during a time window of about 48 h after seed imbibition, and the stress signals can stimulate and maintain *ABI3* and *ABI5* expression, thus resulting in sustained arrest of germination and growth (Lopez-Molina et al., 2002). Kong et al. (2015) demonstrated that AtGLR3.5 mediated seed germination by repressing the expression of *ABI4*. In the present study, we found that seed germination of *atglr3.4* mutants was more sensitive to ABA, while seed germination of *AtGLR3.4* overexpression lines was less sensitive to ABA than wild-type plants (**Fig. 6**). Another observation is higher expression of *ABI3*, *ABI4* and *ABI5* in *atglr3.4* mutants than in the wild type in control medium (Supplementary Fig. S6C), implying the involvement of AtGLR3.4 in ABA-mediated seed germination. However, we found that fluridone, an ABA biosynthesis antagonist, exhibited a similar alleviatory effect on seed germination of *atglr3.4* mutants and the wild type in salt stress. Similar to the *atglr3.5* mutant (SALK_035264) (Supplementary Fig. S7), there was no significant difference in ABA content between wild-type

and *atglr3.4* seeds when treated with NaCl (Supplementary Fig. S6B). Moreover, in contrast to the higher expression levels of *ABI3*, *ABI4* and *ABI5* in *atglr3.5* seeds (Supplementary Fig. S8), the expression levels of *ABI3* and *ABI4* were lower in *atglr3.4-1* and *atglr3.4-2* mutants than in wild-type seeds in the presence of NaCl, while the expression of *ABI5* in *atglr3.4-1* and *atglr3.4-2* mutants was comparable with that of wild-type seeds (Supplementary Fig. S6C). These results may imply that the regulatory role of AtGLR3.4 in seed germination under salt stress is not directly associated with the ABA pathway.

In conclusion, we demonstrated that *atglr3.4-1* and *atglr3.4-2* mutants were hypersensitive to NaCl treatments during seed germination and post-germination growth, with an impaired NaCl-induced elevation in $[Ca^{2+}]_{cyt}$. When exposed to salt stress, the *atglr3.4* mutants accumulated greater amounts of Na^+ than wild-type seeds due to lower expression of *SOS1*, *SOS2* and *SOS3* in the mutants. These results are valuable for dissecting the molecular mechanisms underlying the regulatory roles of AtGLR3.4-dependent Ca^{2+} signaling in seed germination under salt stress.

Materials and Methods

Plant materials and growth conditions

Arabidopsis plants were grown in soil or in Petri dishes in the greenhouse at 25/22°C with a 14/10 h light/dark cycle. The *atglr3.4* T-DNA insertion mutants were kindly provided by Professor Lai-Hua Liu (China Agricultural University, Beijing, China). All the T-DNA insertion mutant lines are in the Col-0 background. Col::*aequorin* seeds were kindly provided by Professor Zhi Qi (Inner Mongolia University, Hohhot, China). The aequorin-expressing *atglr3.4* T-DNA insertion mutants were generated by cross-pollination with Col-0 expressing apoaequorin. Homozygous T_3 *atglr3.4* mutants plants expressing apoaequorin were detected with gene-specific primers (SALK_079842, 5′-GGGTTAATCCGGCTTATGAAG-3′, 5′-GAAGTGAGACTGGCCGTGTAG-3′; and SALK_016904, 5′-TTCAGAGAGGAGCCAACAGAG-3′ and 5′-TGCAAATTCCGTACAGTAGGG-3′) and the T-DNA left border-specific primer (5′-ATTTTGCCGATTTCGGAAC-3′). The presence of active aequorin was validated by recording luminesence.

Plasmid construction and generation of transgenic plants

For the construction of *AtGLR3.4* overexpression transgenic plants, the coding sequence of *AtGLR3.4* was amplified using cDNA by PCR with gene-specific primers (forward, 5′-AAA ACT GCA GAT GGG ATT TGG TGA TAA G-3′; reverse, 5′-GGA CTA GTA ATT TCG CCA TGT GAT-3′). The resulting PCR product was cloned into the *Pst*I and *Spe*I sites of the binary vector pCAMBIA1300-GFP under the control of the *Cauliflower mosaic virus* 35S promoter. For the construction of AtGLR3.4::*GUS* transgenic plants, an 1,811 bp promoter sequence was amplified from genomic DNA by PCR with the following primer pairs (forward, 5′-CCA AGC TTC GTC AGC TTC TGT AAT C-3′; reverse, 5′-CGG ATC CCT TTA TCA CAA TTT CCA G-3′). The PCR fragment was cloned into the *Hin*dIII and *Bam*HI sites of pBI121 to obtain the construct containing the *AtGLR3.4* native promoter fused in the GUS coding region. The resultant plasmids were introduced into Columbia wild-type plants by a floral dip infiltration method using *Agrobacterium tumefaciens* GV3101. T_2 seeds from each selected transgenic plant were plated on 1/2 MS medium containing 50 mg l^{-1} hygromycin (for pCAMBIA1300-GFP) or kanamycin (for PBI121) as selective antibiotics to select the homozygous lines.

Seed germination assay

To minimize biological variation, seeds used in a given experiment were grown under identical conditions and harvested at the same time. Seeds were surface sterilized by incubation for 1 min in 75% ethanol, and rinsed with sterile distilled water, followed by 15 min in 10% (v/v) sodium hypochlorite, and then washed with sterile water. For each comparison, the seeds were sown on the same plate containing MS medium (1/2 MS salts, 0.8% sugar and 0.8% agar) with and without different concentrations of NaCl, mannitol and ABA as indicated. $LaCl_3$, a Ca^{2+} channel blocker, was added to the medium at the indicated concentrations after autoclaving. Germination is defined as the emergence of the radicles through the seed coat. Germination assays were carried out with at least three replicates and containing 30 seeds for each replicate.

Determination of cotyledon greening

To study the effect of NaCl on cotyledon greening, seeds were sown on 1/2 MS medium supplemented with different concentrations of NaCl as described above. The percentage of cotyledon greening was recorded at 7 d after the end of stratification. Cotyledon greening is defined as obvious cotyledon expansion and turning green. The cotyledon greening rate was calculated from the results of three independent experiments.

Measurements of early root elongation

Wild-type and *atglr3.4* mutants seeds were directly sown on 1/2 MS medium supplemented with different concentrations of NaCl after 2 d stratification. Root elongation was measured after 7 d of treatment with Image-J software.

Determination of survival rates

For the survival assays after salt stress, 7 d seedlings of wild-type and *atglr3.4* mutants were transferred to 1/2 MS medium supplemented with and without NaCl. The survival rates were counted on the 14th day. The experiments were repeated three times.

Histochemical GUS staining

Plant materials were harvested and fixed in 90% acetone for 20 min. All samples were rinsed with 100 mM sodium phosphate buffer (pH 7.0). After vacuum infiltration with GUS staining solution (1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-glucuronic acid; 100 mM sodium phosphate buffer, pH 7.0; 10 mM EDTA; 0.5 mM potassium ferrocyanide; 0.5 mM potassium ferricyanid; and 0.1% Triton X-100) for 15 min each, plant materials were incubated overnight at 37°C in the dark (Meyerhoff et al. 2005). The tissues were cleaned with 70% ethanol and observed with a stereoscope. Seed coats were removed under a dissecting microscope before GUS staining.

Measurement of $[Ca^{2+}]_{cyt}$ in Arabidopsis seedlings expressing aequorin

Seeds were sown and grown on 1/2 MS medium as described above. To reconstitute the aequorin with its substrate, 7-day-seedlings were transferred to a 1.5 ml centrifuge tube with 100 µl of control buffer composed of 1 mM KCl, 1 mM $CaCl_2$ and 5 mM MES (pH 5.7). Under dim light, coelenterazine *hcp* (Promega) was added into the buffer to a final concentration of 5 µM. After approximately 8 h incubation in the dark, the tube was left to stand for 5 min to avoid potential disturbances of $[Ca^{2+}]_{cyt}$ triggered by handling. For the treatment, 100 µl of control buffer with twice the final concentration of NaCl 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 seedling. This process avoided directly dropping the treatment solution into control buffer in the tubes. Pre-treatment with EGTA or GLR antagonists was done 2 h prior to NaCl treatment. Luminescence signals were measured at a rate of 1 Hz using s single-tube luminometer (Promega). The remaining aequorin was discharged by delivery of 400 µl of 2 M $CaCl_2$ in 20% ethanol, and luminescence was recorded until values were within 1% of the highest discharge values (Li et al. 2013). The absolute $[Ca^{2+}]_{cyt}$ was calculated according to Rentel and Knight (2004). To validate our experimental system, we have examined the effect of exogenous ddH_2O and H_2O_2 on cytosolic Ca^{2+} concentration. For aequorin bioluminescence-based Ca^{2+} imaging analysis, sterilized seeds were sown on 1/2 MS plates and grown in Petri dishes for 7 d. To reconstitute the aequorin with its substrate, the seedlings in Petri dishes were immersed with control buffer containing 5 µM coelenterazine *hcp* for 6 h. Bright-field images

were taken before aequorin reconstitution, and aequorin images were taken after 1 min recording using a PIXIS CCD camera (Berthold Technologies) before or after seedlings were exposed to control buffer containing 150 mM NaCl (Kong et al. 2015).

Determination of Na^+ and K^+ concentrations in seeds

Seeds of *atglr3.4* mutants and the wild type were harvested and rinsed thoroughly with deionized water. Samples were oven dried at 75°C for 2 d until they reached constant mass. Plant materials were digested completely (approximately 4 h) in 70% (v/v) HNO_3 at 130°C as described by Tian et al. (2016). The concentrations of Na^+ and K^+ were measured by inductively coupled plasma optical emission spectrometry (ICP-OES; ICAP6300, Thermo Scientific).

RNA isolation and quantitative real-time PCR

Seeds were ground in liquid nitrogen and total RNA was extracted using HiPure Plant RNA Kits (Magen). First-strand cDNAs were reverse transcribed from 1 μg of total RNA with the PrimeScript® RT reagent Kit with gDNA Erager (TAKARA). Quantitative real-time PCR was performed in an optical 96-well plate with an ABI StepOne Plus instrument. Each reaction contained 5.0 μl of 2× UltarSYBR Mixture (with ROX) reagent (Cwbio), 1 μl of cDNA samples and 0.6 μl of 10 μM gene-specific primers in a final volume of 10 μl. The thermal cycle program was 95°C for 10 min, 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. The primers pairs used for each gene are listed in Supplementary Table S1. *Arabidopsis thaliana Actin11* (accession No. NM_112046) was used as an internal control with primers 5′-TGTTCTTTC CCTCTACGCT-3′ and 5′-CCTTACGATTTCACGCTCT-3′. The relative expression level was analyzed by the comparative Ct method using Microsoft Excel (Livak and Schmittgen 2001).

Determination of endogenous ABA contents in seeds

The endogenous ABA contents in seeds were determined by gas chromatography–mass spectrometry (GC-MS) following a modification of protocols described by Müller et al. (2002). Briefly, the Arabidopsis seeds (about 10 mg) were collected, instantly frozen and grounded into a fine powder in liquid nitrogen. To better preserve plant hormones in seeds, the ground materials were dried under vaccum (0.08 mbar) at a low temperature. The seeds were weighed, their weight recorded and then extracted with MeOH solvent ($MeOH/H_2O$ = 4:1) containing [$^2H_6$] ABA as internal standards, then the extract was evaporated to dryness. The dried extract was re-dissolved in MeOH solvent and passed through a C18 Sep-pack column, followed by MeOH solvent ($MeOH/H_2O$ = 4:1) elution. The fraction containing plant hormones was collected and dried by a centrifugal concentrator. The plant hormones in extracts were trimethylsilylated with *N*-methyl-*N*-trimethylsilylteifluoroacetamide (MSTFA) at 80°C for 30 min. Samples were taken to dryness and dissolved in hexane before they were analyzed in a GC-QqQ MS 7890/7000 C (Agilent) with a fused silica glass capillary column DB-5 (0.2 5 mm × 30 mm, 0.25 μm film thickness, J&W). Injection and interface temperatures were 260 and 280°C, respectively. The column temperature gradient was maintained at 80°C for 2 min, then increased by 6°C min^{-1} to 250°C, followed by 20°C min^{-1} to 300°C. The identification of ABA was confirmed by monitoring diagnostic ions of both endogenous and deuterated ABA according to Müller et al. (2002). The internal standard [$^2H_6$]ABA (No. 034 2723) was purchased from OlChemIm Company.

Statistical analysis

All data were analyzed by ANOVA using SPSS statistical software. Significant differences were evaluated using Student's *t*-test.

Supplementary Data

Supplementary data are available at PCP online.

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