Environmental and Experimental Botany 130 (2016) 68–78

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Glutamate receptors are involved in mitigating effects of amino acids on seed germination of *Arabidopsis thaliana* under salt stress

Yao Cheng[a,b], Qiuying Tian[a], Wen-Hao Zhang[a,c,*]

[a] State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, The Chinese Academy of Sciences, Beijing 100093, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China
[c] Research Network of Global Change Biology, Beijing Institutes of Life Science, The Chinese Academy of Sciences, Beijing, PR China

ARTICLE INFO

Article history:
Received 29 October 2015
Received in revised form 3 May 2016
Accepted 5 May 2016
Available online 17 May 2016

Keywords:
Amino acids
Arabidopsis thaliana
atglr3.4 and *atglr3.7*
Glutamate receptors
Salt stress
Seed germination

ABSTRACT

Seed germination is an elaborate process during life cycle and regulated by numerous intrinsic and environmental cues. The role of amino acids in seed germination under salt stress has been reported, however the underlying mechanisms by which amino acids mitigate salt stress-induced seed germination remain largely elusive. Here, we reported that seed germination of *Arabidopsis thaliana* wild-type was suppressed by exposure to salt stress, and that the inhibition was markedly alleviated by exogenous application of those amino acids including Gly, Cys, Ser and Met. Calcium channel blocker La^{3+} suppressed the mitigating effect of those amino acids on seed germination under salt stress. We further demonstrated that germination of wild-type seeds was suppressed by the glutamate receptor (GLR) antagonist 6,7-dinitriquinoxaline-2,3-dinoe (DNQX) under salt stress and the alleviative effects of amino acid on seed germination under salt stress were suppressed by DNQX. In addition, mutation of clade 3 GLRs led to lower seed germination rates of *atglr3.4* and *atglr3.7* mutants than those of wild-type seeds when exposed to NaCl and the mitigating effects of amino acids on seed germination rates of *atglr3.4* and *atglr3.7* were less effective than those of wild-type seeds. Moreover, seed germination of *atglr3.4-1*, *atglr3.4-2* and *atglr3.7* mutants were less sensitive to $CaCl_2$ and DNQX than wild-type under salt stress. Taken together, these findings highlight the involvement of GLR-dependent Ca^{2+} influx in alleviation of salt stress-induced inhibition of seed germination by exogenous amino acids.

1. Introduction

Seed germination, a key economic and ecological trait, is considered to be the most critical phase in plant life cycle (Holdsworth et al., 2008; Rajjou et al., 2012). Seed germination includes those events starting with uptake of water by the quiescent dry seed and terminating with two sequential phase of testa and endosperm rupture through the expansion of the embryo and endosperm weakening (Bewley 1997; Holdsworth et al., 2008; Rajjou et al., 2012). This is an elaborate developmental process regulated by various developmental and environmental cues (Finch-Savage and Leubner-Metzger 2006). Seed germination is sensitive to saline environment evoked by osmotic stress and sodium toxicity (Daszkowska-Golec 2011; Zhu 2001). A number of phytohormones and messenger molecules, including gibberellic acid (GA), ethylene (Deng et al., 2009; Wilson et al., 2014; Yuan et al., 2011), nitric oxide (NO) and hydrogen peroxide (Lin et al., 2012; Zhao et al., 2007), have been reported to alleviate the inhibitory effect of salt stress on seed germination.

In addition to the phytohormones, calcium ions can also mitigate the inhibitory effects of salt stress on seed germination (Tobe et al., 2003; Zehra et al., 2012). Calcium is not only an essential mineral nutrient for plant growth and development, but it also acts as a versatile second messenger to participate in numerous physiological processes in plants (Dodd et al., 2010). The transient elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), which in turn elicits downstream gene expression and biological events, is a well-established signaling cascade in response to environmental and/or developmental stimuli in

---

[*] Corresponding author at: State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, The Chinese Academy of Sciences, Beijing 100093, PR China.

E-mail address: whzhang@ibcas.ac.cn (W.-H. Zhang).

http://dx.doi.org/10.1016/j.envexpbot.2016.05.004
0098-8472/© 2016 Elsevier B.V. All rights reserved.

plants (Dodd et al., 2010; Kudla et al., 2010). For example, plants exhibit a rapid increase in $[Ca^{2+}]_{cyt}$ upon exposure to NaCl (Knight et al., 1997). The increased $[Ca^{2+}]_{cyt}$ positively regulates seed germination by antagonizing the inhibitory effect of ABA (Kong et al., 2015). The spatiotemporal changes of $[Ca^{2+}]_{cyt}$ in living cells are accurately regulated by channels, pumps and carriers in the membranes that underlie Ca^{2+} homeostasis (Dodd et al., 2010; Kudla et al., 2010). In plants, several gene families have been suggested to encode Ca^{2+}-permeable channels, among them ionotropic glutamate receptors homologs (GLRs) are potential Ca^{2+}- permeable channels to mediate Ca^{2+} influx (Jammes et al., 2011; Swarbreck et al., 2013).

In Arabidopsis, GLRs consist of 20 members that are grouped into three clades based on their sequence homology with high structural similarity to animal ionotropic glutamate receptors (iGluRs) (Chiu et al., 2002; Lacombe et al., 2001; Lam et al., 1998). Pharmacological and genetic studies have revealed that the plant GLRs play regulatory roles in many physiological and developmental processes, ranging from abscisic acid biosynthesis (Kang et al., 2004) and carbon/nitrogen sensing (Kang and Turano, 2003) to root development (Li et al., 2006; Vincill et al., 2013) and aluminum toxicity (Sivaguru et al., 2003). In mammalian cells, iGluRs are ligand-gated cation channels mediating the excitatory neurotransmission in the central nervous system (Dingledine et al., 1999). Similar to the iGluRs in mammalian cells, exogenous application of Glu and Gly triggers large and rapid increases in $[Ca^{2+}]_c$ and a concomitant membrane depolarization in plants (Dennison and Spalding, 2000). The rise in $[Ca^{2+}]_{cyt}$ triggered by six different amino acids (Glu, Ala, Asn, Cys, Gly, Ser and Met) in wild-type Arabidopsis disappeared in the *aglr3.3* mutants (Qi et al., 2006). Moreover, a patch-clamp study in the transfected human embryonic kidney (HEK) cells showed that AtGLR3.4 is a Ca^{2+}-permeable cation channel activated by Asp, Ser and Gly, but not by Glu (Vincill et al., 2012). Moreover, AtGLR1.4 also has been demonstrated to function as a ligand-gated, nonselective, Ca^{2+}-permeable cation channel that is responsive to amino acids by heterologous expression of *AtGLR1.4* in Xenopus oocytes (Tapken et al., 2013). These results suggest that glutamate receptors can act as sensors and mediators to mediate Ca^{2+} influx across plasma membranes in response to exogenous amino acids.

Amino acids play critical roles in plants, ranging from assembly of proteins to being an exogenous signal to modulate physiological and developmental processes (Michard et al., 2011; Sivaguru et al., 2003; Walch-Liu et al., 2006). Physiological studies have shown that amino acids were implicated in the regulation of seed germination, such that exogenous application of amino acids can alleviate salt-induced inhibition of seed germination (Chang et al., 2010; King and Gifford, 1997). However, the underlying mechanism by which exogenous amino acids modulate seed germination remain largely elusive. In the present study, the mechanistic hypothesis that Ca^{2+}-influx mediated by GLRs is involved in amino acids-induced alleviation of seed germination under salt stress was tested by pharmacological and genetic approaches. Our results showed that exogenous application of the amino acids, including Gly, Cys, Ser and Met, significantly mitigated the salt stress-induced inhibition of seed germination in wild-type Arabidopsis, and that the mitigating effect was markedly suppressed by Ca^{2+} channel blockers $LaCl_3$ and DNQX, an iGLRs antagonist. We also demonstrated that seed germination of the *glr3.4* and *glr3.7* T-DNA insertion mutants was more sensitive to salt stress than their wild-type counterpart. We further showed that the knockout of *Atglr3.4* and *Atglr3.7* rendered the germination of mutants seeds less responsive to amino acids, $CaCl_2$ and DNQX under salt stress, implying that the involvement of GLRs in the alleviative effects of amino acids on salt-induced inhibition of seed germination.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis plants were grown in soil or in petri dishes in the green house at 25/22 °C with a 14/10 light/dark cycle. Seeds of *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) were obtained from the Arabidopsis Biological Resource Center and *glr3.2*, *glr3.3*, *glr3.4-1*, *glr3.4-2*, *glr3.5*, *glr3.6*, *glr3.7* 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.

2.2. Seed germination assays

To minimize biological variation, seeds used in a given experiment were 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. After stratification for 2 d at 4 °C in darkness, the seeds were placed on half-strength MS medium containing 0.8% (w/v) sugar, 0.8% (w/v) agar and NaCl. The amino acids, $LaCl_3$ and 6,7-dinitriquinoxaline-2,3-dinoe (DNQX) that are Ca^{2+} channel blocker and antagonist of GLRs, respectively, were added to the medium at the indicated concentrations after autoclaving. Seed germination is defined as the emergence of the radicles through the seed coat. For the determination of seed germination rate, 30 seeds per sample in each treatment were tested, and at least three biological replicates were performed.

2.3. Genotyping of mutants

To screen individual homozygous mutants, genomic DNA was extracted from leaf tissues as described by Edwards et al. (1991). PCR was performed to verify the presence of wild-type or the T-DNA insertion in homozygous mutants plants using a T-DNA specific border primer (ATTTTGCCGATTTCGGAAC) in combination with gene-specific primers (Table S1). PCR products were evaluated through agarose gel electrophoresis.

2.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted with Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Promega). The 2 μg total RNAs were reverse transcribed into first-strand cDNA in a 20-μl volume with M-MLV reverse transcriptase (Promega) by random primers. Real-time PCR was carried out using SYBR green PCR master mix and an ABI 7900 sequence detection system (Applied Biosystems). Each reaction contained 12.5 μl of the SYBR Green Master Mix reagent, 5 μl diluted cDNA and 2 μl of 10 μM gene-specific primers in a final volume of 25 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 corresponding specific primers were: for *Atglr3.4*, 5′-CTGGAGGGTTTTCTGGCA-3′ and 5′-TGAAACTCGGTGCTCGTG-3′; for Atglr3.7, 5′-CCCGAAGACAACTCAGCACA-3′ and 5′-GCGGTTAGAACCATCA ATAGG. *Arabidopsis thaliana Actin11* (accession No. NM_112046) was used as an internal control with primers 5′-TGTTCTTTCCCTCTACGCT-3′ and 5′- CCTTACGATTTCACGCTCT-3′. The relative expression level was analyzed by the comparative Ct method using the Microsoft Excel (Livak and Schmittgen, 2001).