Alleviation of salt stress-induced inhibition of seed germination in cucumber (*Cucumis sativus* L.) by ethylene and glutamate

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ABSTRACT

Ethylene is an important plant gas hormone, and the amino acid Glu is emerging as a messenger molecule in plants. To evaluate the role of ethylene and Glu in seed germination and radicle growth under salt stress, effects of 1-aminocyclopropane-1-carboxylic acid (ACC), Ethephon and Glu on germination and radicle growth of cucumber (*Cucumis sativus* L.) seeds in the absence and presence of 200 mM NaCl were investigated. Seed germination was markedly inhibited by salt stress, and this effect was alleviated by ACC and Ethephon. In contrast to seed germination, ACC and Ethephon had little effect on radicle growth under salt stress. In addition to ethylene, we found exogenous supply of Glu was effective in alleviating the salt stress-induced inhibition of seed germination and radicle growth. The effect of Glu on the seed germination and radicle growth was specific to L-Glu, whereas D-Glu and Gln had no effect. There was an increase in ethylene production during seed imbibition, and salt stress suppressed ethylene production. Exogenous L-Glu evoked ethylene evolution from the imbibed seeds and attenuated the reduction in ethylene evolution induced by salt stress. The alleviative effect of L-Glu on seed germination was diminished by antagonists of ethylene synthesis, aminoethoxyvinylglycine (AVG) and CoCl₂, suggesting that L-Glu is likely to exert its effect on seed germination by modulation of ethylene evolution. These findings demonstrate that ethylene is associated with suppression of seed germination under salt stress and that L-Glu interacts with ethylene in regulation of seed germination under salt stress.

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Introduction

Seed germination is a complicated process and is sensitive to many hormonal and environmental cues (Finch-Savage and Leubner-Metzger, 2006). Among the phytohormones, ABA inhibits seed germination, while ethylene and gibberellin acid (GA) antagonize the ABA-induced inhibitory effect on seed germination (Finch-Savage and Leubner-Metzger, 2006; Matilla and Matilla-Vazquez, 2008; Linkies et al., 2009). It has been widely reported that ethylene production is stimulated during seed imbibition and that peak ethylene production is correlated with radicle protrusion (Fu and Yang, 1983). However, it remains to be established whether the ethylene production acts as a trigger to elicit seed germination or the ethylene production is a consequence of seed germination and radicle protrusion. Ethylene is not only involved in seed germination under optimal conditions (Matilla and Matilla-Vazquez, 2008), but may also play an important role in alleviating the inhibitory effect of the stressed environment on seed germination in many species (Kepczynski and Kepczynska, 1997). There have been numerous reports demonstrating that seed germination of both glycophytes (Zhu, 2003) and halophytes (Khan and Huang, 1988; Gul and Weber, 1998; Li et al., 2005) is inhibited by salinity. The inhibitory effect of salt stress on seed germination is alleviated by phytohormones, including cytokinin (Khan and Huang, 1988), ethylene (Khan and Huang, 1988; Kepczynski and Kepczynska, 1997; Gul and Weber, 1998), GA (Khan and Ungar, 1998; Khan et al., 2004) and messenger molecules such as nitric oxide (NO) (Zhao et al., 2007).

In addition to phytohormones, seed germination is also sensitive to external nitrogen (N) in general and nitrate/nitrite in particular (Bethke et al., 2006) and glucose (Zhao et al., 2009). Recent studies have also revealed that organic N, particularly L-Glu, plays a regulatory role in modulation of root growth and development (Sivaguru et al., 2003; Walch-Liu et al., 2006; Walch-Liu and Forde, 2008). Glu has also been shown to interact with signaling cascades of Ca²⁺ (Dennison and Spalding, 2000) and ABA in plants (Kang et

al., 2004). There is evidence suggesting the involvement of glutamate receptors (GLRs) in plant development and stress responses (Ford and Lea, 2007; Roy et al., 2008). Given that seed germination is sensitive to ABA (Finch-Savage and Leubner-Metzger, 2006) and Glu is associated with ABA signaling cascades (Kang et al., 2004), it is conceivable that Glu may also be involved in seed germination. However, to our knowledge, there have been no studies to evaluate the role of Glu in seed germination and the interactions between ethylene and L-Glu in physiological processes. In the present study, we investigated the effect of Glu and ethylene on seed germination and radicle growth of cucumber under salt stress. Our results indicate that both Glu and ethylene alleviate salt stress-induced inhibition of seed germination. The possible interactive mechanisms underlying the alleviative effect of ethylene and Glu on seed germination exposed to salt stress are discussed.

Materials and methods

Determination of seed germination and radicle length

Seeds of cucumber (*Cucumis sativus* L., cv Zhongnong 8) were surface-sterilized for 15 min in 10% (v/v) sodium hypochlorite solution, and rinsed thoroughly with deionized water. Seeds were then used to investigate the effect of NaCl on seed germination. Cucumber seeds were placed randomly in Petri dishes (9.0 cm diameter) containing filter paper soaked with 0.5 mM $CaCl_2$ solution containing either 0 (control) or different concentrations of NaCl (100 and 200 mM) at 28 °C for 48 h in a growth chamber in the dark. There were 40 seeds in each Petri dish and the seeds were soaked with 5 mL treatment solution. Seeds were considered to be germinated at the emergence of the radicle and scored. The effects of Glu and ethylene on seed germination in the absence and presence of NaCl were investigated by treating the seeds with the solutions containing Glu (both L-Glu and D-Glu), ethylene synthesis precursor (1-aminocyclopropane-1-carboxylic acid, ACC), ethylene releaser (Ethephon) and antagonists of ethylene synthesis (aminoethoxyvinylglycine, AVG and $CoCl_2$). All the compounds were freshly prepared as stock solutions in sterilized deionized water for each experiment, and diluted to the appropriate concentrations just before use.

Measurements of ethylene evolution

Ten cucumber seeds that were exposed to solutions with and without 10 mM L-glutamate in the absence and presence of 200 mM NaCl for varying time periods were put in to 5 mL gas-tight vials. One milliliter of the headspace was taken from the vials, and then injected into a gas chromatograph equipped with an alumina column (GDX502) and a flame ionization detector (GC-7AG; Shimadzu Japan) for measuring the ethylene concentration as described previously (Sun et al., 2007).

Statistical analysis

Analysis of variance (ANOVA) was conducted between different treatments. Significant differences between treatments were evaluated by ANOVA, and the dates are expressed as the mean values ± SD of at least 4 replicates for seed germination percentage.

Results

Effect of NaCl on seed germination and radicle growth

The cucumber seeds used in the present study had an average germination rate of 96.9% after 48 h imbibition under control
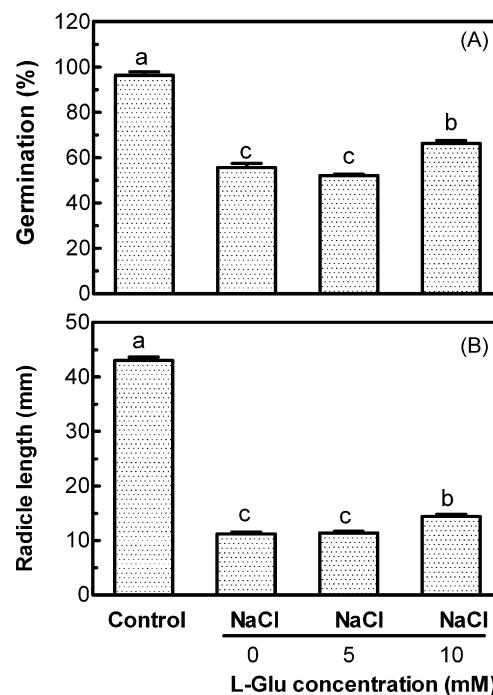

Fig. 1. Effects of L-Glu on seed germination (A) and radicle length (B) in the presence of NaCl. Seed germination rates and radicle length were codetermined after imbibition for 48 h under various treatments: control; treatment with 200 mM NaCl plus 0, 5 and 10 mM L-Glu. Data are mean ± SE for 4 replicates for calculation of seed germination, and values for radicle length were obtained from measurements of 40 radicles. Different letters shown in the error bars mean significant differences among control and treatments.

conditions. The germination rate was reduced to 90.0% and 55.7% when 100 and 200 mM NaCl were added to the incubation solution, respectively (data not shown). The mean radicle length was 38.7.0 ± 0.75 mm ($n = 40$) after 48 h imbibition in the control medium. The radicle length was reduced to 23.1 ± .0.43 and 7.72 ± 0.30 mm ($n = 40$), respectively after 48 h imbibition in the presence of 100 and 200 mM NaCl in the medium, suggesting that radicle growth is more sensitive to NaCl than seed germination. Given that 200 mM NaCl significantly inhibited seed germination and radicle growth, this concentration of NaCl was chosen for further study, and results are reported throughout the remainder of the paper.

Glu alleviated the inhibitory effect of NaCl on seed germination

Glu, particularly L-Glu, plays an important regulatory role in both animal and plant cells (Ford and Lea, 2007). To examine whether Glu is involved in NaCl-induced inhibition of seed germination and radicle growth, the effect of Glu on seed germination and radicle growth in the absence and presence of NaCl was investigated. Neither seed germination and radicle length were affected by L-Glu up to 20 mM in the absence of NaCl (Data not shown). In contrast, L-Glu at 10 mM enhanced seed germination in the presence of NaCl, while 5 mM L-Glu did not have any effect on germination of cucumber seeds exposed to NaCl (Fig. 1A). Like seed germination, 10 mM L-Glu, but not 5 mM L-Glu, increased the radicle length in the presence of NaCl (Fig. 1B). To test whether the observed effect of L-Glu on NaCl-induced inhibition of seed germination and radicle growth is specific, we also investigated the effect of D-Glu and Gln on seed germination and radicle growth in the presence of NaCl. As shown in Table 1, 10 mM D-Glu and Gln did not alleviate NaCl-induced reduction in seed germination and radicle length. Rather, both D-Glu and Gln further inhibited seed germination and radicle

Table 1
Effect of L-Glu (10 mM), D-Glu (10 mM) and Gln (10 mM) on seed germination and radicle length in the presence of 200 mM NaCl. Data are mean ± SE with number of replicates for seed germination and radicle numbers given in the brackets.

Treatments	Germination (%)	Radicle length (mm)
Control	97.08 ± 1.48^a ($n = 12$)	39.30 ± 0.61^a ($n = 102$)
NaCl	47.78 ± 1.98^b ($n = 12$)	10.88 ± 0.47^b ($n = 102$)
NaCl + L-Glu	66.25 ± 1.25^c ($n = 4$)	14.41 ± 0.39^c ($n = 30$)
NaCl + D-Glu	41.25 ± 0.72^d ($n = 4$)	8.13 ± 0.21^d ($n = 40$)
NaCl + Gln	38.12 ± 4.83^d ($n = 4$)	9.03 ± 0.33^d ($n = 32$)

Means with different letters within a column are significantly different ($P < 0.05$) with regards to treatments.

growth in the presence of NaCl. These results indicate that the alleviative effect of Glu on seed germination is unlikely to result from a general effect of amino acids, but that it is specific to L-Glu.

Ethylene stimulated seed germination in the presence of NaCl

Treatment of cucumber seeds with the ethylene precursor ACC and ethylene donor Ethephon did not affect seed germination in the absence of NaCl (data not shown). However, radicle length was markedly reduced by treatment with ACC and Ethephon in the control medium without NaCl (data not shown). Furthermore, seed germination was enhanced when ACC and Ethephon were used to treat those seeds exposed to NaCl (Fig. 2A), suggesting that ethylene alleviates the NaCl-induced suppression of cucumber seed germination. In contrast to seed germination, the NaCl-induced reduction in radicle length was insensitive to both ACC and Ethephon (Fig. 2B). To further evaluate the role of ethylene in seed germination under conditions of NaCl, we studied effect of antagonists of ethylene biosynthesis (Co^{2+} and AVG) on seed germination and radicle length. As shown in Fig. 2C and D, Co^{2+} and AVG did not affect seed germination in the presence of NaCl, while AVG, but not Co^{2+}, significantly promoted radicle growth in the presence of NaCl.
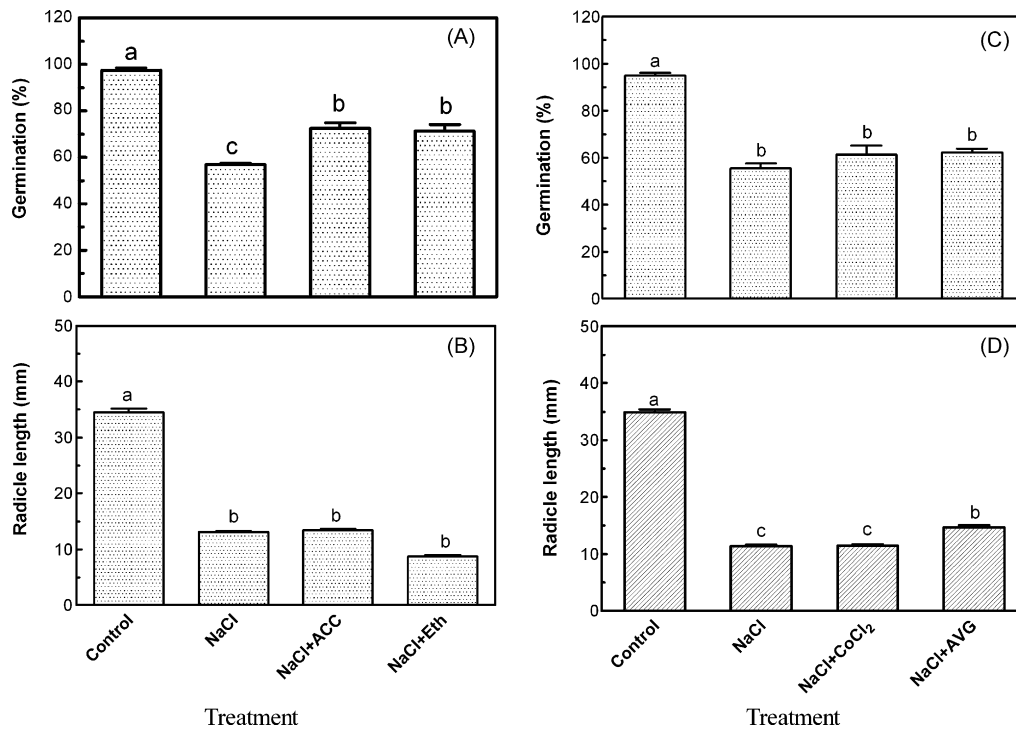

Fig. 3. Ethylene evolution from the cucumber seeds incubated in control, 200 mM NaCl, 10 mM L-Glu and NaCl and L-Glu together for 2 and 12 h. Data are means ± SE for 4 replicates.

Effect of NaCl and L-Glu on ethylene evolution during seed germination

The alleviative effect of ACC and Ethephon on NaCl-induced inhibition of seed germination prompted us to hypothesize that the inhibitory effect of NaCl on seed germination may result from reduction in ethylene production. To test this hypothesis, we directly measured the effect of NaCl on ethylene evolution during seed germination. Fig. 3 shows that ethylene evolution from seeds increased with imbibition duration in the control medium. For instance, ethylene evolution increased from 0.91 ± 0.04 to 3.2 ± 0.46 ppm g^{-1} FW h^{-1} as the imbibition extended from 2 to 12 h in the control solution. Addition of NaCl to the imbibition medium significantly suppressed the ethylene evolution, and the inhibitory effect was more pronounced after 12-h imbibition than after 2-h imbibition (Fig. 3). In contrast to NaCl, treatment with L-Glu stimulated ethylene evolution from seeds in the absence of NaCl (Fig. 3). When L-Glu was added to incubation medium contain-

Fig. 2. Effects of ACC, Ethphon (Eth) (A and B), AVG and $CoCl_2$ (C and D) on germination and radicle length of cucumber seeds in the presence of NaCl. Germination rates and radicle length were determined after 48-h imbibing cucumber seeds in the medium containing 200 mM NaCl plus 100 μM ACC, 100 μM Ethphon, 10 μM AVG and 10 μM $CoCl_2$, respectively. Data are mean ± SE for 4 replicates for seed germination and 40 radicles for determination of radicle length. Different letters shown in the error bars mean significant differences among control and treatments.
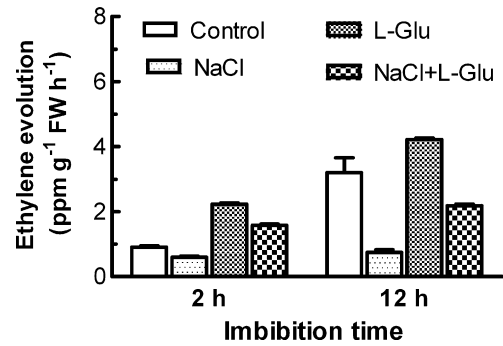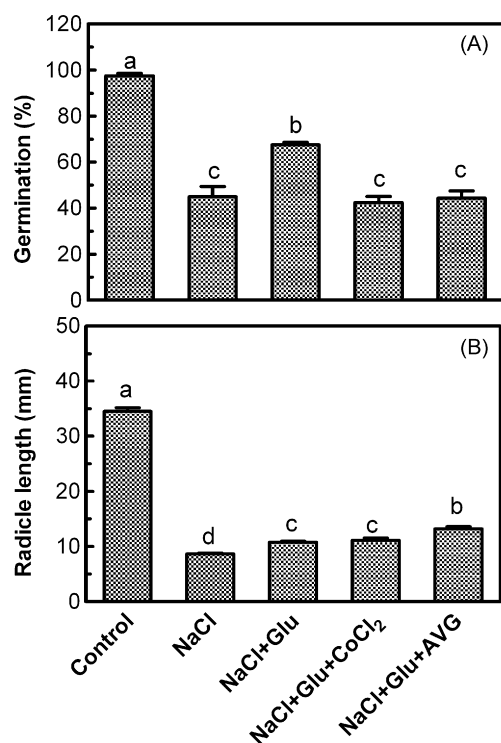
Fig. 4. Seed germination and radicle length exposed to control and medium containing 200 mM NaCl, 200 mM plus 10 mM L-Glu, 200 mM NaCl plus 10 mM L-Glu and 10 μM CoCl₂, and 200 mM NaCl plus 10 mM L-Glu and 10 μM AVG for 48 h, respectively. Data are mean ± SE for 4 replicates for seed germination and 40 radicles for determination of radicle length. Different letters shown in the error bars mean significant differences among control and treatments.

ing NaCl, the reduction in ethylene evolution induced by NaCl was substantially reversed by addition of L-Glu (Fig. 3). These findings indicate that the alleviative effect of L-Glu on salt stress-induced suppression of seed germination is likely to result from alleviation of NaCl-induced reduction in ethylene production.

AVG and Co²⁺ counteracted L-Glu action on seed germination

To test whether the alleviative effect of L-Glu on seed germination exposed to NaCl occurs by modulating ethylene production during seed germination, we investigated the effect of ethylene biosynthesis antagonists (AVG and Co²⁺) on seed germination in the presence of both NaCl and L-Glu. As shown in Fig. 4A, CoCl₂ and AVG completely reversed L-Glu-induced increase in seed germination in the presence of NaCl. These results indicate that the alleviative effect of L-Glu on NaCl-induced reduction in seed germination is likely to occur through its effect on ethylene production. In contrast to seed germination, AVG promoted radicle growth in the presence of NaCl and Glu, while no effect of Co²⁺ on radicle length was observed (Fig. 4B).

Discussion

In the present study, we found that germination of cucumber seeds was significantly inhibited by 200 mM NaCl and that the salt stress-induced suppression of seed germination was alleviated by ACC and Ethephon (Fig. 2). We further demonstrated that the salt stress-induced inhibition of seed germination was related to its suppression of ethylene production during imbibition (Fig. 3). In addition to ethylene, we found that L-Glu was also effective in alleviating the inhibitory effect of salt stress on seed germination. More importantly, we demonstrated that L-Glu evoked ethylene

production from the imbibed cucumber seeds and that the alleviative effect of L-Glu on seed germination lay in its interaction with ethylene such that inhibition of ethylene production by ethylene synthesis inhibitors diminished the alleviative effect of L-Glu (Fig. 4). Taken together, these findings reveal, for the first time, that crosstalk between ethylene and L-Glu exists in modulation of seed germination under conditions of salt stress.

Ethylene has been reported to stimulate germination in non-dormant seeds and break dormancy in seeds that display embryo dormancy (Kepczynski and Kepczynska, 1997; Matilla, 2000; Kucera et al., 2005). A positive correlation exists between seed germination and ethylene production (Machabee and Saini, 1991; Petruzzelli et al., 2000; Calvo et al., 2004), suggesting that ethylene plays a role in modulation of seed germination. Petruzzelli et al. (2000) reported that the onset of seed germination in pea is accompanied by ethylene evolution from the embryonic axis. However, the mechanisms by which ethylene stimulates seed germination is unclear. It has been suggested that ethylene production during imbibition interrupts dormancy maintained by ABA, thus stimulating seed germination (Beaudoin et al., 2000). Previous studies have shown that seed germination in cucumber (Ramakrishna and Amriphale, 2005) and muskmelon (Welbaum et al., 1990) is controlled at least in part by weakening perisperm. Very recently, Linkies et al. (2009) reported that ethylene enhances seed germination by promoting endosperm cap weakening and endosperm rupture in *Lepidium sativum* and *Arabidopsis thaliana* seeds and that ethylene counteracts the inhibitory action of ABA on these two processes. As salt stress may induce increases in ABA levels in seeds, a similar mechanism may also account for our findings.

Ethylene has also been reported to be involved in modulation of seed germination under stressed conditions. For instance, ethylene alleviates thermoinhibition of germination of lettuce seeds (Saini et al., 1986). Ethylene has also been shown to alleviate salt stress-induced seed dormancy (Kepczynski and Karssen, 1985; Khan and Huang, 1988; Li et al., 2005). Our results showing that ACC and Ethephon alleviated salt stress-induced inhibition of seed germination are consistent with reported findings in the literature. The alleviative effect of ACC and Ethephon on salt stress-induced seed germination may be accounted for by the suppression of ethylene production by slat stress (Fig. 3) such that treatment with ACC and Ethephon maintains an ethylene level essential for seed germination. A similar reduction in ethylene evolution from lettuce seeds by NaCl was reported by Khan and Huang (1988).

In addition to ethylene, we found that Glu was also effective in alleviating the inhibitory effect of seed germination induced by salt stress (Fig. 1A). Moreover, we demonstrated that the alleviative effect of Glu was specific to L-Glu. This finding suggests that the act of L-Glu on salt-stressed seed germination is not due to general effects of amino acids, but rather, it is indicative that L-Glu is an important signaling molecule involved in seed germination process. Given that both ethylene and Glu were effective in alleviating the inhibitory effect of NaCl on seed germination, we further examined the interactions between ethylene and L-Glu in the alleviation of salt stress-induced inhibition of seed germination. We found crosstalk between L-Glu and ethylene in alleviating salt-stressed seed germination. Several observations suggest that L-Glu is likely to act upstream of ethylene during their effect on seed germination under conditions of salt stress. First, the mitigating effect of L-Glu on salt-stressed seed germination was abolished by antagonists of ethylene biosynthesis (AVG and Co²⁺) (Fig. 4). Secondly, salt stress suppressed ethylene production from the imbibed seeds and L-Glu evoked ethylene production from the imbibed seeds (Fig. 3). This mitigated the salt stress-induced decrease in ethylene evolution, thus leading to the observed alleviative effect of L-Glu on seed germination in the presence of NaCl (Fig. 1). The L-Glu-induced

ethylene evolution from the salt-stressed seeds occurred earlier than its alleviative effect on seed germination (12 h vs. 48 h). Therefore, the less reduction in ethylene production from the seeds in the presence of L-Glu may be a cause for the alleviative effect on seed germination. The involvement of ethylene in alleviation of salt stress-induced lettuce seed germination by kinetin has been reported in the literature (Khan and Huang, 1988). Similar to L-Glu, kinetin can relieve the inhibitory effect of salt stress on germination of lettuce seeds, and this effect was prevented by the inhibitor of ethylene action (Khan and Huang, 1988).

In addition to seed germination, our results also demonstrated that radicle growth was sensitive to salt stress and that the inhibitory effect of salt stress on radicle growth was also attenuated by glutamate (Fig. 1B). In contrast to seed germination, the inhibition of radicle growth by salt stress was not alleviated by treatment with ACC and Ethephon (Fig. 2B), suggesting that the inhibitory effect of NaCl on radicle growth may not be related to ethylene. As inhibition of cell elongation is one of the most distinct effects of ethylene on plants (Pierik et al., 2006), the inhibition of ethylene production by salt stress would enhance radicle growth. However, we found that radicle growth in the presence of NaCl was independent of ethylene, as evidenced by treatment with ACC and Ethephon (Fig. 2B). In addition, the observed alleviative effect of L-Glu on radicle growth in the presence of NaCl may not be directly accounted for by enhancing ethylene evolution. Future studies to unravel the crosstalk between ethylene and L-Glu in modulation of radicle growth are warranted.

Acknowledgements

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