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Letter

Salt stress releases extracellular ATP to activate purinergic signaling and inhibit plant growth

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Dear Editor,

Intracellular ATP is essential for all living organisms; however, extracellular ATP (eATP) is important in danger-associated molecular pattern (DAMP) signaling in eukaryotes (Kim et al. 2006; Weerasinghe et al. 2009; Cao et al. 2014; Choi, Tanaka, Cao et al. 2014). In response to biotic and abiotic stresses, ATP is released into the extracellular environment where it is sensed by purinoreceptors that activate secondary signaling cascades (Choi, Tanaka, Cao et al. 2014; Choi, Tanaka, Liang et al. 2014; Chen et al. 2017, 2021; Cho et al. 2022; Duong et al. 2022). Purinergic signaling is well characterized in animals, which possess two purinoreceptor types: ligand-gated ion channels (P2X) and G protein-coupled receptor (P2Y) (Faria et al. 2012). Purinoreceptors are involved in many physiological processes, including tumor recognition, inflammation, cell death, and neurotransmission (Cekic and Linden 2016; Feng et al. 2020; Kitajima et al. 2020). In contrast to an extensive animal literature, relatively little is known about purinergic signaling in plants.

The plant purinoreceptor P2K1 (purinergic 2-type receptor kinase 1)/DORN1 (does not respond to nucleotides 1) was identified via screening of EMS-mutagenized plants that failed to elevate intracellular calcium when exposed to eATP (Choi, Tanaka, Cao et al. 2014). The plant purinoreceptors are termed P2K, consistent with nomenclature in animals and reflecting their kinase nature (Choi, Tanaka, Cao et al. 2014; Pham et al. 2020). P2K1 regulates downstream signaling associated with a reactive oxygen species (ROS) burst, cytoplasmic calcium response, mitogen-activated protein kinase activation, and defense gene regulation—all important in the plant immune response (Chen et al. 2017; Duong et al. 2022; Kim et al. 2023). eATP also triggers an ROS wave during a wound response in Arabidopsis (*Arabidopsis thaliana*) (Myers et al. 2022). S-acylation regulates the temporal dynamics of P2K1, and mevalonate kinase, a key enzyme in the mevalonate pathway, is a target for P2K1-mediated phosphorylation. Thereby, eATP regulates many plant secondary products (Chen et al. 2021; Cho et al. 2022).

P2K1 (L-type lectin receptor kinase I.9; LecRK I.9) was identified as playing a role in plant defense against fungal pathogens (Cao et al. 2014; Choi, Tanaka, Liang et al. 2014). Recent literature attributes eATP to immunity regulation in plants (Chen et al. 2017, 2021; Cho et al. 2022). However, purinergic signaling in mammals is also involved in abiotic stress responses. For example, the ATP/UTP-mediated P2Y2 purinoreceptor regulates renal salt and water homeostasis in mice (Vallon and Rieg 2011). The P2X receptor in mice interferes with NaCl absorption (Svendsen et al. 2017). Purinergic signaling under high salinity was suggested in a few earlier plant studies (Kim et al. 2009; Hou et al. 2018). Therefore, we conducted experiments to conclusively associate purinergic signaling with plant salt stress response.

To visualize the in vivo eATP release caused by NaCl, we utilized the firefly luciferase-based D-luciferin assay. In this assay, ATP interacts with D-luciferin, undergoes catalytic oxidation by luciferase, and emits bioluminescence (Fig. 1A). The intensity of emitted light in the luciferase/D-luciferin assay is directly proportional to the ATP concentration when luciferase, D-luciferin, and oxygen concentrations are maintained (Kim et al. 2006; Chen et al. 2017). Using GST-Luciferase protein isolated from rosetta bacterial cells (Fig. 1B and Supplemental Fig. S1), we observed bioluminescence in the presence of ATP. Higher concentrations of GST-Luciferase protein and D-luciferin displayed gradual changes in bioluminescence in

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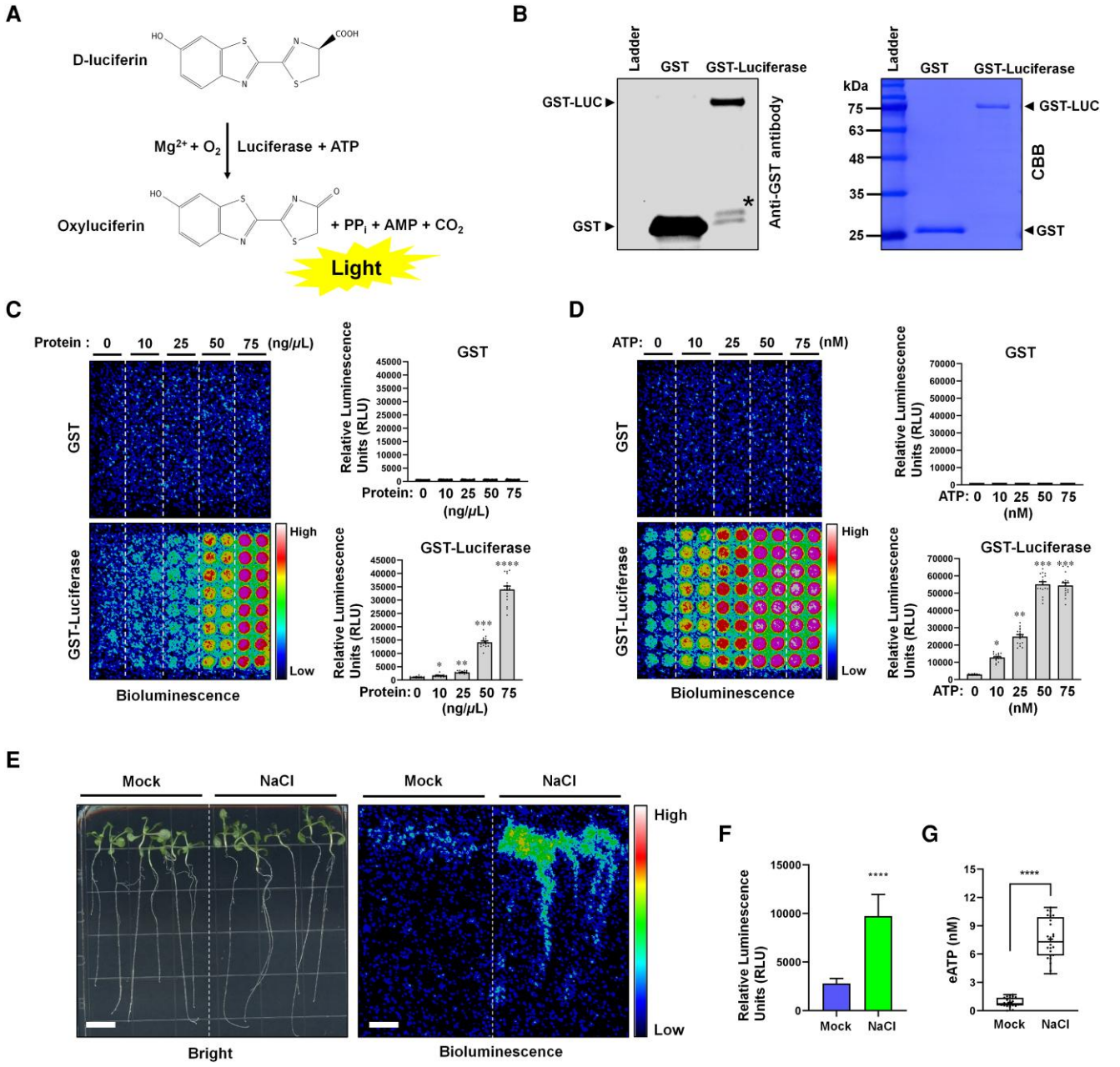

Figure 1. Extracellular ATP is increased by high NaCl. **A)** Theoretical reaction of Luciferase for detecting eATP. PPi and AMP represent pyrophosphate and adenosine monophosphate, respectively. **B)** Detection of purified glutathione S-transferase tagged luciferase (GST-Luc) protein using anti-GST antibody. Coomassie Brilliant Blue (CBB) staining was performed to visualize proteins. The asterisk (*) indicates the cleaved Luciferase protein. **C and D)** Bioluminescence signals in the presence of different levels of GST-Luc protein (**C**) and different ATP concentration (**D**). Bioluminescence signals were monitored, images were captured, and luciferase signal intensities were quantified using C-vision/Im32. The data were analyzed using GraphPad Prism 8 software. Data are shown as mean ± SEM, $n = 16$. Statistical significance is denoted as $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. The *P*-value indicates significant differences relative to the GST protein treatment and was determined by one-way ANOVA followed by Tukey's multiple comparisons. **E)** Extracellular ATP (eATP) is increased by salt stress. The luminescence was monitored and images were captured using a low light imaging CCD camera (Photek; Photek, Ltd.) after treatment with 200 mM NaCl. Scale bars = 1 cm. **F)** Luciferase intensities were monitored, and luciferase signal intensities were quantified using C-vision/Im32 and analyzed using the GraphPad Prism 8. Data are shown as mean ± SEM, $n = 24$ (biological replicates). Statistical significance is denoted as $^{****}P < 0.0001$. The *P*-value indicates significance relative to Mock treatment and was determined by unpaired Student's *t*-test, two-way ANOVA. **G)** The eATP concentration in salt-treated plants was calculated using the equation derived from the measured values of Luciferase (Supplemental Fig. S3). "+" indicates the mean, and the graph style is Appearance box and whiskers, where the whiskers represent the min to max range. The graph was generated using GraphPad Prism 8 software, with a sample size of $n = 24$ (biological replicates). Statistical significance is denoted as $^{****}P < 0.0001$ indicates statistical significance relative to the Mock treatment and was determined using an unpaired Student's *t*-test, two-way ANOVA.

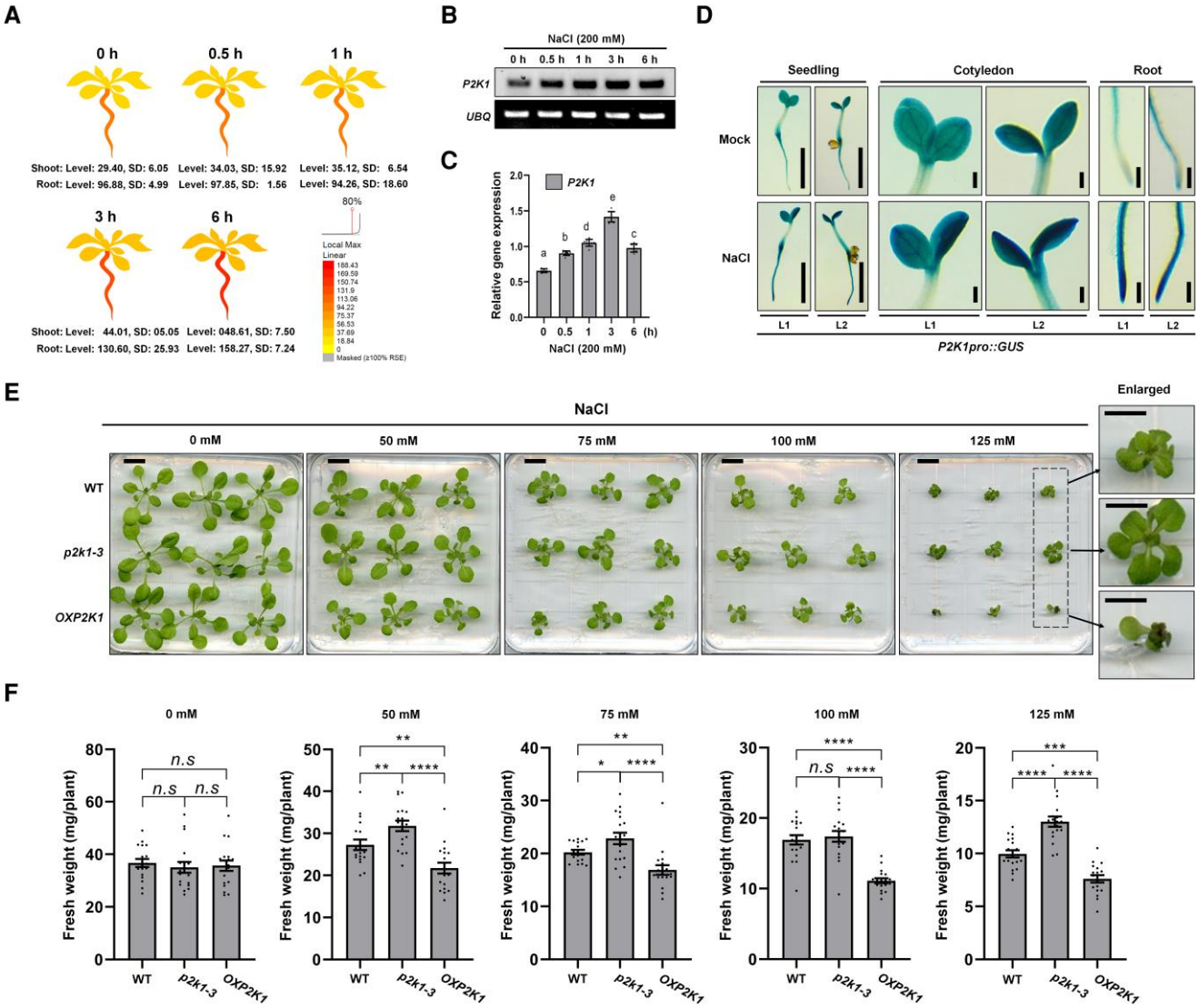

Figure 2. NaCl induces *P2K1* gene expression and inhibits plant growth. **A)** Expression of *P2K1* transcripts in the root under NaCl conditions was analyzed using in silico data obtained from BAR ePlant (http://bar.utoronto.ca/eplant). Data are shown as mean ± SD, $n = 2$ (biological replicates). **B)** RT-PCR analysis of *P2K1* transcript levels in Col-0 backgrounds after NaCl (200 mM) treatment. Total RNA was isolated from 1-week-old plants, and 2 μg of total RNA was used for this experiment. RNA levels were normalized against the expression of *ubiquitin* (*UBQ*). **C)** Analysis of *P2K1* transcript levels in Col-0 backgrounds after NaCl (200 mM) treatment using reverse transcription quantitative polymerase chain reaction (RT-qPCR). The *SAND* reference gene was used for RT-qPCR data normalization. Data are shown as mean ± SEM ($n = 4$), different letters above the bars indicate significant differences ($P < 0.05$) relative to wild-type plants. The *P*-value was determined and analyzed using the GraphPad Prism 8 by one-way ANOVA followed by Tukey's multiple comparison test. All above experiments were repeated two times (biological replicates) with similar results. **D)** Histochemical analysis of *P2K1* gene expression after NaCl treatment. *P2K1promoter::GUS* (β-Glucuronidase) transgenic plants (L1 and L2) were generated in wild-type Arabidopsis plants transformed with a chimeric *P2K1promoter::GUS* construct containing 2 kb of the putative *P2K1* promoter (5′ region of the *P2K1* gene) fused to the *GUS* coding sequence. Expression patterns of the *P2K1promoter::GUS* transgenic plant were detected by histochemical staining in the rosette leaf and root of a 5-day-old seedling after treatment with 200 mM NaCl for 1 h. Scale bars = 2 mm (seedling), 0.2 mm (cotyledon and root). **E)** Photograph of Arabidopsis plants after 2 weeks of growth under different treatments. *Arabidopsis thaliana* ecotype Columbia (Col-0), T-DNA insertion mutant (*p2k1-3*, Salk_042209), *P2K1*-overexpressing transgenic plants (*OXP2K1*) seeds were germinated and grown for 1 week, then transferred to petri dishes containing half-strength MS media (0 mM, untreated control) and half-strength MS media supplemented with the indicated concentrations of NaCl. Plants were grown for 2 weeks and fresh weight of each plant shoot was measured. Scale bars = 1 cm (NaCl 0 to 125 mM), 0.5 cm (enlarged images). **F)** Mean fresh weight of shoot per plant of Col-0, *p2k1-3*, and *OXP2K1* after 2 weeks growth under different treatments. Data are shown as mean ± SEM ($n = 18$). Statistical significance is denoted as $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. The *P*-value indicates significance relative to mock treatment and was determined and analyzed using the GraphPad Prism 8 software by two-way ANOVA followed by Tukey's multiple comparisons. All experiments were repeated two times (biological replicates) with similar results.

response to ATP (Fig. 1C). Thereby, a standard curve of bioluminescence intensity as a function of ATP concentration was generated (Fig. 1D). We used this to measure eATP released from 10-day-old *A. thaliana* seedlings. Under mock treatment, low background bioluminescence was observed; however, under NaCl treatment, a significantly higher signal was observed (Fig. 1E). Relative bioluminescence signal intensities were quantified (Fig. 1F and Supplemental Fig. S2) and eATP concentration was calculated using the calibration curve (Supplemental Fig. S3). This revealed that approximately 8 nM of ATP can be detected following NaCl treatment compared to only 0.9 nM under mock conditions (Fig. 1G). This demonstrates that ATP is released into the extracellular compartment after adding NaCl in planta, suggesting a connection between salt signaling and purinergic signaling.

We analyzed in silico data to examine the expression of *P2K1* transcripts in response to abiotic stresses like cold and heat, osmotic, genotoxic, and oxidative conditions, salinity, drought, UV-B, and wounding. Among these, the strongest transcriptional response was to NaCl treatment, under which *P2K1* transcript levels increased significantly within 6 h (Fig. 2A and Supplemental Fig. S4). To confirm this, reverse transcription quantitative PCR (RT-qPCR) was performed on *A. thaliana* plants treated with 200 mM NaCl. The expression of *P2K1* transcripts significantly increased after 30 min and reached their peak after 3 h (Fig. 2B and C). To confirm that *P2K1* is induced by NaCl, GUS activity was measured after NaCl treatment in transgenic plants expressing a *P2K1promoter::GUS* construct (Kim et al. 2023). Higher histochemical GUS staining was observed in NaCl-treated seedlings compared to mock treatment (Fig. 2D).

eATP plays an important role in P2K1 purinoreceptor activation and DAMP signaling in plants. DAMPs have the potential to adversely influence plant growth; therefore, we treated *P2K1* mutant (*p2k1-3*; Salk_042209) and *P2K1*-overexpressing transgenic plants (*OXP2K1*) with NaCl to determine whether plant growth inhibition by NaCl is mediated by P2K1. Wild-type, *p2k1-3*, and *OXP2K1* transgenic seedlings were allowed to germinate on Murashige and Skoog (MS) medium. After 1 week, they were transferred to MS medium with a range of NaCl concentrations, and plant growth was monitored for 2 weeks. Interestingly, *p2k1-3* knock-out mutant was less sensitive to NaCl, with less stunting compared to wild type (Fig. 2E). *OXP2K1*-overexpressing plants were hypersensitive compared to wild type, with severely shortened leaves (Fig. 2E). The effect on growth was quantified by shoot weight, which showed a significant difference compared to wild type (Fig. 2F). Results suggest that plant growth inhibition under NaCl is affected by P2K1 and that eATP accumulation in response to NaCl is important in plant growth regulation that is partially mediated by the P2K1 purinoreceptor, indicating an important link between salinity stress responses and purinergic signaling cascades.

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Author Contributions

D.K. contributed to conceptualization, data curation, validation, investigation, methodology, writing—original draft, project administration; S.Y. contributed to validation, methodology, writing; G.S. contributed to conceptualization, data curation, supervision, funding acquisition, project administration, writing—revisions. All authors reviewed the manuscript.

Supplemental data

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

Supplemental Figure S1. Detection of purified glutathione S-transferase tagged luciferase (GST-Luc) protein using anti-Luciferase antibody.

Supplemental Figure S2. Bioluminescence signals in the presence of NaCl (200 mM).

Supplemental Figure S3. A representative calibration curve generated to measure extracellular ATP levels using the Microsoft Excel program.

Supplemental Figure S4. *In-silico* gene expression of *P2K1* under various stress conditions.

Supplemental Table S1. Sequence of primers used in this study.

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Conflict of interest statement. None declared.

Data availability

The authors declare that all other data supporting the findings of this study are available within the manuscript and its supplementary files or are available from the corresponding author on request.

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