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Release of extracellular purines from plant roots and effect on ion fluxes

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Extracellular purine nucleotides appear capable of regulating plant development, defense and stress responses by acting in part as agonists of plasma membrane calcium channels. Factors stimulating ATP release include wounding, osmotic stress and elicitors. Here we show that exogenous abscisic acid and L-glutamate can also cause ATP accumulation around *Arabidopsis thaliana* roots. Release of ADP from root epidermis would trigger ionotropic receptor-like activity in the plasma membrane, resulting in transient elevation of cytosolic free calcium. Root epidermal protoplasts (expressing aequorin as a cytosolic free calcium reporter) can support an extracellular ADP-induced cytosolic calcium elevation in the presence of an extracellular reductant. This confirms that ADP could elicit calcium-based responses distinct to those of ATP, which have been shown previously to involve production of extracellular reactive oxygen species.

Keywords: ATP, ADP, abscisic acid, calcium, channel, glutamate, root

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Extracellular purine nucleotides can regulate plant cell growth, stress responses, immunity and symbiotic events.[1,2] Their activity at the plasma membrane elicits increased production of nitric oxide (NO), activation of NADPH oxidases to produce reactive oxygen species (ROS), and transient elevations of cytosolic free calcium ($[Ca^{2+}]_{cyt}$).[3-9] Although there are no equivalents to animal ionotropic or metabotropic purinoreceptor genes apparent in higher plant genomes, plant responses to extracellular purine nucleotides are sensitive to antagonists of animal purinoceptors.[1,10-12] *Arabidopsis thaliana* plasma membrane Ca^{2+} channel activity was recently found to be activated rapidly by extracellular ADP, consistent with the presence of an ionotropic receptor.[13] Parallels with animal cells also extend to a conserved mechanism of ATP and ADP hydrolysis by extracellular apyrase,[14] plus release of ATP by exocytosis[15] and an ABC transporter.[16]

ATP is released at plant wound and growth points, in response to touch, elicitors and hyperosmotic stress imposed by salts.[1,17,18] Here, wounding, hyperosmotic or sodicity stress resulted in distinct levels and time courses of ATP accumulation around excised roots of *A. thaliana* (**Fig. 1**). Recovery of ATP levels to control values was evident at the 30 min time point after wounding but remained significantly elevated in response to abiotic stresses (30 min time point; NaCl, $p < 0.001$; sorbitol, $p < 0.036$, Student's t-test). The stress response hormone abscisic acid (ABA) at 1 and 10 µM also promoted ATP accumulation around excised roots (**Fig. 2A**), albeit to a lower level. Accumulation above the ethanol control was significant for both concentrations at 10 and 15 min. Application of 10 µM ABA inhibits *A. thaliana* primary root growth, a response involving a plasma membrane NADPH oxidase and plasma membrane proline-rich extensin-like receptor kinase (PERK4).[19,20] PERK4 directs the activation of plasma membrane Ca^{2+} influx channels.[20] As extracellular ATP activates root epidermal plasma membrane Ca^{2+} influx channels via activation of an NADPH oxidase, it becomes feasible that ABA inhibition involves ATP release and sensing upstream of NADPH oxidases and PERKs, resulting in an inhibitory Ca^{2+} influx. High $[Ca^{2+}]_{cyt}$ can act as a "brake" to root hair elongation

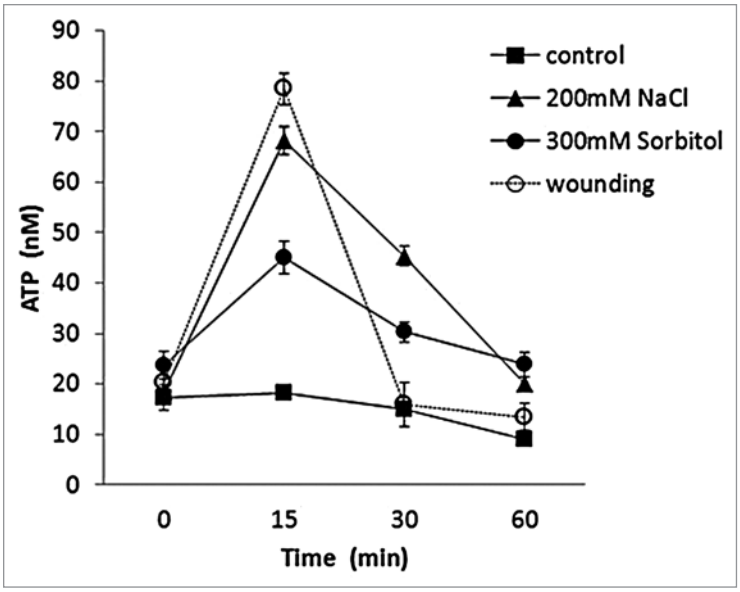

Figure 1. Wounding and abiotic stress cause ATP accumulation around *A. thaliana* roots. *A. thaliana* (Col-0) was grown for 7 d as described previously on MS medium with 1% (w/v) sucrose.[13] Eight roots of similar lengths were placed in the well of a 96-well plate containing sterile liquid MS/1% (w/v) sucrose, with or without test substances. ATP content of samples was determined with a luciferin/luciferase assay (Molecular Probes), using a Fluostar Optima plate reading luminometer. Wounding was inflicted by moving a gloved finger up and down the roots to damage root hairs prior to immersion in assay medium. Results are mean ± SEM from three independent trials.

and perhaps this may also hold for elongation of primary roots.[21] Production of ROS by NADPH oxidases also promotes root cell elongation by activating plasma membrane Ca^{2+} channels.[22] Thus, the dose-dependent stimulation or inhibition of growth by extracellular ATP may be effected by the levels of ROS production and channel activation.

Extracellular glutamate can also elevate root $[Ca^{2+}]_{cyt}$ by activating plasma membrane glutamate receptor-like (GLR) channels.[23,24] As shown in **Figure 2B**, 1 µM L-glutamate caused transient ATP accumulation but equimolar D-glutamate did not, indicating stereospecificity of the response. Exposure to 10 µM L-glutamate was less effective than 1 µM, suggesting a biphasic response. At these concentrations, glutamate is unlikely to be transported into roots by the low affinity amino acid permease AAP1 or by H^+-coupled symport[25,26] and so should be acting extracellularly. L-glutamate at 10 µM and above inhibits *A. thaliana* primary root growth,[27] and it will be interesting to see how this relates, if at all, to ATP functions. Perception of extracellular glutamate at the epidermis upstream of ATP release could be mediated by GLR2.1, 3.3, 3.5 and 5 as the most highly expressed of the gene family in this cell file. Across the meristematic region GLR2.4, 3.3, 3.6,5 are the most highly expressed while across the elongation zone, GLR 1.4, 2.1, 3.3, 3.5 and 3.6 predominate.[28]

Once purine nucleotides are released from roots, they can activate plasma membrane Ca^{2+}-permeable channels directly (ADP)[13] or via production of extracellular ROS (ATP).[11] Accordingly, ATP activation of root epidermal plasma membrane Ca^{2+}-permeable channels can be prevented by applying dithiothreitol (DTT) as a reductant.[11] Here, ADP-induced $[Ca^{2+}]_{cyt}$ elevation of *A. thaliana* root epidermal protoplasts (measured as in ref. 13) was unaffected by incubation in DTT (1 mM) (0.1 mM ADP; 0.45 ± 0.04 µM $[Ca^{2+}]_{cyt}$, n = 6: ADP + DTT; 0.52 ± 0.03 µM $[Ca^{2+}]_{cyt}$, n = 9), confirming that extracellular ROS are not involved in ADP activation of Ca^{2+} influx.

Addition of purine nucleotides to *A. thaliana* root epidermis causes transient changes in net Ca^{2+} and K^+ fluxes downstream of the initial sensing event.[13] ADP promotes net Ca^{2+} influx but ATP and the weakly hydrolysable analog adenosine 5′-(α,β-methylene)triphosphate (αβATP) only promote influx when applied up to 100 µM, above which Ca^{2+} efflux is promoted.[13] Under control conditions used in reference 13, there is a net K^+ efflux from both elongation zone (-235 ± 16 nmol m^{-2} s^{-1}; n = 33) and mature epidermis (-44 ± 3 nmol m^{-2} s^{-1}; n = 25) detected by extracellular vibrating K^+-selective microelectrodes. From the data presented in reference 13, this indicates that in the elongation zone, ADP, ATP and αβATP up to approximately 10 µM would decrease net K^+ efflux and promote Ca^{2+} influx. Above 10 µM, K^+ efflux would be promoted. At the mature epidermis ADP up to 10 µM and ATP/αβATP up to approximately 100 µM would decrease K^+ efflux while promoting Ca^{2+} influx. Above these levels, K^+ efflux would be promoted. At present the contribution of underlying cells and the walls are unknown. If the origin were the epidermal plasma membrane, the results indicate involvement of K^+ efflux pathways and it will be interesting to test whether the epidermal K^+ efflux channel AtGORK is involved.[29] How the cation fluxes are generated and what their consequences are now need to be elucidated.

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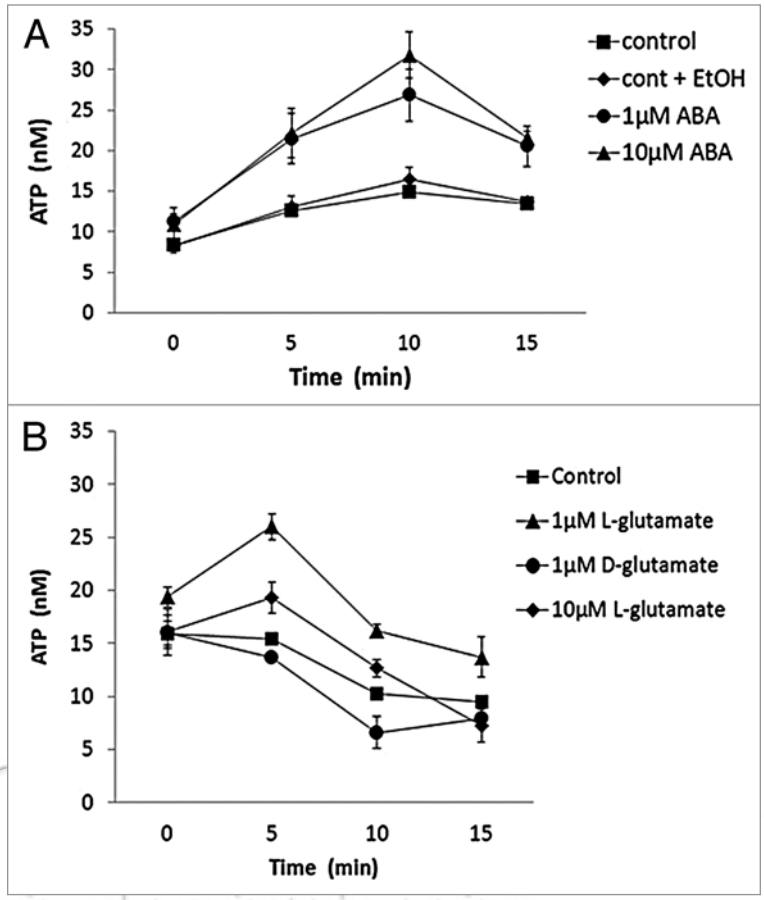

Figure 2. ABA and L-glutamate cause ATP accumulation around *A. thaliana* roots. Roots were exposed to (A) ABA with 0.025% (v/v) ethanol (EtOH) as the solvent carrier control and (B) L- or D-glutamate. Conditions as in **Figure 1**. Results are mean ± SEM from three independent trials.

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