LETTER

Arabidopsis DORN1 extracellular ATP receptor; activation of plasma membrane K^+-and Ca^{2+}-permeable conductances

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The first reports in the 1970s of extracellular ATP (eATP) effects on algal cytoplasmic streaming and Venus fly trap closure received little attention (Jaffe, 1973; Williamson, 1975). By the time interest in plant eATP revived, work on animals had revealed the existence of plasma membrane (PM) receptors for eATP that function in such processes as pain perception and vasodilation (Burnstock, 2016). Plant research is now catching up. eATP effects on roots (gravitropism, growth and development), hypocotyl (elongation), pollen (germination and tube growth), stomatal aperture, and cell viability are now documented (reviewed by Clark *et al*., 2014, Cho *et al*., 2017). Furthermore, eATP is implicated in immunity, abiotic stress responses and nodulation (reviewed by Clark et al., 2014, Cho *et al*., 2017). eATP increases in response to wounding, mechanical stimulation, abiotic stress, abscisic acid, glutamate and chitin (Dark *et al*., 2011; Cho *et al*., 2017). eATP can increase plant free Ca^{2+} (cytosolic, nuclear, mitochondrial), phosphatidic acid, nitric oxide, and reactive oxygen species (ROS) as potential second messengers in signalling (Demidchik *et al*., 2003, 2009; Loro *et al*., 2012; Cho *et al*., 2017). Until recently, perception of eATP was very much a "black box" mechanism as no equivalents of animal receptors were apparent in higher plant genomes. A breakthrough came with the discovery of *Arabidopsis thaliana* DORN1 (Does not Respond to Nucleotides1) as a PM eATP receptor (Choi *et al*., 2014). Analysis of *dorn1* mutants has shown that this receptor governs eATP-induced $[Ca^{2+}]_{cyt}$ elevation in young seedlings and also a specific transcriptional response enriched in wound-responsive genes (Choi *et al*., 2014).

Delineation of the first higher plant eATP receptor will now spur on attempts to identify the immediate targets of its serine/threonine kinase activity and downstream components of the signalling pathway it commands. eATP affects plasma membrane Ca^{2+}, K^+ and Na^+ fluxes (Demidchik *et al*., 2011; Zhao *et al*., 2016) but none have yet been shown to rely on DORN1. eATP-activated Ca^{2+} fluxes are mediated by channel proteins in *Arabidopsis* root epidermal and guard cell PM but their genetic identities are unknown (Demidchik *et al*., 2009; Wang *et al*., 2014; Zhu *et al*., 2017). eATP- and eADP-induced K^+ fluxes have been detected at the *Arabidopsis* root epidermis using extracellular K^+-selective microelectrodes (Demidchik *et al.,* 2011). eADP was found (by using patch clamp electrophysiology) to activate channel-mediated K^+ efflux and Ca^{2+} influx conductances (Demidchik *et al.,* 2011). Patch clamp electrophysiology has been used here to test whether DORN1 is required for activation of *Arabidopsis* root epidermal PM channel conductances by exogenous ATP. Roots were chosen as they have greater eATP-induced $[Ca^{2+}]_{cyt}$ elevations than leaves (Tanaka *et al*., 2010) and robust *DORN1* expression (Cho *et al*., 2017).

Plasma membrane cation-permeable conductances under control conditions

DORN1 expression is high at the root apex and is maintained there as the root ages; expression declines with age in distal epidermis (Cho *et al*., 2017). Therefore epidermal protoplasts were isolated from excised root apices (<3 mm) of 9-16 days old Col-0 (parental wild type) and two *dorn1* mutants. The *dorn1-1* mutant has a point mutation in its cytosolic serine-threonine kinase domain while the *dorn1-3* mutant has a T-DNA insertion in the extracellular legume-type lectin domain; both lesions result in failure in eATP-induced $[Ca^{2+}]_{cyt}$ elevation (Choi *et al*., 2014). Both mutants were confirmed as homozygous. Mean (± SE) protoplast diameter was 16 ± 0.5 μm (n = 40), smaller than those in a previous study from the mature epidermis (20 μm) isolated using the same method (Demidchik *et al.,* 2011). The "whole cell" mode of patch clamping was applied, permitting measurement of net current as ions are conducted through PM channels at different trans-membrane voltages, and reported as the overall current/voltage (*I/V*) relationship. Under control conditions, one type of overall non-linear conductance was most commonly observed in Col-0 (Figure 1a, left panel and Supplemental Figure S1a) and both *dorn1* mutants (Supplemental Figure S1b, c). The time constant of half-activation of Col-0 outward currents at 23 mV was 88.4 ± SE 8.5 ms (n = 12). This is reminiscent of the depolarization-activated non-selective cation channels (NSCC) in different plant species (Wegner and de Boer, 1997; Zhang *et al*., 2002; Shabala *et al*., 2006). In all genotypes, both

inward currents (evoked by hyperpolarised voltages) and outward currents (evoked by depolarised voltages) were greatly inhibited by the cation channel blocker, Gd^{3+} (Supplemental Figure S1a-c). The Gd^{3+}-insensitive conductance was not investigated further. Tail-current analysis of the Col-0 outward conductance revealed a reversal potential (E_{rev}) of -90.2 ± 8.9 mV (n = 8). Thus, permeability ratios P_K/P_{Cl} and P_{Ca}/P_K of 13.7 ± 5.4 and 0.04 ± 0.01, respectively, were estimated using the Goldman equation. A high selectivity for K^+ was also found in the NSCC studies and also for PM K^+ channels in root cells of *Arabidopsis* (Maathuis and Sanders, 1995). Voltage ramping minimised activation of the Col-0 outward conductance to delineate the inward conductance (Supplemental Figure 2) and yielded an E_{rev} of -8.2 ± 4.7 mV (n = 6) and P_{Ca}/P_K of 1.9 ± 0.7. This indicates Ca^{2+} influx contributing to the inward conductance. Single channel studies are now required to delineate channel contribution to the Gd^{3+}-sensitive K^+- and Ca^{2+}-permeable conductances.

eATP activates K^+- and Ca^{2+}-permeable conductances in wild type root plasma membrane

Before addition of ATP, at least two recordings were performed to confirm the stability of currents (Figure 1b, -3 and 0 mins). ATP was added as its disodium salt and in controls with an equivalent Na^+ concentration applied as NaCl, there was no current activation (Supplemental Figure 3), confirming that any responses would be caused by ATP. Addition of 300 µM ATP increased the outward currents and initiated small 'spiky' inward currents at hyperpolarized voltage after 3 minutes (Figure 1a, b). The kinetics of the inward conductance resembled the hyperpolarization-activated Ca^{2+} conductance (HACC) found in previous studies on epidermal PM (*e.g.*, Demidchik *et al.*, 2009). Current activation was transient (Figure 1c), as was previously observed with eATP- and eADP-activated PM conductances in protoplasts from mature epidermis (Demidchick *et al.*, 2009, 2011). Eight minutes after ATP application, maximum current values (at +43 and -217 mV) were significantly greater than those prior to treatment (p < 0.01, Student's t-test; Figure 1c, d) and inward rectification of the hyperpolarization-activated currents became apparent. Analysis of the eATP-activated currents (control I/V subtracted from eATP I/V at 8 minutes; Figure 1d, insert) revealed a positive shift of E_{rev} to -64.5 ± 16.8 mV, indicating a greater Ca^{2+} permeability in eATP-induced inward currents (E_{Ca} at + 161 mV). It is therefore likely that eATP transiently increased Ca^{2+} conductivity to deliver Ca^{2+} to the cytosol, although increased Cl^- permeation is also possible.

However, neither outward nor inward currents in Col-0 were stimulated by the same concentration of eADP (300 µM; Supplemental Figure 4).

eATP-induced current activation does not occur in dorn1 mutants

Under control conditions, there was no significant difference between the overall Col-0 conductance and that of *dorn1-1* (Figure 2a,b: Tail current analysis of outward current, E_{rev} -88.8 ± 9.3 mV; P_K/P_{Cl} 9.4 ± 3.0; n=5). Consistent with Col-0, the epidermal PM currents recorded from both *dorn1* mutants were blocked by Gd^{3+} (Supplemental Figure S1b, c). In contrast to Col-0, *dorn1-1* did not respond to 300 µM ATP (Figure 2a,b,c), neither did *dorn1-3* (Figure 2d,e). As DORN1 binds ATP with a K_d of 45.7 nM (Choi *et al*., 2014), the lack of response to this high concentration of ATP supports the currents' lying downstream of this receptor in Col-0.

DORN1 is involved in eATP activation of *Arabidopsis* root epidermal PM currents

DORN1 governs eATP's transient activation of a Ca^{2+}-permeable inward conductance at hyperpolarized voltages (Figure 1c, d; Figure 2). With resting PM voltage in epidermal cells from *Arabidopsis* roots varying between -153 and -129 mV (Maathuis and Sanders, 1993), eATP-induced HACC-like currents could initiate $[Ca^{2+}]_{cyt}$ elevation for wound signalling (Choi *et al*., 2014) and promote the eATP-induced depolarization of *Arabidopsis* PM (Lew and Dearnaley, 2000). DORN1 also governs eATP's transient activation of a K^+-permeable outward conductance (probably NSCC) at depolarized voltages (Figure 1c, d; Figure 2). In whole *Arabidopsis* roots, the same concentration of eATP (300 µM) was shown by Demidchik *et al.* (2011) to induce K^+ efflux at the root epidermis. The K^+ efflux conductance found in the present study would be a strong candidate for the root eATP-induced K^+ efflux pathway. This may have significance for eATP and DORN1 function in pathogen- or stress-induced K^+ loss in cell death (Demidchik, 2014).

DORN1 commands $[Ca^{2+}]_{cyt}$ elevation in response to both eATP and eADP (Choi et al., 2014). The eADP concentration used here (300 µM) was found previously to activate PM K^+ efflux and Ca^{2+} influx conductances in Col-0 protoplasts from mature root epidermis (Demidchik *et al.*, 2011) but in the present study it had no effect on those from the root apex (Supplemental

Figure 4). A possible explanation is a cell-specific secondary effect of ADP that negates DORN1's activation of channels. Nevertheless, the relationship between DORN1 and PM conductances found here for eATP may well prove valuable in the search for the molecular identities of the contributory channels. Root PM conductances activated by eATP independently of DORN1 may also yet be discovered.

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Figure 1. Exogenous ATP activates K^+ and Ca^{2+} currents in Col-0 root epidermal plasma membrane.

a. Representative current traces from whole cell recordings of *Arabidopsis thaliana* Col-0 before (left panel) and after 3 minutes' ATP treatment (300 µM; right panel). Baseline membrane voltage was held at -137 mV prior to a step-wise voltage protocol of 20 mV increments. b. Effect of 300 µM eATP on *I/V* relationships. Data are means ± SE (n = 6), recorded 3 minutes before ATP addition, immediately before addition (0 minutes) and 3 minutes after. Inward current below the *V* axis is mainly Ca^{2+} influx. Outward current above the *V* axis is mainly K^+ efflux from cytosol. Equilibrium potentials for K^+ (E_K) and Cl^- (E_{Cl}) are annotated. E_{Ca} = + 161 mV c. eATP transiently increased inward and outward currents. Data are means ± SE of current recorded at -217 and +43 mV respectively (n = 3 to 6). ** marks significant difference from control ($p < 0.01$, Student's t-test). d. *I/V* relationships 8 minutes after ATP addition and (insert) difference *I/V* to reveal the eATP-activated currents (n = 4). Bath solution comprised 20 mM $CaCl_2$, 0.1mM KCl, 20 µM NaCl, 5 mM MES-Tris, pH 5.6. Pipette solution comprised 40 mM K-gluconate, 10 mM KCl, 0.4 mM $CaCl_2$, 1mM BAPTA, 2mM MES-Tris, pH 7.2 (Demidchik *et al*., 2011).

Figure 2. *dorn 1-1* and *dorn 1-3* failed to respond to exogenous ATP.

a. Representative current traces from *Arabidopsis thaliana dorn 1-1* before (left panel) and after 3 minutes' ATP treatment (300 µM; right panel). b. There was no effect of eATP on *I/V* relationships of *dorn 1-1* 3 minutes after eATP application and (c) no effect over an extended time period. d. There was no effect of eATP on *I/V* relationships of *dorn 1-3* 3 minutes after eATP application and (e) no effect over an extended time period. Data are presented as means ± SE (n = 5 in b and c; n = 3 in d and e). Bath solution comprised 20 mM $CaCl_2$, 0.1mM KCl, 20 µM NaCl, 5 mM MES-Tris, pH 5.6. Pipette solution comprised 40 mM K-gluconate, 10 mM KCl, 0.4 mM $CaCl_2$, 1mM BAPTA, 2mM MES-Tris, pH 7.2 (Demidchik *et al*., 2011).

Fig. S1. Gd^{3+} inhibits whole-cell currents from epidermal plasma membrane of Col-0 and *dorn1* mutants.

Fig. S2. Voltage ramping to determine Ca^{2+} permeability of the Col-0 inward conductance.

Fig. S3. Exogenous NaCl has no effect on Col-0 currents.

Fig. S4. Exogenous ADP does not induce currents in Col-0 apical root epidermal plasma

membrane.

Key words *Arabidopsis*, ATP, calcium, channel, DORN1, extracellular, potassium

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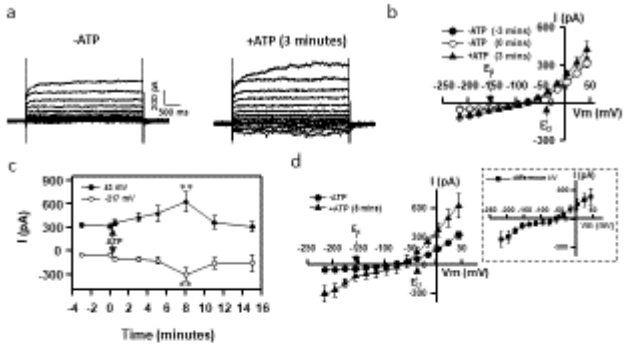
Author contributions The study was conceived by LW and JMD. LW and KAW generated and analysed the data. LW and JMD wrote the paper.



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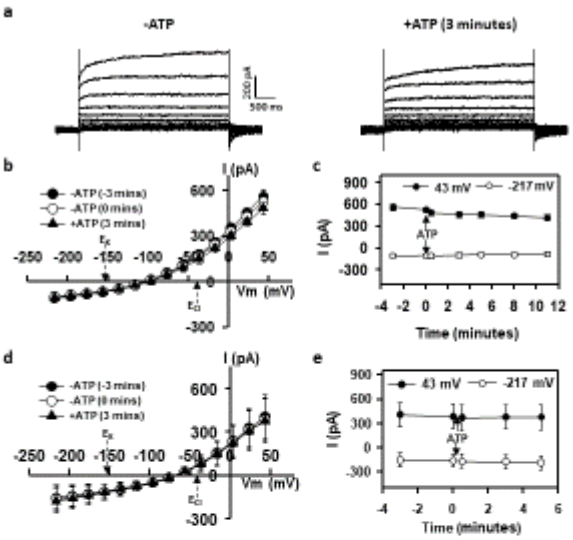

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