RESEARCH IN CONTEXT

DORN1/P2K1 and purino-calcium signalling in plants: making waves with extracellular ATP

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INTRODUCTION

ATP is the universal cellular energy currency. In plant cells, cytosolic ATP occurs at 0.5–2 mM (Gout et al., 2014; De Col et al., 2017). Whilst the mechanisms of ATP release into the plant cell’s extracellular space remain under investigation (Kim et al., 2006; Song et al., 2006; Rieder and Neuhaus, 2011; Wu et al., 2011), accumulation of extracellular ATP (eATP) to nanomolar levels occurs during growth and possibly to higher levels in response to abiotic and biotic stimuli (Jeter et al., 2004; Kim et al., 2006; Song et al., 2006; Weerasinghe et al., 2009; Clark et al., 2010; Dark et al., 2011; Zhu et al., 2017; Nizam et al., 2019). Levels of eATP are controlled through ATP-hydrolysing enzymes such as nucleotidases and most possibly by apyrases, although results on the latter are contended (Wu et al., 2007; Riewe et al., 2008; Lim et al., 2014; Massalski et al., 2015; Nizam et al., 2019). Damage to the plasma membrane rapidly releases large amounts of intracellular ATP into the extracellular space (Song et al., 2006; Weerasinghe et al., 2009; Dark et al., 2011). Extracellular ATP has therefore been termed a ‘danger signal’ (Choi et al., 2014a). Basal levels of eATP are, however, needed for optimal plant growth, with both depletion and augmentation of eATP triggering plant stress responses and too low or high a level promoting cell death (Chivasa et al., 2005; Kim et al., 2006; Clark et al., 2010; Sun et al., 2012; Jia et al., 2019). Thus, eATP has the hallmarks of a tightly regulated plant cell regulator.

eATP causes accumulation of reactive oxygen species (ROS), nitric oxide (NO), Ca²⁺ and phosphatidic acid, all of which are significant plant signalling intermediates (Demidchik et al., 2003a, 2003b, 2009; Tonón et al., 2010; Salmi et al., 2013; Clark and Roux, 2018; Q. W. Wang et al., 2019a). These may be involved in the changes in gene expression and protein abundance upon eATP perception (Jeter et al., 2004; Demidchik et al., 2009; Choi et al., 2014b; Lim et al., 2014; Lang et al., 2017; Tripathi et al., 2018; Rueda et al., 2019).

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Jewett et al. — DORN1-mediated calcium signalling

Studies to date on Arabidopsis thaliana seedlings support enrichment of defence- and wound-response genes in the suite regulated by eATP (Choi et al., 2014b; Tripathi et al., 2018; Jewell et al., 2019). The use of Arabidopsis receptor and signalling mutants has revealed that subsets of the eATP-regulated genes also require input from the jasmonate, ethylene and salicylic acid pathways (Tripathi et al., 2018; Jewell et al., 2019). The receptor on which eATP research currently hinges was identified through a forward genetic screen as a plasma membrane-spanning legume-like lectin serine–threonine receptor kinase termed ‘DOes not Respond to Nucleotides’ (DORN1; Choi et al., 2014b). The first higher plant eATP receptor, DORN1 (also known as P2K1 to align with animal purino-receptor nomenclature) has been characterised as a plasma membrane–cell wall continuum and is important for pathogen resistance (Bouwmeester et al., 2011; Balague et al., 2017; Tripathi et al., 2018).

DORN1 came to light as an eATP receptor through its role in increasing cytosolic free Ca2+ ([Ca2+]cyt) in seedlings (Choi et al., 2014b). This ability to direct [Ca2+]cyt as a second messenger is pivotal to plant purino signalling. Elevation of [Ca2+]cyt in plants is held to be stimulus-specific with the dis- mension of the plasma membrane–cell wall continuum and is important for pathogen resistance (Bouwmeester et al., 2011; Balague et al., 2017; Tripathi et al., 2018).

Leaf eATP: implications for abiotic stress and wounding

Hou et al. (2018) reported that hypertonic salt stress increases Arabidopsis leaf eATP and that eATP may then protect photosystem II (PSII) activity. The dornl-3 mutant was compromised in eATP protection of PSII (Hou et al., 2018), which suggests that eATP effects on PSII may run through [Ca2+]cyt as a second messenger. Applying gadolinium or lanthanum as blockers of plasma membrane Ca2+ influx channels inhibits eATP stimulation of PSII activity (Feng et al., 2015), from which an eATP-induced [Ca2+]cyt increase in leaves can be inferred. More recently, Hou et al. (2019) found that DORN1 is needed for the protective effects of eATP on PSII under high light. Whether eATP affects Ca2+ signalling in the chloroplast itself also remains to be determined. Certainly, eATP causes a plastidial Ca2+ increase in roots (Loro et al., 2016). Additionally, in leaves DORN1 is implicated in transducing the eATP signal induced in response to cadmium stress (Hou et al., 2017). This would be an interesting test case for the involvement of [Ca2+]cyt as cadmium can interfere with Ca2+ transport and signalling due to its similar size (Wilkins et al., 2016).

Given that eATP increases on wounding, and governs wound-induced/jasmonate-dependent transcription through DORN1 (Choi et al., 2014b; Tripathi et al., 2018; Jewell et al., 2019), it is timely to consider this receptor’s position in wound-induced [Ca2+]cyt signalling. Blocking plasma membrane Ca2+ influx channels impairs eATP induction of the jasmonate-dependent transcripts implicated in leaf wounding and necrotroph attack (Tripathi et al., 2018), potentially putting DORN1 upstream of channel opening. Leaf wounding (including by insect feeding) triggers a local [Ca2+]cyt elevation (Kiep et al., 2015; Vincent et al., 2017) and a vascular [Ca2+]cyt ‘wave’ that signals to other leaves, evoking a distal transcriptional response (Kiep et al., 2015; Nguyen et al., 2018; Toyota et al., 2018). The local [Ca2+]cyt signal involves two glutamate receptor-like Ca2+ influx channels (GLR 3.3 and 3.6; Vincent et al., 2017) and the TPC1 (two pore channel1) vacuolar Ca2+ release channel (Kiep et al., 2015; Vincent et al., 2017), while the wave is underpinned by GLR 3.3 and 3.6 (Nguyen et al., 2018; Toyota et al., 2018). At present, there is some doubt as to whether those GLRs operate at the plasma membrane (Toyota et al., 2018) or in endomembranes (Nguyen et al., 2018) in the vascular [Ca2+]cyt wave. Termination of the wound-induced [Ca2+]cyt signal is mediated in part by the plasma membrane ACA8 Ca2+-ATPase efflux pump (Costa et al., 2017).

Leaf wounding causes accumulation of extracellular glutamate (Toyota et al., 2018). It may be that DORN1 plays a part in the local wound [Ca2+]cyt signal or wave, especially given the finding that glutamate can cause eATP accumulation (Song et al., 2006; Dark et al., 2011). This could also help to explain the occurrence of apyrase in caterpillar saliva, which can suppress plant jasmonate-dependent wounding responses (Wu et al., 2012). The caterpillar apyrase could hydrolyse plant eATP to prevent a [Ca2+]cyt -driven wound response, a scenario supported by the finding that insect oral secretions suppress wound-induced [Ca2+]cyt elevation in leaves (Kiep et al., 2015). Recent work on kidney bean (Phaseolus vulgaris) leaves suggests that local wound-induced eATP elevation causes ROS elevation not only locally but also in other leaves, potentially through an ROS ‘wave’ (Q. W. Wang et al., 2019a). In Arabidopsis roots, [Ca2+]cyt and ROS work together in wave propagation for abiotic stress signalling (Evans et al., 2016). High-resolution studies (using leaf cell-specific aequorin lines; Marti et al., 2013) or more sensitive [Ca2+]cyt reporters are needed to determine the
spatial extent of DORN1’s operation in leaf eATP Ca^{2+} signatures and waves. Cell-specific lines would be particularly relevant to testing for DORN1’s role in guard cells.

**DORN1 and eATP operate in stomatal aperture regulation**

Guard cells use [Ca^{2+}]_{cyt} as a second messenger in aperture control (a system that involves plasma membrane NADPH oxidases and Ca^{2+} channels; Jezec and Blatt, 2017), and are now known to respond to eATP. Clark et al. (2011) reported eATP-induced production of ROS and NO with closure of Arabidopsis stomata in the light, but opening in the dark. Perhaps high light-driven ATP production results in greater eATP to close stomata and protect from evapotranspiration. Certainly, light levels control the triggering of cell death by eATP depletion in tobacco (Nicotinia tabacum; Chivasa et al., 2009). eATP promotion of stomatal opening was found to require the heterotrimeric G protein alpha subunit (GPA1) and plasma membrane NADPH oxidases RBOHD and F, with plasma membrane Ca^{2+} influx detected (Hao et al., 2012). In Vicia faba guard cells, plasma membrane Ca^{2+} channel activity was enhanced by eATP, which also promoted opening (Wang et al., 2014).

Stomatal closure by eATP involves DORN1. dorn1 mutants do not close in response to eATP yet can still close when challenged by abscisic acid (ABA) (Chen et al., 2017), even though exogenous ABA can induce eATP accumulation by guard cells (Clark et al., 2011). The RBOHD NADPH oxidase is a common component of both eATP and ABA pathways (Kwak et al., 2003; Chen et al., 2017). RBOHD directly interacts with DORN1 and eATP-induced stomatal closure fails in rbohD mutants (Chen et al., 2017). A recent transcriptional study of the rbohD mutant under high light stress found that some eATP-dependent transcripts were misregulated, also indicating that eATP signalling may run through this NADPH oxidase (Zandalinas et al., 2019). The DORN1 pathway is also proposed to operate in stomatal closure in response to pathogen attack (Chen et al., 2017). Whether DORN1 can contribute to the guard cell [Ca^{2+}]_{cyt} oscillations that occur in defence signalling (Thor et al., 2014) could be tested using ratiometric fluorescence imaging of [Ca^{2+}]_{cyt}.

Clearly, understanding how eATP can induce both stomatal opening and closure (potentially through RBOHD) requires further investigation, with the premise of dose-dependency (Clark et al., 2011) as a logical entry point. The mechanism of release of ATP to the extracellular space is central to further work in this area. Guard cell eATP accumulation is impaired in Arabidopsis mutants lacking the MRPs/5 ABC transporters (Wang et al., 2015). These have been proposed to be at the plasma membrane, suggesting that they mediate efflux of ATP (Wang et al., 2015). This could in turn relate to the impaired guard cell plasma membrane Ca^{2+} channel activity of the mrp5 mutant (Suh et al., 2007), with the possibility that the channel lesion is due to lower eATP levels leading to a failure in channel activation. However, a green fluorescent protein (GFP) localization study using MRPS’s native promoter (rather than constitutive expression) revealed its vacuolar origin, and heterologous expression showed MRPS to be an inositol hexakisphosphate (IP_{6}) transporter (Nagy et al., 2009). As IP_{6} regulates endomembrane Ca^{2+} release (Lemtiri-Chlieh et al., 2003) and plasma membrane K^{+} influx (Lemtiri-Chlieh et al., 2000), a more complicated picture of guard cell eATP accumulation emerges that potentially involves inositol signalling and with a black box still at the plasma membrane for ATP efflux.

**eATP at work in roots: growth and navigation**

Ratiometric imaging has revealed that cytosolic ATP (as the MgATP^{2-} species) is heterogeneously distributed through the Arabidopsis root (de Col et al., 2017). This is true also for eATP, with greatest levels at the root cap and root cell expansion points, particularly the root hair apex (Kim et al., 2006; Weerasinghe et al., 2009). High concentrations of exogenous (experimentally applied) ATP inhibit root elongation, probably through elevation of auxin, disruption of vesicular trafficking and increased cell wall lignification (Tang et al., 2003; Liu et al., 2012; Lim et al., 2014; Deng et al., 2015; Yang et al., 2015; Zhu et al., 2018). Prolonged exposure to high exogenous ATP (0.8 mM for 12 h) causes root cell death (Deng et al., 2015), so in planta eATP must be tightly regulated. Downregulation of apoplastic apyrase (which could elevate eATP) increases potato (Solanum tuberosum) tuber number and alters their shape (Riewe et al., 2008). eATP concentration appears critical to growth regulation of root hairs, with 7.25–25 μM exogenously added ATP or ADP stimulating elongation but ≥150 μM inhibiting elongation (Clark et al., 2010; Terrile et al., 2010). It is tempting to speculate that the low cytosolic MgATP^{2-} that correlates with high root hair growth rate (de Col et al., 2017) reflects release of ATP to the apex to drive elongation. Chelation of extracellular Ca^{2+} or application of plasma membrane Ca^{2+} channel inhibitors lowers root cell eATP levels and indicates a reliance on Ca^{2+} influx (Kim et al., 2006). It may be that plasma membrane mechanosensitive Ca^{2+}-permeable channels are responsible for the influx, opening in response to membrane stretch during cell expansion. As increased [Ca^{2+}]_{cyt} can stimulate exocytosis (Carroll et al., 1998), release of ATP as possible cargo of exocytotic vesicles (Yang et al., 2015) would help explain why root cell eATP levels are lowered by the exocytosis inhibitor breflidin A (Kim et al., 2006). The growth effects of eATP on root hairs involve the production of NO and ROS, with RBOHD and F implicated in the latter (Clark et al., 2010). Whether DORN1 is the root hair eATP receptor remains unknown. Neither is it clear yet whether extracellular purine nucleotides affect root hair [Ca^{2+}]_{cyt}, even though the latter is an important component of elongation and is increased by ROS (Foreman et al., 2003). Lew and Dearnaley (2000) found that eATP and eADP could depolarize the plasma membrane potential of Arabidopsis root hairs, which would be consistent with Ca^{2+} influx, but when testing eADP found no effect on [Ca^{2+}]_{cyt}.

Touch causes a transient, asymmetric increase in root eATP (Weerasinghe et al., 2009; Dark et al., 2011). Subjecting Arabidopsis roots to a mechanical stress typical of that experienced on growth through soil leads to increased concentrations of eATP, approximately 60 nM at 1 min after application (Weerasinghe et al., 2009), with higher levels on the side touched. The distal elongation zone was the most responsive root part, with both heterotrimeric G protein alpha (GPA1) and beta subunits (AGB1) found to be necessary for limiting the refractory period of eATP accumulation on repeated stimulation.
eATP at work in roots: symbiosis, mutualism and adaptation

Few studies have addressed whether eATP operates in root symbioses. Tanaka et al. (2015) proposed that legumes contain a specific type of extracellular apyrase. In soybean (Glycine max), silencing of the GS52 extracellular apyrase suppressed nodule development and maturation (Govindarajulu et al., 2009). This could be offset by the application of ADP as the product of the apyrase activity. The Dolichos biflorus LNP root hair extracellular apyrase binds to the Rhizobium lipo-chitin Nod factor and inhibiting the activity of this apyrase impairs both root hair formation and nodulation (Kalsi and Etzler, 2000). As eATP accumulates at the apex of Medicago truncatula root hairs (Kim et al., 2006), this suggests that eATP concentrations at the root hair apex must be lowered (or those of eADP increased) to allow symbiotic signalling. Sensing the presence of fungi could involve eATP. A fungal polysaccharide extract caused eATP accumulation by Salvia miltiorrhiza hairy root culture (Wu et al., 2011). In that study, eATP accumulation was prevented by anion channel antagonists, implicating efflux through plasma membrane anion channels. Whether symbionts (or pathogens) export ATP or ADP to communicate with or confound the plant root will be a challenging but interesting line of enquiry. Very little is known of eATP in fungal biology (Cai, 2012). For example, inhibiting the activity of ecto-nucleotidases of the rice blast fungus Magnaporthe oryzae inhibited germination of conidia and appresorium formation, implicating eATP as a regulator (Long et al., 2015). Recently, eATP levels of Arabidopsis and barley (Hordeum vulgare) roots were found to increase in the early (biotrophic) phase of colonization by the endophytic fungus Serendipita indica (formerly known as Piriformospora indica) (Nizam et al., 2019). The fungus secretes an ecto-5′-nucleotidase with which to lower eATP levels and permit greater colonization. The inability to sense eATP in the Arabidopsis dorn1-3 mutant also allows extensive colonization (Nizam et al., 2019). eATP activates conidiation in Trichoderma viride; conidiation is a wound response and intriguingly can be mimicked by eATP, acting upstream of [Ca^{2+}]_{cyt} elevation (Medina-Castellanos et al., 2014, 2018). eATP as a wound signal therefore appears ancient and conserved.

Wounding of roots can be envisaged to occur through herbivory, and indeed mechanical wounding of Arabidopsis roots causes a transient increase in eATP (Dark et al., 2011). As extracellular glutamate can also induce eATP accumulation by Arabidopsis roots (Dark et al., 2011), there may be parallels to be drawn with the herbivore-induced GLR-eATP-DORN1 signalling hypothesized in a previous section. Experimentally, it will be important in further exploration to mimic herbivore-induced wounding of roots accurately to deduce membrane-based signalling. Gross mechanical damage of a root is not appropriate as the resultant ionic fluxes (including of Ca^{2+}) are insensitive to channel blockers, indicating catastrophic membrane damage (Meyer and Weisenseel, 1997).

Abiotic stresses are good stimuli of eATP accumulation by roots. Copper stress causes eATP accumulation by wheat (Triticum aestivum) roots and protects against cell death (Iia et al., 2019). Cold, salt and hyperosmotic stress all cause transient increases in Arabidopsis root eATP, as does ABA which would imply the involvement of eATP in acute and longer term stress responses (Dark et al., 2011; Deng et al., 2015). Additionally, salt stress increases eATP of Glycrrhiza uralensis (licorice) roots (Lang et al., 2014). For salt stress, eATP can play a positive role in Na+/K+ homeostasis in Populus euphratica suspension cells (Sun et al., 2012) and also for mangrove and licorice roots (Lang et al., 2014, 2017). Cold, salt, hyperosmotic stress and ABA all cause elevation of [Ca^{2+}]_{cyt} in Arabidopsis roots as a second messenger in adaption (Wilkins et al., 2016). Could eATP be a part of this? Salt-induced [Ca^{2+}]_{cyt} elevation in dorn1 seedlings did not differ from that in the wild type, leading to the conclusion that this receptor had no part to play (Choi et al., 2014b). However, these measurements were made with aquorin and would be the average of a variety of cell populations, potentially masking root cell-specific responses. In the next sections, the evidence for eATP elevation of root cell [Ca^{2+}]_{cyt} will be reviewed and DORN1 hypothetically placed in that context.

eATP increases Ca^{2+} in specific root regions and multiple compartments of Arabidopsis root cells

The first demonstration of [Ca^{2+}]_{cyt} increase by extracellular purine nucleotides was in excised Arabidopsis roots, using
acquorin (Demidchik et al., 2003a). Since then, exogenous ATP has come to be used as a standard stimulus to test the efficacy of more powerful genetically encoded [Ca\textsuperscript{2+}]\textsubscript{cyt} indicators in roots and the resolution of different imaging methods (Rincón-Zachary et al., 2010; Krebs et al., 2012; Loro et al., 2012, 2016; Bonza et al., 2013; Waadt et al., 2017; Kelner et al., 2018). Such reporters have resolved spatially distinct regions of [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation in Arabidopsis roots that are superimposed experimentally with ATP (Rincón-Zachary et al., 2010; Loro et al., 2016; Waadt et al., 2017; Matthys et al., 2019). The initial [Ca\textsuperscript{2+}]\textsubscript{cyt} response occurs at the apex, with a significant contribution by the lateral root cap and meristem, followed by sub-apical elevation (Rincón-Zachary et al., 2010; Tanaka et al., 2010; Shi et al., 2015; Behera et al., 2018; Matthys et al., 2019). With two spatially and temporally distinct [Ca\textsuperscript{2+}]\textsubscript{cyt} elevations evident at the root apex, the question becomes ‘does DORN1 underpin them both?’.

Using such reporters, non-synchronized single-cell [Ca\textsuperscript{2+}] oscillations have been found to underlie the ‘averaged’ signal that acquorin generates (Tanaka et al., 2010; Krebs et al., 2012; Costa et al., 2017). Yellow Cameleon 3.6 (YC3.6) targeted to the plasma membrane revealed differing [Ca\textsuperscript{2+}] increases within a single cell, suggesting hotspots of local [Ca\textsuperscript{2+}]\textsubscript{cyt} maxima (Krebs et al., 2012).

Targeting reporters to various subcellular compartments has also uncovered links between cytosolic and organelar Ca\textsuperscript{2+} dynamics upon eATP perception. Targeting YC3.6 to the nucleus revealed non-synchronous oscillations in nuclear-free [Ca\textsuperscript{2+}] in response to eATP that lagged behind the [Ca\textsuperscript{2+}]\textsubscript{cyt} response (measured in different plants) by 7 min (Krebs et al., 2012), while another nuclear targeted YC3.6 reported much faster Ca\textsuperscript{2+} increases in response to eATP (Loro et al., 2012). However, dual targeting of GECO reporters to both the nucleus and the cytoplasm enabling simultaneous measurements from both compartments revealed only a [Ca\textsuperscript{2+}]\textsubscript{cyt} response to eATP in Arabidopsis root elongation zone cells (Kelner et al., 2018).

eATP treatment has also prompted [Ca\textsuperscript{2+}] increases in root mitochondria, plastids and endoplasmic reticulum (ER) (Loro et al., 2012, 2016; Bonza et al., 2013). Mitochondrial, plastid and ER increases in [Ca\textsuperscript{2+}] were found to be strictly related to increases of [Ca\textsuperscript{2+}]\textsubscript{cyt}, i.e. the larger the [Ca\textsuperscript{2+}]\textsubscript{cyt} increase, the larger the mitochondrial/plastid/ER increase (Loro et al., 2012, 2016; Bonza et al., 2013). Recovery of mitochondrial Ca\textsuperscript{2+} levels back to pre-stimulus values was much slower (more than 20 min) than the cytosol, ER and plastids (Loro et al., 2012, 2016; Bonza et al., 2013). Therefore, each compartment has its own Ca\textsuperscript{2+} signature, implying signal specificity and distinct operations of Ca\textsuperscript{2+} transporters and binding proteins. So far, it has been found that an increase in mitochondrial free Ca\textsuperscript{2+} is regulated by the MICU Ca\textsuperscript{2+} transporter (Wagner et al., 2015). The coordination of these various signatures downstream of eATP perception promises to be a fascinating area for future research.

At the mechanistic level, antagonists of plasma membrane Ca\textsuperscript{2+} influx channels or chelation of extracellular Ca\textsuperscript{2+} abolishes almost all of the eATP- or eADP-induced elevation of Arabidopsis root [Ca\textsuperscript{2+}]\textsubscript{cyt} (Demidchik et al., 2003a, 2009, 2011; Loro et al., 2016; Behera et al., 2018). This implies that the apoplast is the predominant source of Ca\textsuperscript{2+}, but it does not rule out any downstream involvement of intracellular Ca\textsuperscript{2+} stores which might rely on an initial trigger of apoplastic Ca\textsuperscript{2+}. Inhibition of intracellular phospholipase C signalling was shown to affect only the later stages of [Ca\textsuperscript{2+}]\textsubscript{cyt} signalling in response to eATP, leading the authors to conclude that both intracellular and apoplastic Ca\textsuperscript{2+} stores were involved in generating the later signal (Tanaka et al., 2010). As none of the organelle-targeted Ca\textsuperscript{2+} reporters showed a decrease of [Ca\textsuperscript{2+}] upon onset of the [Ca\textsuperscript{2+}]\textsubscript{cyt} response, it was reasoned that the organelles examined so far do not function as the Ca\textsuperscript{2+} source for the observed initial [Ca\textsuperscript{2+}]\textsubscript{cyt} increases (e.g. Bonza et al., 2013). However, the involvement of other organelles such as the vacuole and Golgi in roots remains to be tested. It is noteworthy that salt-stress-induced vacuolar Ca\textsuperscript{2+} release in Populus euphratica suspensions cells requires eATP and Ca\textsuperscript{2+} influx across the plasma membrane (Zhang et al., 2015). If the root vacuole were involved, then TPC1 would be a likely candidate for mediating Ca\textsuperscript{2+} efflux to the cytosol as its cytosol-facing EF hands would respond to increased [Ca\textsuperscript{2+}]\textsubscript{cyt} to enable Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release. Alternatively, the vacuole could be responding to cyclical ADPR (adenosine diphosphate-ribose). This is known to promote Ca\textsuperscript{2+} efflux from the guard cell vacuole (Leckie et al., 1998) and is synthesized in response to NO increase (Abdul-Awal et al., 2016). Root NO can increase in response to eATP and may rely on Ca\textsuperscript{2+} influx across the plasma membrane (Wu and Wu, 2008; Clark et al., 2010).

**Signalling components at the root plasma membrane**

Patch clamp electrophysiology has shown that both eATP and eADP can stimulate hyperpolarization-activated Ca\textsuperscript{2+} channels (HACCs) in the plasma membrane of Arabidopsis mature epidermal cells that could mediate the [Ca\textsuperscript{2+}]\textsubscript{cyt} increase (Demidchik et al., 2009, 2011; Shang et al., 2009). These results are supported by studies using extracellular ion-selective microelectrodes, which have shown net Ca\textsuperscript{2+} influx to the root epidermis induced by eATP and eADP (Demidchik et al., 2009, 2011). Root plasma membrane HACC activation by eATP also requires the heterotrimeric G protein alpha subunit (Zhu et al., 2018). A more recent patch clamp analysis demonstrated that DORN1 is required for eATP activation of a root elongation zone plasma membrane channel conductance that could permit Ca\textsuperscript{2+} influx to the cytosol at hyperpolarized membrane voltage (Wang et al., 2018). The need for a hyperpolarized (very negative) membrane voltage to permit eATP-activated Ca\textsuperscript{2+} influx is supported by a study on seedlings of the Arabidopsis aha2 mutant, expressing acquorin. As mentioned previously, AHA2 is the root’s predominant plasma membrane H\textsuperscript+-ATPase that generates the majority of the hyperpolarized membrane potential. AHA2’s absence caused significant diminution of the eATP-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} response (Haruta and Sussman, 2012).

eATP rapidly triggers production of both intra- and extracellular ROS in roots (Kim et al., 2006; Demidchik et al., 2009, 2011). Blocking plasma membrane Ca\textsuperscript{2+} channels with Gd\textsuperscript{3+} can effectively abolish eATP-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} increase in Arabidopsis roots but only inhibits intracellular ROS accumulation by approximately 50 % (Demidchik et al., 2003a, 2009). This implies that plasma membrane Ca\textsuperscript{2+} channel activity lies downstream of DORN1 and is required for a significant proportion of the ROS produced. The interaction of DORN1 with RBOHD (Chen et al., 2017) may yet help to explain the involvement of ROS in the root eATP-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} signature.
To date, only the RBOHC isoform is implicated experimentally for roots and some intracellular ROS accumulation was still observed in response to eATP in roots of the Arabidopsis rbohc mutant (Demidchik et al., 2009), potentially pointing to the activation of other isoforms. Certainly, RBOH D and F are expressed in roots and operate in Ca\(^{2+}\)-based ABA and salt stress signalling (Wilkins et al., 2016).

Modelling studies suggest that the ‘decay time’ of a calcium signature plays an important role in the regulation of gene expression (Lenzoni et al., 2018). However, very little is known mechanistically about how the eATP-induced [Ca\(^{2+}\)]\(_{cyt}\) increase might be limited or terminated. The heterotrimeric G protein beta subunit AGB1 negatively regulates eATP-induced [Ca\(^{2+}\)]\(_{cyt}\) increase in Arabidopsis seedlings, but its role in roots is unknown (Tanaka et al., 2010). In contrast, the plasma membrane Ca\(^{2+}\)-ATPase exporters ACA8 and ACA10 have been shown to play a role in ending the [Ca\(^{2+}\)]\(_{cyt}\) signal, through Ca\(^{2+}\) imaging of mutant roots (Costa et al., 2017; Behera et al., 2018). Whether post-translational modification of DORN1 or its retrieval from the plasma membrane plays a part remains unknown.

The eADP conundrum and DORN1-independent channel activation

A conundrum evident in the literature is that eADP can evoke extracellular superoxide anion production by roots, but unlike eATP it cannot induce intracellular ROS accumulation (Kim et al., 2006; Demidchik et al., 2009, 2011). The inability of eADP to evoke intracellular ROS accumulation has also been noted in leaves (Song et al., 2006). eADP and eATP are not always equivalent in effect. eADP has also been found to fail in inhibiting endocytosis (Deng et al., 2015). It can, however, promote nodule formation in soybean roots whereas eATP does not (Govindarajulu et al., 2009). For ROS, it has been suggested previously that eADP may somehow prevent entry of extracellular H\(_2\)O\(_2\) into the cytosol (Wang et al., 2018). At fine resolution, differences appear between eADP and eATP in their relationship with root [Ca\(^{2+}\)]\(_{cyt}\) increase. Protoplasts from Arabidopsis mature epidermis expressing aequorin show an eADP-induced [Ca\(^{2+}\)]\(_{cyt}\) increase that is resistant to the reductant dithiothreitol (Dark et al., 2011). This suggests that, in contrast to eATP, there is no oxidation-dependent component. Furthermore, the eATP-activated [Ca\(^{2+}\)]\(_{cyt}\) increase in such protoplasts is enhanced by neutral extracellular pH but this does not occur with eADP (Demidchik et al., 2011). Patch clamping has shown that eADP can activate HACC activity in Arabidopsis plasma membrane from the mature epidermis, independently of G protein activity (Demidchik et al., 2009). This appears to be in contrast to the GPA1-dependent eATP-activated HACC reported by Zhu et al. (2018). Finally, in patch clamp trials, the DORN1-dependent eATP-activated plasma membrane Ca\(^{2+}\) influx pathway in the elongation zone did not respond to eADP, even at a concentration orders of magnitude above DORN1’s \(K_a\) (Wang et al., 2018). This begs the question of whether there is a DORN1-independent eADP pathway in some epidermal cells. There is no a priori argument against a cell’s having more than one type of purinoreceptor. As described earlier, the Arabidopsis root’s eATP avoidance response is independent of DORN1 (Zhu et al., 2018). Indeed, given the evidence for multiple purinoreceptors in mammals, it might be considered surprising if plants do not also contain multiple eATP/eADP receptors. Further patch clamp analysis at the single channel rather than population level is now needed, combined with high-resolution single cell imaging of DORN1 mutants.

Interplay of eATP with other signals

In animals, eATP can modulate other signalling pathways by interacting with their receptors (Kim et al., 2018). Recently, ATP has been identified as a hydrotrope, capable of maintaining protein solubility and preventing protein aggregates (Patel et al., 2017). Whether it operates in this way outside of the plant plasma membrane is an interesting possibility. As such, the interplay between eATP and other regulators in plants is an understudied area of interest. eATP can still increase root [Ca\(^{2+}\)]\(_{cyt}\) after [Ca\(^{2+}\)]\(_{cyt}\) has been previously elevated by auxin or glutamate (Costa et al., 2013; Waadt et al., 2017; Behera et al., 2018) but it is not clear whether such pre-exposure to these regulators has an effect on the eATP Ca\(^{2+}\) signature or requires DORN1. In sensory synapses, eATP can cause glutamate release through purinergic receptor activation (Gu and MacDermott, 1997) and while glutamate can cause eATP accumulation in plants (Dark et al., 2011), the reverse case has not been reported. This could have consequences for our understanding of glutamate-based regulation of wound signalling or other processes such as carbon/nitrogen balance and root development (Toyota et al., 2018; Wudick et al., 2018). eATP has been found to modulate animal N-methyl-d-aspartate (NMDA)-type glutamate receptors, acting as a competitive antagonist of glutamate binding and a positive modulator at a separate allosteric site (Klodà et al., 2004). Plant GLRs have significant structural homology with NMDA receptors (Wudick et al., 2018), and it will be interesting to see whether eATP can influence their activity. There may also be an intricate relationship between eATP, salicylic acid and ethylene given that treating Arabidopsis roots with ethylene weakens the eATP-induced [Ca\(^{2+}\)]\(_{cyt}\) elevation (Waadt et al., 2017). This may prove relevant to the coordination of growth and immune responses and it will be interesting to see whether ethylene lowers DORN1 abundance.

Testing DORN1’s involvement and the possibility of an eATP ‘wave’

This review of extracellular purines and [Ca\(^{2+}\)]\(_{cyt}\) in leaves and roots provides a prima facie case for DORN1’s underpinning eATP- and eADP-induced [Ca\(^{2+}\)]\(_{cyt}\) in a variety of processes, but has also highlighted instances where DORN1 may be redundant. A critical first step in understanding the extent of DORN1’s involvement is to move from whole seedling studies to leaves and roots. To address this, aequorin-expressing seedlings of wild type Arabidopsis and its dornl-1 mutant have been dissected. DORN1’s abundance at the Arabidopsis root apex has been examined through GFP as a first test of whether it is present in root regions that undergo spatially discrete
eATP-induced $[Ca^{2+}]_{cyt}$ elevations. Finally, the relationship between the two spatially distinct eATP-induced $[Ca^{2+}]_{cyt}$ elevations at the root apex have been examined to test whether they are independent of each other and could form the basis of an eATP-induced $[Ca^{2+}]_{cyt}$ ‘wave’.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

Arabidopsis Col-0, dorn1-1 and dorn1-3 constitutively expressing cytosolic (apo)aequorin were as described by Matthus et al. (2019) with the exception that roots were bathed in 10 mM CaCl$_2$, 0.1 mM KCl, 2 mM MES/Tris, pH 5.8. Plant tissues were allowed to equilibrate with 5× growth medium agar. Twenty seconds after the start of image recording, light source and camera. ImageJ Fiji was used to process the GCaMP3 GFP signal intensities. Z-axis profiles were plotted for each region of interest, and background signal was subtracted. The protocol for normalizing GFP fluorescence ($ΔF/F_0$) was taken from Vincent et al. (2017). For DORN1-GFP determination, root tips were imaged on a Leica SP5 DM6000B confocal microscope, using a 20× objective (HC PL APO 20×/0.7×). GFP was excited at 488 nm using an argon laser, set to 30% laser power. GFP fluorescence emission was measured at 500–540 nm. Throughout the experiments the settings were kept constant (pinhole: 221.9 μm, gain set to 600 and speed of scanning to 400 Hz). Brightfield images were also taken of every imaged root section.

**RESULTS**

**Leaf eATP-dependent $[Ca^{2+}]_{cyt}$ elevation requires DORN1**

The Arabidopsis leaf eATP $[Ca^{2+}]_{cyt}$ signature has not been shown in the literature. Here, addition of control solution caused a brief monophasic $[Ca^{2+}]_{cyt}$ increase due to mechanical disturbance (‘touch response’) in 11-d-old and 14-d-old Col-0 leaves (Fig. 1A, B). In contrast, eATP caused a sustained $[Ca^{2+}]_{cyt}$ increase in Col-0, regardless of plant age (Fig. 1A, B). This $[Ca^{2+}]_{cyt}$ signature had a markedly different time course and lower magnitude than the typically biphasic root $[Ca^{2+}]_{cyt}$ response (Demidchik et al., 2003a; Matthus et al., 2019). Baseline $[Ca^{2+}]_{cyt}$ was not recovered during the recording. The whole seedling eATP-induced $[Ca^{2+}]_{cyt}$ increase appears to be entirely dependent on DORN1, as loss of function mutants do not respond (Choi et al., 2014b). Results here show that this is not as clear cut for leaves. Those of the dorn1-1 kinase-domain mutant (Fig. 1B, D) and the dorn1-3 ATP-binding-domain mutant (Fig. 1D) still supported a significant eATP-induced $[Ca^{2+}]_{cyt}$ increase, but not to the same level as the Col-0 wild type. At the level of resolution afforded by aequorin, DORN1 therefore appears important to the leaf’s eATP-dependent $[Ca^{2+}]_{cyt}$ response but is potentially partially redundant.

**The root eATP-induced and eADP-induced $[Ca^{2+}]_{cyt}$ signatures are DORN1-dependent**

No studies to date have tested whether dorn1 mutants can sustain eATP- or eADP-induced $[Ca^{2+}]_{cyt}$ elevation in their roots. Figure 2A shows that single excised roots of Col-0 and dorn1-1 did not differ in their baseline $[Ca^{2+}]_{cyt}$ and were indistinguishable in their response to control solution. Addition of 0.1 mM ATP caused an initial touch response in both genotypes but only Col-0 then sustained the typical root biphasic $[Ca^{2+}]_{cyt}$ response after the initial touch response (Matthus et al., 2019) (Fig. 2B). It is interesting to note that a biphasic response to eATP was sustained by Col-0 in the much simplified assay solution used here (10 mM CaCl$_2$, 0.1 mM KCl; Laohavisit et al., 2013) compared to the nutrient solution used by Matthus et al. (2019). This suggests a robust signalling system that remains unperturbed by environmental changes. The first and second peak $[Ca^{2+}]_{cyt}$ elevations of Col-0 were significantly greater than the $[Ca^{2+}]_{cyt}$ of dorn1-1 at the equivalent time point (Fig. 2B) and the total $[Ca^{2+}]_{cyt}$
mobilized by eATP was also significantly greater in Col-0 (Fig. 2E). Addition of 1 mM ATP to Col-0 gave a less distinct biphasic \([\text{Ca}^{2+}]_{\text{cyt}}\) increase compared to 0.1 mM (in agreement with an earlier study by Demidchik et al., 2003a) but this was still significantly greater than dorn1-1 (Fig. 2C, E). The Col-0 response to 1 mM ADP was also biphasic and significantly greater than dorn1-1 (Fig. 2D, E). Critically, while Col-0 \([\text{Ca}^{2+}]_{\text{cyt}}\) responses to purine nucleotides were all significantly higher than to control solution, those of dorn1-1 were not (Fig. 2E) indicating that at this level of resolution (and in contrast to leaves; Fig. 1) the DORN1 receptor is essential.

**DORN1 abundance declines as root cells mature but is evident in trichoblasts**

A GFP study previously confirmed DORN1’s plasma membrane localization (Choi et al., 2014a). The same construct was used here. Abundance of DORN1 in the root epidermis declined as the cells matured from the transition zone (Fig. 3A, D, G), through to the elongation zone (Fig. 3B, E, G) and mature zone (Fig. 3C, F, G). Abundance was greatest in the first apical millimetre of root, which corresponds to the region that supports the apical and initial \([\text{Ca}^{2+}]_{\text{cyt}}\) increase by eATP (Matthus et al., 2019). Of additional note is the appearance of DORN1 in the trichoblasts of the mature zone epidermis (Fig. 3F), which has not been reported previously.

eATP may generate a \([\text{Ca}^{2+}]_{\text{cyt}}\) wave in the root

Superfusion of a root with eATP causes apical and then sub-apical \([\text{Ca}^{2+}]_{\text{cyt}}\) responses that have the appearance of a \([\text{Ca}^{2+}]_{\text{cyt}}\) wave (noted by, for example, Rincón-Zachary et al., 2010; Loro et al., 2016; Matthus et al., 2019). However, the sub-apical \([\text{Ca}^{2+}]_{\text{cyt}}\) increases could simply be the result of direct cellular responses to eATP that are delayed in time rather than a consequence of the initial apical increase. Hence, it is unknown if eATP induces a \([\text{Ca}^{2+}]_{\text{cyt}}\) wave which propagates away from locally treated areas. Here, a single root expressing cytosolic GCaMP3 (Vincent et al., 2017) was placed over a gap in the underlying agar medium. The gap started after approximately the first millimetre of the root apex, so that it began after the region supporting the first \([\text{Ca}^{2+}]_{\text{cyt}}\) increase in response to eATP noted by Matthus et al. (2019) and after the greatest abundance of DORN1 (Fig. 3). eATP or control solution was applied sequentially to the root apex and then the mature region (Fig. 4A); the gap in the agar prevented capillarity-driven ATP movement between test regions (judged by previously testing fluorescence movement).

Regions of interest (‘Roi’) A, B and C were set for quantification of the GCaMP3 signal and corresponded to the apex, the region over the air gap and the mature zone respectively (Fig. 4A). Application of control solution did not lead to a fluorescence increase (Fig. 4B–J). Applying eATP first to the root apex (‘Phase 1’) revealed an immediate, largely monophasic fluorescence increase in the apex that recovered within 180 s (Roi
A; Fig. 4B, E, H). This resembled the apical \([\text{Ca}^{2+}]_{\text{cyt}}\) increase reported by Tanaka et al. (2010) and Matthus et al. (2019) using YC3.6. Lower but significant transient increases were later also detected in Roi B (over the air gap) and Roi C (mature zone) (Fig. 4C–J). This indicated that local application of eATP treatment to the apical root tip triggered a \([\text{Ca}^{2+}]_{\text{cyt}}\) increase in tissue that did not come in contact with the treatment, travelling ~0.3–0.5 mm of untreated tissue before being detected in Roi C.

A second eATP addition to the mature zone (‘Phase 2’) evoked a significant fluorescence increase there (Roi C; Fig. 4D, G, J) that did not evoke an increase at the apex (Fig. 4A, E, H).

In a separate set of experiments, eATP was first added to the mature zone of the root (‘Phase 1’, Roi C) followed by application to the apex (‘Phase 2’, Roi A; Fig. 5A–G). Application to the mature zone caused a significant increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) there compared to control solution (Fig. 5G, J) but although \([\text{Ca}^{2+}]_{\text{cyt}}\) increased in the root above the air gap (Roi B), it was not significant (Fig. 5F, I). The apex (Roi A) did not respond...

**Fig. 2.** DORN1 governs the root eATP-induced and eADP-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increase. (A) Mean ± s.e.m. \([\text{Ca}^{2+}]_{\text{cyt}}\) time course of individual excised roots from 7-d-old *Arabidopsis thaliana* Col-0 and *dorn1-1* constitutively expressing cytosolic aequorin. Control medium (10 mm \(\text{CaCl}_2\), 0.1 mm KCl, 2 mm Tris/MES, pH 5.8) was added at 35 s (black triangle). No significant difference between genotypes was found. (B) Response of Col-0 and *dorn1-1* to 0.1 mm ATP. (C) Response to 1 mm ATP. (D) Response to 1 mm ADP. (E) Total \([\text{Ca}^{2+}]_{\text{cyt}}\) mobilized (estimated as the area under the curve, after subtraction of mean pre-stimulus baseline) in response to control medium, 0.1 or 1 mm ATP, 1 mm ADP. \(n = 9–15\) roots per genotype and treatment in three independent trials. Analysis of variance (ANOVA) with post-hoc Tukey test was used to assess statistical differences. Significance levels (\(P\)-values) in B–D: *** (<0.001); D: different lower-case letters indicate \(P\) < 0.05.

**Fig. 3.** DORN1 abundance declines as primary root cells mature. (A–F) Confocal microscopy images of the different zones of 14-d-old *Arabidopsis* root expressing a DORN1-GFP fusion from the native promoter. Epidermis in the transition zone (TZ) is marked by brown rectangle, elongation zone (EZ) by pink, and mature zone (MZ) by yellow. Scale bar = 100 μm for all. (G) Background-subtracted DORN1-GFP fluorescence of epidermis in the transition zone (TZ, brown), elongation zone (EZ, pink) and mature zone (MZ, yellow) of 14-d-old *Arabidopsis* roots. GFP-signal was measured from the plasma membrane of epidermal cells and normalized to cells not expressing GFP; \(n = 24\) (TZ/MZ) and \(n = 44\) (EZ) from six individual plants. Analysis of variance (ANOVA) with post-hoc Tukey test was used to assess statistical differences, and different letters indicate significant difference (\(P\) < 0.001).
significantly when eATP was added to the mature zone. This confirms the previous result (Fig. 4E, H) that addition of eATP to the mature zone fails to evoke an apical response. When eATP was subsequently added to the apex (‘Phase 2’, Roi A), a significant $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation occurred there. This showed that this region was competent to respond to a local stimulus (Fig. 5E, H) and perhaps was not in a refractory period that prevented it from responding to the eATP that had previously been added to the mature zone. Moreover, in contrast to the previous experiment in which eATP was added to the apex first (Fig. 4C–J),
in this case addition of eATP to the apex after its addition to the mature zone did not cause significant [Ca$^{2+}$]$_{cyt}$ elevation in Roi B over the air gap or in the Roi C mature zone (Fig. 5E–J). Perhaps these zones were in a refractory period that prevented their response.

**DISCUSSION**

Leaf and root [Ca$^{2+}$]$_{cyt}$ signatures differ

The findings presented here clearly show that Arabidopsis leaf
and root eATP-induced \([Ca^{2+}]_{\text{cyt}}\) signatures differ in their time courses and amplitude. Seedling responses would be an average of these. The leaf \([Ca^{2+}]_{\text{cyt}}\) response qualitatively resembled that reported for eATP-treated \textit{Nicotiana benthamiana} leaf discus (DeFalco \textit{et al.}, 2017). Furthermore, while the root signature appears to be entirely dependent on DORN1, other receptors may be operating in the leaf. This may be use of the identification of other receptors and also in further studies on the possible roles of DORN1 in leaf purino-signalling. Certainly, the results here support the premise that eATP could affect response to cadmium, PSI activity and leaf jasmonate-dependent transcription through a \([Ca^{2+}]_{\text{cyt}}\) increase (Feng \textit{et al.}, 2015; Hou \textit{et al.}, 2017, 2018). More detailed, higher resolution studies on cell-specific \([Ca^{2+}]_{\text{cyt}}\) responses are now needed for leaves and roots, such as those performed with YC3.6 on root responses to auxin (Shi \textit{et al.}, 2015).

**An eATP-induced root wave?**

So far, exogenous ATP has been applied by superfusion of roots or their total immersion. This does not allow us to distinguish between local or systemic effects of eATP perception. For example, locally applied salt treatment of Arabidopsis roots led to increases in \([Ca^{2+}]_{\text{cyt}}\), which propagated away and into areas of the plant that had not been in direct contact with the salt treatment, termed a ‘Ca\(^{2+}\) wave’ (Choi \textit{et al.}, 2014c). Wave propagation involved both RBOHD and TPC1 (Evans \textit{et al.}, 2016). A resultant transcripional response was evident in the leaves. The same study found no such signal propagation upon mechanical stimulation, H\(_2\)O\(_2\), or cold treatment, and did not test for the response to eATP (Choi \textit{et al.}, 2014c). The results in Fig. 4 show that application of eATP to the root apex results in a sub-apical \([Ca^{2+}]_{\text{cyt}}\) elevation, consistent with a wave. However, the results in Fig. 5 show that this fails if the sub-apical region has already responded to a direct application of eATP, suggesting that there is a refractory period for the sub-apical response to apical stimulation. Furthermore, apical and mature region responses to eATP can be generated independently of one another and the mature region can still respond even though DORN1 abundance is lower there (Fig. 3). The response of the mature region to direct application of eATP may implicate the operation of another eATP sensing mechanism. The loss of DORN1 as the epidermis matures could also help to explain why eATP- and eADP-dependent net fluxes of Ca\(^{2+}\) and K\(^+\) also decline as the epidermis ages (Demidchik \textit{et al.}, 2011). However, it should be noted that receptor abundance may not positively correlate with the magnitude of the response; much would depend on the levels of signal amplification and positive feedback downstream of the receptor. The results in Figures 4 and 5 suggest strongly that a reverse Ca\(^{2+}\) wave (sub-apical to apical) cannot be generated. Whether eATP application to the apex results in a systemic transcriptional response now needs to be examined.

**Placing DORN1 in the context of root ROS**

Activation of RBOHs could be directly by DORN1-dependent phosphorylation and/or through their cytosolic EF hands (Takeda \textit{et al.}, 2008) responding to an initial \([Ca^{2+}]_{\text{cyt}}\) elevation caused by DORN1-dependent plasma membrane Ca\(^{2+}\) channels. The latter would be consistent with the inhibitory effects of Gd\(^{3+}\) on eATP-induced ROS accumulation (Demidchik \textit{et al.}, 2009). RBOHs would produce an extra-cellular superoxide anion that could readily be converted to \(H_{2}O_{2}\), and from that to hydroxyl radicals (Richards \textit{et al.}, 2015). From the guard cell paradigm, \(H_{2}O_{2}\) could then enter into the cytosol through aquaporins (Rodrigues \textit{et al.}, 2017) to be detected as part of the intracellular ROS accumulation. Both of those ROS are known to activate Arabidopsis root epidermal plasma membrane Ca\(^{2+}\) channels (Demidchik \textit{et al.}, 2003b, 2007; Foreman \textit{et al.}, 2003). This would place some part of the plasma membrane Ca\(^{2+}\) influx downstream of RBOH activation in the eATP cascade, potentially acting to amplify the initial \([Ca^{2+}]_{\text{cyt}}\) elevation. This would be consistent with the inhibition of the eATP-activated HACC by the reductant dithiothreitol in mature epidermal plasma membrane (Demidchik \textit{et al.}, 2009). As emerging root hairs are capable of ROS- and \([Ca^{2+}]_{\text{cyt}}\)-dependent growth (Foreman \textit{et al.}, 2003), the finding that DORN1 is present in trichoblasts suggests that DORN1 could be involved in root hair elongation through ROS- and \([Ca^{2+}]_{\text{cyt}}\)-signalling. The finding that DORN1 can underpin both purine nucleotide-induced \([Ca^{2+}]_{\text{cyt}}\) increases in roots and leaves justifies this receptor as a logical starting point in the search for the proteins mediating plasma membrane Ca\(^{2+}\) fluxes. Direct phosphorylation of plasma membrane Ca\(^{2+}\) channels by DORN1 is a distinct possibility but, with channels likely to have a low copy number, approaches such as pull-down assays may not be fruitful in their identification. Publicly available protein–protein interaction data (MIND database; Jones \textit{et al.}, 2014) revealed few interaction partners of DORN1, as only one uncharacterized leucine-rich repeat receptor kinase (At3g02880) was found to interact reliably. However, Chen \textit{et al.} (2017) reported that in total 23 peptides were phosphorylated by DORN1 upon eATP perception, one of which was the RBOHD NADPH oxidase (the remaining 22 peptides were not further identified). By extension of the leaf wound response, the root plasma membrane Ca\(^{2+}\) influx channels underpinning what may be a Ca\(^{2+}\) wave in the root could include GLR3.3 and 3.6 (Vincent \textit{et al.}, 2017). Members of the cyclic nucleotide-gated channel (CNGC) family could also be involved, although so far Arabidopsis CNGC14 has been reported not to be involved in lateral root cap eATP-induced \([Ca^{2+}]_{\text{cyt}}\) elevation (Shi \textit{et al.}, 2015). Annexins could also participate as channel regulators or unconventional ROS-activated channels and have been proposed to operate downstream of RBOHD (Laohavisit and Davies, 2011; Laohavisit \textit{et al.}, 2012; Zandalinas \textit{et al.}, 2019). Recently, Arabidopsis Annexin4 expressed in HEK cells was found to support \([Ca^{2+}]_{\text{cyt}}\) elevation in response to eATP (Ma \textit{et al.}, 2019) but the operation of this annexin \textit{in planta} in eATP signalling was not reported. Work needs to extend beyond Arabidopsis, as the legume root hair plasma membrane Ca\(^{2+}\) channels implicated in eATP/ADP-related symbiotic signalling remain to be discovered. Candidate root hair channel genes have been identified...
CONCLUSIONS

Overall, DORN1 still provides an important experimental gateway into the further dissection of purine–calcium signalling in roots and leaves. However, comparisons with animal purino-signalling and some plant studies (including this) suggest that it is unlikely that DORN1 is the only purine nucleotide receptor in Arabidopsis. Other receptors may lie outside the leucine kinase family and may not even rely on the presence of an ATP-binding pocket. Novel ATP-binding sites have been reported for Arabidopsis peptides using acyl-ATP probes (Villamor et al., 2013). Future studies also need to consider as yet unexplored areas of the plant. For example, as mechanical stress can result in release of ATP, does this occur during the shoot apical meristem? Does this link to its mechanically induced [Ca\(^{2+}\)]\(_i\) increase that is mediated by Ca\(^{2+}\) influx (Li et al., 2019)? While major breakthroughs on eATP signalling have come from Arabidopsis, it remains imperative that other plants (particularly monocot crops) continue to be studied. Apart from Arabidopsis, only tobacco has been reported to sustain an eATP-induced [Ca\(^{2+}\)]\(_i\)\(\rightarrow\) signature (DeFalco et al., 2017). [Ca\(^{2+}\)]\(_i\) indicators have been introduced into Medicago and rice (Behera et al., 2015; Kelner et al., 2018) but as yet there are no reports of the effect of eATP. At present, another potential eATP receptor has been identified in Camelina sativa as an orthologue of DORN1 (csLecRK-1.9; Li et al., 2016), but this is another brassica. Are there other DORNs?

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