Damage signalling by extracellular nucleotides; a role for cyclic nucleotides in elevating cytosolic free calcium?

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Abstract

Extracellular ATP (eATP) is now held to be a constitutive Damage Associated Molecular Pattern (DAMP) that is released by wounding, herbivory or pathogen attack. The concentration of eATP must be tightly regulated as either depletion or overload leads to cell death. In *Arabidopsis thaliana*, sensing of eATP is by two plasma membrane legume-like lectin serine-threonine receptor kinases (P2K1 and P2K2), although other receptors are postulated. The transcriptional response to eATP is dominated by wound- and defence-response genes. Wounding and pathogen attack can involve the cyclic nucleotides cyclic AMP (cAMP) and cyclic GMP (cGMP) which, in common with eATP, can increase cytosolic free Ca^{2+} as a second messenger. This Perspective on DAMP signalling by eATP considers the possibility that the eATP pathway involves production of cyclic nucleotides to promote opening of Cyclic Nucleotide Gated Channels and so elevates cytosolic free Ca^{2+}. *In silico* analysis of P2K1 and P2K2 reveals putative adenylyl and guanylyl kinase sequences that are the hallmarks of "moonlighting" receptors capable of cAMP and cGMP production. Further, an *Arabidopsis* loss of function *cngc* mutant was found to have an impaired increase in cytosolic free Ca^{2+} in response to eATP. A link between eATP, cyclic nucleotides and Ca^{2+} signalling therefore appears credible.

Keywords: ATP, calcium, CNGC, cyclase, cyclic nucleotide, DAMP.

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Introduction

Extracellular ATP (eATP) is now recognised as a plant cell regulator, with the ability to affect growth, development and stress responses (reviewed by Matthus *et al*., 2019a). With resting levels thought to be in the nanomolar range, eATP concentration must be tightly regulated as either depletion or overload leads to cell death (Chivasa et al., 2005; Sun *et al*., 2012; Deng *et al*., 2015). This extracellular nucleotide is now thought to be involved in the response to wounding and microbial attack, such that it is classified as a constitutive DAMP (Damage Associated Molecular Pattern; Choi *et al*., 2014a; Tanaka and Heil, 2021). It is readily envisaged that a breach of the plasma membrane would allow release of cytosolic ATP to the apoplast. Increased eATP concentration has indeed been measured from *Arabidopsis thaliana* roots, plus *Arabidopsis* and kidney bean (*Phaseolus vulgaris L*) leaves in response to

mechanical wounding (Song et al., 2006; Weerasinghe *et al.*, 2009; Dark et al., 2011; Wang *et al*., 2019a). Wounding can also result in accumulation of extracellular glutamate, which in turn can cause eATP accumulation (Dark *et al*., 2011; Toyota *et al*., 2018). Non-damaging mechanical disturbance generated by touch can lead to increased eATP of roots (Weerasinghe *et al.*, 2009), suggesting that microbial growth that does not breach the plasma membrane could cause eATP increase by putting pressure on walls. If the plasma membrane were to remain intact, studies suggest that ATP release mechanisms include exocytosis and a diversity of transport proteins (Kim *et al*., 2006; Choi *et al*., 2014a; Matthus *et al*., 2019a).

Microbial presence has indeed been found to influence eATP levels, which in turn may affect colonisation. The early phase of root colonisation of both *Arabidopsis* and barley (*Hordeum vulgare*) by the endophytic fungus *Serendipita indica* is associated with significant eATP increase (Nizam *et al.*, 2019). However, secretion of an eATP hydrolysing ecto-5'-nucleotidase by the fungus lowers eATP and allows greater colonisation (Nizam *et al*., 2019). *Arabidopsis* roots expressing the *S. indica* nucleotidase became more susceptible to colonization by the pathogenic fungus *Colletotrichum incanum*, suggestive of a role for eATP depletion (Nizam *et al*., 2019). Presence of *Pseudomonas syringae* (and to a lesser extent flg22) results in increased eATP levels in *Arabidopsis* leaf, particularly around guard cells. The high eATP causes stomatal closure, limiting bacterial entry (Chen *et al*., 2017). The mycotoxin beauvericin (BEA) increases eATP of wheat (*Triticum aestivum*) coleoptiles and causes cell death (Šrobárova *et al*., 2009). BEA is most notably produced by *Fusarium* species and acts in wheat head blight: Šrobárova *et al*., 2009). As a Ca^{2+} ionophore (Caloni *et al*., 2020), BEA could act to increase Ca^{2+} influx into cells. In *Arabidopsis* roots, promoting Ca^{2+} influx results in increased eATP most likely by stimulating exocytotic ATP release (Kim *et al*., 2006), which may help explain BEA's ability to elevate wheat eATP. In contrast the mycotoxin Fumonisin B1 (FB1) causes eATP depletion in *Arabidopsis* leading to cell death (Chivasa *et al*., 2005). Delineating how cells sense and respond to varying eATP levels is therefore critical to understanding the role of eATP in regulating what may be a continuum between growth, immunity and death (Choi *et al*., 2014a).

The first angiosperm eATP receptor was identified through an *Arabidopsis* forward genetic screen as a plasma membrane legume-like lectin serine-threonine receptor kinase, "DOes not Respond to Nucleotides1" (DORN1), now also known as P2K1 (consistent with animal eATP receptor terminology) and formerly known as LecRK1.9 (Gouget *et al*., 2006; Bouwmeester *et al*., 2011; Choi *et al*., 2014b; Balagué *et al*., 2016). It is thought to form part of the plasma membrane-cell wall continuum (Bouwmeester *et al*., 2011). Structurally, DORN1/P2K1 differs markedly from the animal eATP receptors, which are eATP-binding Ca^{2+} channels or G-protein-coupled receptors (Choi *et al*., 2014a, b; Nguyen *et al*., 2016; Verkhratsky, 2021). DORN1/P2K1 is important for defence against *Botrytis cinerea* (necrotrophic fungus: Tripathi et al., 2018), *Phytophthora brassicae* and *P. infestans* (biotrophic oomycetes: Gouget et al., 2006; Bouwmeester et al., 2011, 2014), *Pseudomonas syringae* (hemibiotrophic bacterium: Balagué *et al*., 2016; Chen *et al*., 2017), *Rhizoctonia solani* (necrotrophic fungus: Kumar *et al*., 2020) and *S. indica* (Nizam *et al*., 2019). A second *Arabidopsis* eATP receptor, P2K2, was identified by complementation of a *p2k1* mutant. P2K2 is also a plasma membrane legume-like lectin receptor kinase which is trans-phosphorylated by P2K1 and is involved in defence against *P. syringae* (Pham *et al*., 2020). There is now evidence that DORN1/P2K1 and P2K2 are unlikely to be the only *Arabidopsis* eATP receptors (Zhu *et al*., 2017, 2020; Matthus *et al*., 2019a; Smith *et al*., 2021). Nevertheless, activation of DORN1/P2K1 by eATP leads to phosphorylation of Mitogen-Activated Protein Kinase3 (MPK3: Choi *et al*., 2014b. MPK3 can phosphorylate Calmodulin-binding Transcription Activator3 (CAMTA3) in flg22 signalling,

which results in CAMTA3 breakdown and the release of its repression of defence gene transcription (Jiang *et al*., 2020). It is not clear whether this also occurs in eATP signalling but an estimated 99.8% of seedling eATP-responsive transcriptome requires DORN1/P2K1 and the CAM-box CAMTA3 binding target is highly enriched in promoters of eATP-regulated genes (Jewell *et al*., 2019). The eATP transcriptome is enriched in wound response and defence-related genes, with some also requiring regulation by the ethylene, jasmonate and salicylic acid pathways (Choi *et al*., 2014b; Tripathi *et al*., 2018; Jewell *et al*., 2019).

Whilst receptor characterization and analyses of receptor mutants has established eATP as a DAMP, the signalling systems downstream of receptors remain less well understood. A transient increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) as a second messenger is one of the earliest detectable changes downstream of eATP (Demidchik *et al*., 2003; Choi *et al*., 2014b; Behera *et al*., 2018). Mechanical wounding and insect feeding also increase $[Ca^{2+}]_{cyt}$ (Hander *et al*., 2019; Malabarba *et al*., 2021), suggesting the involvement of eATP. It can be noted in passing that saliva from insect herbivores can contain eATP hydrolyzing enzymes such as ecto-apyrase, suggesting that eATP is an important target to control (Su *et al*., 2012; Wu *et al*., 2012). Studies on how eATP increases $[Ca^{2+}]_{cyt}$ in *Arabidopsis* have focused on plasma membrane Ca^{2+}-permeable channels. Pharmacological block of such channels prevents $[Ca^{2+}]_{cyt}$ elevation (Demidchik *et al*., 2003; Behera *et al*., 2018) and adversely affects transcription of jasmonate-dependent genes involved in wounding and defence (Tripathi *et al*., 2018). Patch clamp electrophysiological analyses of root epidermis have revealed eATP- and DORN1/P2K1-dependent Ca^{2+} influx channel conductances that would be competent to elevate $[Ca^{2+}]_{cyt}$ (Demidchik et al., 2009; Zhu *et al*., 2017; Wang *et al*., 2018, 2019b). eATP-activated Ca^{2+} influx conductances have also been found in tobacco pollen tube and *Vicia faba* guard cell plasma membranes (Wang *et al*., 2014; Wu *et al*., 2018). The identity of the underpinning Ca^{2+} channels in all of these cell types remains under investigation. To date, the plasma membrane NADPH oxidase RBOHC (Respiratory Burst Oxidase HomologueC; Demidchik *et al*., 2009) and the heterotrimeric G protein alpha subunit (GPA1; Zhu *et al*., 2017) are implicated in regulating *Arabidopsis* root eATP-activated channels. Annexin1 (which supports a hydroxyl radical-activated Ca^{2+} channel activity; Laohavisit *et al*., 2012) is implicated in eATP-induced $[Ca^{2+}]_{cyt}$ increase of *Arabidopsis* roots and transcriptional regulation of wound- and defence-response genes but its mode of action has not been determined (Mohammad-Sidik *et al*., 2021). It also underpins wounding- and herbivory-induced $[Ca^{2+}]_{cyt}$ increases (Malabarba *et al*., 2021). Annexin4 supports an eATP-induced $[Ca^{2+}]_{cyt}$ increase when expressed in *Xenopus* oocytes but its *in planta* activity has not yet been reported (Ma *et al*., 2019). Recently, a patch clamp study on *Arabidopsis* pollen grains identified two Cyclic Nucleotide Gated Channel (CNGC) subunits (CNGC2 and CNGC4) as underpinning a plasma membrane eATP-activated Ca^{2+} influx conductance that may be relevant to germination (Wu *et al*., 2021). The conductance was lost in the *dorn1* loss of function mutants suggesting the channels act downstream of this receptor although it was not checked if *CNGC2* and *CNGC4* expression were downregulated in *dorn1*. The CNGC2 and CNGC4 channel subunits form a heterotetrameric plasma membrane Ca^{2+} influx channel in flg22 PAMP-triggered immunity (Pathogen Associated Molecular Pattern; Tian *et al*., 2019), indicating a possible common point in DAMP and PAMP signalling. Furthermore, the results of Wu *et al.* (2021) imply that the cyclic nucleotides cAMP (3', 5'-cyclic adenosine monophosphate) or cGMP (3', 5'-cyclic guanosine monophosphate) act downstream of eATP and DORN/P2K1. Although eATP has long been known to increase animal cell cAMP (Choi and Kim, 1997; Castro *et al*., 1998) there appear to be no reports to date of eATP's having an effect on cAMP or cGMP levels in plants, but these nucleotides can increase $[Ca^{2+}]_{cyt}$ through CNGC activation (reviewed by Jarratt-Barnham *et al*., 2021). This

Perspective will consider the possibility that cyclic nucleotides and CNGCs act in eATP-DAMP $[Ca^{2+}]_{cyt}$ signalling.

Cyclic nucleotide production by moonlighting proteins in response to pathogens and wounding

cAMP and cGMP are involved in growth and development, abiotic stress responses, stomatal kinetics, control of photorespiration and photosynthesis and immunity (reviewed by Jarratt-Barnham *et al*., 2021). Production of cAMP and cGMP is now held to be by adenylyl cyclase (AC) and guanylyl cyclase (GC) centres embedded in a diverse range of soluble and membrane-integral proteins (Ludidi and Gehring, 2003; Ruzvidzo *et al*., 2019; Al-Younis *et al*., 2021; Turek and Irving, 2021). There is clear evidence supporting cytosolic cyclic nucleotide production by a "moonlighting" function of members of the Receptor-Like Kinase (RLK) superfamily, relevant to response to pathogens, where the catalytic centre is present in the cytosolic kinase domain (Qi *et al*., 2010; Kwezi *et al*., 2011; Turek and Irving, 2021). For example in *Arabidopsis*, LLRAC1 (Leucine-Rich Repeat Adenylyl Cyclase1) is implicated in defence against *P. syringe* and the biotrophic fungus *Golovinomyces orontii* (Bianchet *et al*., 2019). Wall-Associated Kinase-LIKE (WAKL) proteins in *Arabidopsis*, wheat and *Brassica napus* are implicated in cGMP production and pathogen defence (reviewed by Turek and Irving, 2021). Most recently, expression of rice WAKL21.2 (which has GC activity) in *Arabidopsis* resulted in transcription of SA-related defence genes and conferred resistance to *P. syringae* (Mulkani *et al*., 2020).

In terms of DAMP signalling, wounding was found to result in a rapid, five-fold increase in *Arabidopsis* leaf cAMP and cGMP by van Damme *et al*. (2014). In this instance, the 2',5' isomers were detected but the mode of production and downstream consequences remain unknown. Larval oral secretions from the moth *Spodoptera littoralis* can increase cAMP content of *Arabidopsis* leaves within 3 minutes, which may relate to herbivory damage (Kumar *et al*., 2019). An initial decreased transcription followed by longer-term increased transcription of *Hippeastrum hybridum Guanylyl Cyclase1* (*HpGC1*, most likely encoding a soluble enzyme) was found in wounded and infected scales (fungal infection by *Peyronellaea curtisii*). Although a causal relationship was not demonstrated, HpGC1 activity may relate to downstream production of cGMP in the challenged scales (Świeżawska *et al*., 2015). Since then, Triphosphate Tunnel Metalloenzyme 3 (TTM3) from *Brachypodium distachyon* has been found to have both ATP hydrolyzing and AC activities, with upregulation of transcription by wounding (Świeżawska *et al*., 2019). There remains a significant knowledge gap when it comes to whether eATP as a DAMP (or in its role as growth regulator) has an effect on cAMP or cGMP levels. However, increasing cAMP through expression of an AC increases transcription of wound-related genes (Xu *et al*., 2021) which could place eATP (as a DAMP governing wound-related transcription) upstream of cAMP production. A key question is whether the eATP receptors could themselves generate cAMP or cGMP.

Could eATP receptors generate cAMP or cGMP?

To date neither DORN1/P2K1 nor P2K2 have been identified as harbouring AC/GC domains. However the ACPred AC prediction tool sequence [KSR]X[DE]X{10}[KR]X{0,3}[DE] (Xu *et al*., 2018a; http://gcpred/acpred/; Figure 1A) does reveal possible sequences. Two overlapping sequences are present in DORN1/P2K1 (Figure 1B) that harbour an aspartic acid residue ([D]) at position 3 that should confer ATP binding specificity but the sequences are *extracellular*, lying close to the transmembrane domain and beyond the residues already implicated in eATP binding in the lectin domain (Nguyen *et al*., 2016; Cho *et al*., 2017) (Figure. 1C). The first sequence starting at S259 has an aspartic acid residue predicted for cation binding

that should follow the 14 amino acid catalytic centre motif (Al-Younis *et al.*, 2021) but after an interval of 2 amino acids. The second sequence (starting at R267) lacks a cation binding residue but overlaps at position 4 with the AC catalytic centre motif used recently by Al-Younis *et al.* (2021) ([KSR][YFW][DE][VIL]X{4[Y]X{4}[KR]X{0,3}[DE]; Figure 1A). Curiously, this second sequence contains the PHPR found previously to be an RGD binding sequence, relevant to the RGD-containing IPI-O protein secreted by *Phytopthora infestans* (Gouget *et al*., 2006). Potentially, therefore, DORN1/P2K1 has more than one way to bind eATP. P2K2 habours a putative AC sequence in its cytosolic serine/threonine kinase domain and this sequence conserves an aspartic acid residue for cation binding following the catalytic centre motif (Figure 1D). The GCPred GC prediction tool sequence [KS]X[SCG]X{10}[KR]X{0,3}[DHSE] (Xu *et al*., 2018b; http://gcpred; Figure 1A) revealed two intracellular sequences in DORN1/P2K1, with one harboured in its serine/threonine kinase domain (Figure 1C). For P2K2, two sequences were found in the extracellular lectin domain and one in the intracellular serine/threonine kinase domain (Figure 1D). All of these have the conserved [KS]X[SCG]X{10}[KR] sequence that confers GC function (Wong and Gehring, 2013) and is present in the *Pharbitis nil* GC1 enzyme which has been shown to catalyse cGMP production *in vitro* (Szmidt-Jaworska *et al*., 2009). The [DHSE] sequence was absent or present after a 2 amino acid gap. The intracellular sequence and proximal extracellular sequence also had limited conservation with the more detailed GC motif [RKS][YFW][CTGH][VIL][FV]G[DNA]X[VIL]X{4}[KR] used to identify AtGC1 (Ludidi and Gehring, 2003; Wong *et al*., 2018). Both eATP receptors therefore appear to have the requisite sequences to generate cyclic nucleotides and this now requires experimental validiation.

CNGC2 is implicated in cotyledon eATP-induced $[Ca^{2+}]_{cyt}$ increase

While there remains a question mark between eATP and cyclic nucleotides, critically there is a precedent of a DAMP-cytosolic cGMP-CNGC2 pathway. Wounding of *Arabidopsis* increases $[Ca^{2+}]_{cyt}$ resulting in activation of metacaspase 4. The latter then catalyses production of Plant elicitor petide1 (Pep1) from its inactive precursor PROPEP1 (Hander *et al*., 2019). Pep1 can be perceived as a DAMP in a neighbouring cell by its cognate plasma membrane receptors PEPR1 and PEPR2. Both of these LRR RLKs are predicted to have cytosolic GC activity, with PEPR1 confirmed experimentally. The cytosolic cGMP activates CNGC2 to effect a $[Ca^{2+}]_{cyt}$ increase and downstream defence transcriptional response (Qi *et al*., 2010). eATP is now reported to act upstream of CNGC2. Wu *et al*. (2021) reported that eATP (0.1 mM) failed to elicit a plasma membrane Ca^{2+}-permeable influx conductance in *Arabidopsis* pollen grain protoplasts of a *cngc2* mutant. This concentration of eATP stimulated pollen germination in the wild type but not *cngc2* (Wu *et al*., 2021) but it remains unknown if activation of CNGC2 by eATP would increase $[Ca^{2+}]_{cyt}$. Here, the *cngc2* loss of function mutant *dnd1* (defence not death1; which constitutively expresses cytosolic (apo)aequorin as a luminescent $[Ca^{2+}]_{cyt}$ reporter, Qi *et al*., 2010) was tested for impaired eATP-induced $[Ca^{2+}]_{cyt}$ increase. The *dnd1* mutant of *CNGC2* has a single point mutation causing a stop codon in the third exon that would result in a truncated protein lacking the pore region for ion conduction. *dnd1* has a dwarf rosette phenotype that was also observed here (Supplemental Figure 1). Addition of control solution alone to single, excised cotyledons of Col-0 and *dnd1* only induced the transient "touch response" caused by mechanical disturbance, which did not differ between genotypes (Figure 2A, B). In contrast, addition of 0.1 mM eATP caused a monophasic increase in $[Ca^{2+}]_{cyt}$ after the touch response (Figure 2C). A similar pattern was observed in a previous study by Mohammad-Sidik *et al*. (2021). The response of *dnd1* to 0.1 mM eATP was significantly lower than the Col-0 wild type, both for the maximum $[Ca^{2+}]_{cyt}$ increase and estimated total $[Ca^{2+}]_{cyt}$ mobilized (estimated as Area Under the Curve; AUC, Figure 2D). Increasing eATP to 1 mM

(Figure 2E,F) also revealed a significant impairment in the response of *dnd1* (Figure 2G) although clearly at both eATP concentrations loss of CNGC2 function allows the majority of the $[Ca^{2+}]_{cyt}$ increase to occur. Overall these results show that CNGC2 could be involved in an eATP-induced $[Ca^{2+}]_{cyt}$ increase in leaf tissue.

Discussion

At present, it can only be inferred from existing studies that eATP could increase cAMP or cGMP synthesis to act as second messengers in $[Ca^{2+}]_{cyt}$-based DAMP signalling. However, it is now timely to assess experimentally whether eATP does elicit an increase in cyclic nucleotides and whether this is driven by known eATP receptors. Verification of the putative AC/GC sequences in DORN1/P2K1 and P2K2 would mean that phosphorylation of cytosolic targets is not the only way these receptors can relay the eATP signal. Increasing $[Ca^{2+}]_{cyt}$ inhibits the kinase activity of the *Arabidopsis* plasma membrane phytosulphokine receptor 1 (PSKR1) and promotes its GC activity (Muleva *et al*., 2014). It is therefore feasible that the receptors' initial function is to relay by phosphorylation (which could activate plasma membrane Ca^{2+} channels to increase $[Ca^{2+}]_{cyt}$) but increasing $[Ca^{2+}]_{cyt}$ switches the receptors' mode to cyclic nucleotide production. A further layer of regulation could be effected by levels of H_2O_2, given the findings of Kuzakova *et al*. (2020) of dose-dependent stimulation or inhibition of pea (*Pisum sativum*) AC activity. The finding here of putative extracellular AC sequences in DORN1/P2K1 begs several questions. How does the affinity of eATP binding compare to that of the legume lectin domain (K_d 46 nM; Choi *et al*., 2014b)? Under what circumstances would extracellular cAMP be synthesized and to what purpose? Here it should be noted that plant plasma membrane moonlighting ACs and GCs have low productivity and are held to be within distinct membrane domains containing their target in order to be efficient (Wong *et al*., 2018; Turek and Irving, 2021). Could the plant plasma membrane harbour a receptor for extracellular cAMP in close proximity to those for eATP? Two decades ago, there were no signs of a receptor for eATP in the *Arabidopsis* genome but if a measurable output for sensing extracellular cyclic nucleotides could be established then there is a way forward through forward genetic screens. Similar questions and future directions can also be posed for the extracellular putative cGMP synthesis sites of P2K2.

The finding here that a small but significant component of the eATP-induced $[Ca^{2+}]_{cyt}$ elevation requires CNGC2 (at eATP levels consistent with wounding) helps fill a gap in our knowledge on how $[Ca^{2+}]_{cyt}$ elevation is achieved and helps indirectly make the case for a role for cAMP or cGMP in DAMP signalling by eATP (summarised in Supplemental Figure 2). However, it remains feasible that CNGC2 could be phosphorylated by DORN1/P2K1 or P2K2 (if downstream of these) to promote Ca^{2+} influx. CNGC2 is already known to act downstream of PEPR1 and PEPR2 in DAMP signalling (Qi *et al*., 2010) and downstream of flg22 in PAMP signalling (Tian *et al*., 2019). CNGC2 therefore could be a critical component in co-ordinating the response to pathogens, which requires both damage and PAMPs to elicit the strongest immunogenic transcriptional outcome (Zhou *et al*., 2020). CNGC2 has recently been reported to be involved auxin signalling (Chakraborty *et al.*, 2021) so it could possibly play a part in the restorative growth response of cells surrounding damage sites as these sustain an increase in auxin as a longer-term response (Hoermayer *et al*., 2020). Much will depend on which cells harbour CNGC2, what its possible sub-unit partners in heterotetrameric channels are to help generate specific $[Ca^{2+}]_{cyt}$ signals and their position in the membrane relative to receptors. There are another nineteen CNGCs in *Arabidopsis* that now warrant consideration as components of eATP DAMP signalling, although CNGC14 appears not to be involved in the root's $[Ca^{2+}]_{cyt}$ elevation by eATP (Shih *et al*., 2015).

Author Contributions

JS and KAW designed and performed the aequorin experiments; YN and LW analysed the data. LW designed, performed and analysed the biomass determinations. JMD analysed sequences. JMD conceived the project and wrote the manuscript with contributions from the other authors.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure legends

Figure 1. Putative AC and GC sequences in the eATP receptors DORN1/P2K1 and P2K2. A. Top: The ACPred AC prediction tool sequence (Xu *et al*., 2018a). "X" denotes any amino acid, "{}" denotes the number of undetermined amino acids. Centre: The AC catalytic centre sequence used by Al-Younis *et al*. (2021). Bottom: The GCPred GC prediction tool sequence (Xu *et al*., 2018b). B. The two extracellular, overlapping putative AC sequences from DORN1/P2K1 shown separately for clarity. C. Putative AC and GC sequences of DORN1/P2K1. Overlapping AC sequences are highlighted in light grey. The extracellular lectin domain is underlined with a wave, the intracellular serine/threonine kinase domain is denoted with a straight underline. The transmembrane domain is annotated in ochre. Amino acids found to be important for kinase activity are bold in yellow on a grey background (Choi *et al*., 2014a; Nguyen *et al*., 2016; Cho *et al*., 2017). D. Putative AC and GC sequences of P2K2. The AC sequence is highlighted in light gray. Domains are identified as for (B). Amino acids found to be important for kinase activity are bold in yellow on a grey background (Pham *et al*. 2020).

Figure 2. The *dnd1* mutant has an impaired $[Ca^{2+}]_{cyt}$ response to eATP. Cotyledons were excised from 14 day old Col-0 and *dnd1*; cytosolic aequorin was reconstituted with coelenterazine (in 10 mM $CaCl_2$, 0.1 mM KCl, 2 mM Tris/MES, pH 5.8) as described by Mohammad-Sidik *et al*. (2021). Luminescence was recorded from individual samples bathed in 10 mM $CaCl_2$, 0.1 mM KCl, 2 mM Tris/MES, pH 5.8 as described by Mohammad-Sidik *et al*. (2021). As with flg22, ATP causes pronounced acidification of solutions and so in this study, pH of eATP test solutions was maintained by buffers to avoid acidic pH artefactual responses which can impair interpretation (Westphal *et al*., 2019). $[Ca^{2+}]_{cyt}$ values were calculated as described by Matthus *et al*., (2019b). A. Mean ± SEM time course of $[Ca^{2+}]_{cyt}$ response of Col-0 and *dnd1* to control solution (10 mM $CaCl_2$, 0.1 mM KCl, 2 mM Tris/MES, pH 5.8) added at 35 s (inverted triangle). B. Touch-induced $[Ca^{2+}]_{cyt}$ increase from pre-stimulus baseline and estimated total $[Ca^{2+}]_{cyt}$ mobilized (AUC; Area Under the Curve, after baseline subtraction). C. Mean ± SEM time course of $[Ca^{2+}]_{cyt}$ response of Col-0 and *dnd1* to 0.1 mM eATP added at 35 s (inverted triangle). D. Touch-induced $[Ca^{2+}]_{cyt}$ increase from pre-stimulus baseline, peak eATP-induced $[Ca^{2+}]_{cyt}$ increase from baseline and estimated AUC). E. As (C) but with 1 mM eATP. F. Mean ± SEM time course of the eATP response shown in (E). G. As (D) but for 1 mM eATP. Results are from 3 independent trials with 9 samples in total per genotype in control

trials and 19-21 samples in total per genotype in eATP trials. Each dot in box plots represents an individual recording. The middle line and the triangle in the box plot are the median and mean, respectively. Student's *t*-test was used for analysing statistical difference (***, $p < 0.001$; *, $p < 0.05$; n.s, not significant).

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641 Figure 1

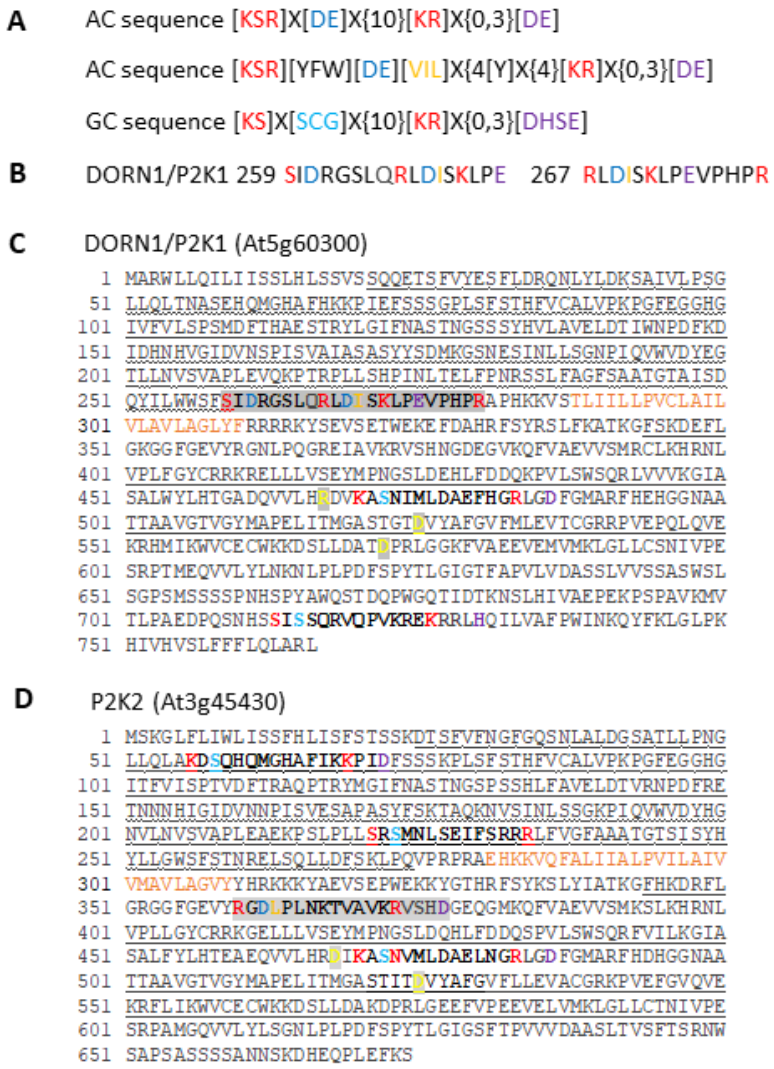

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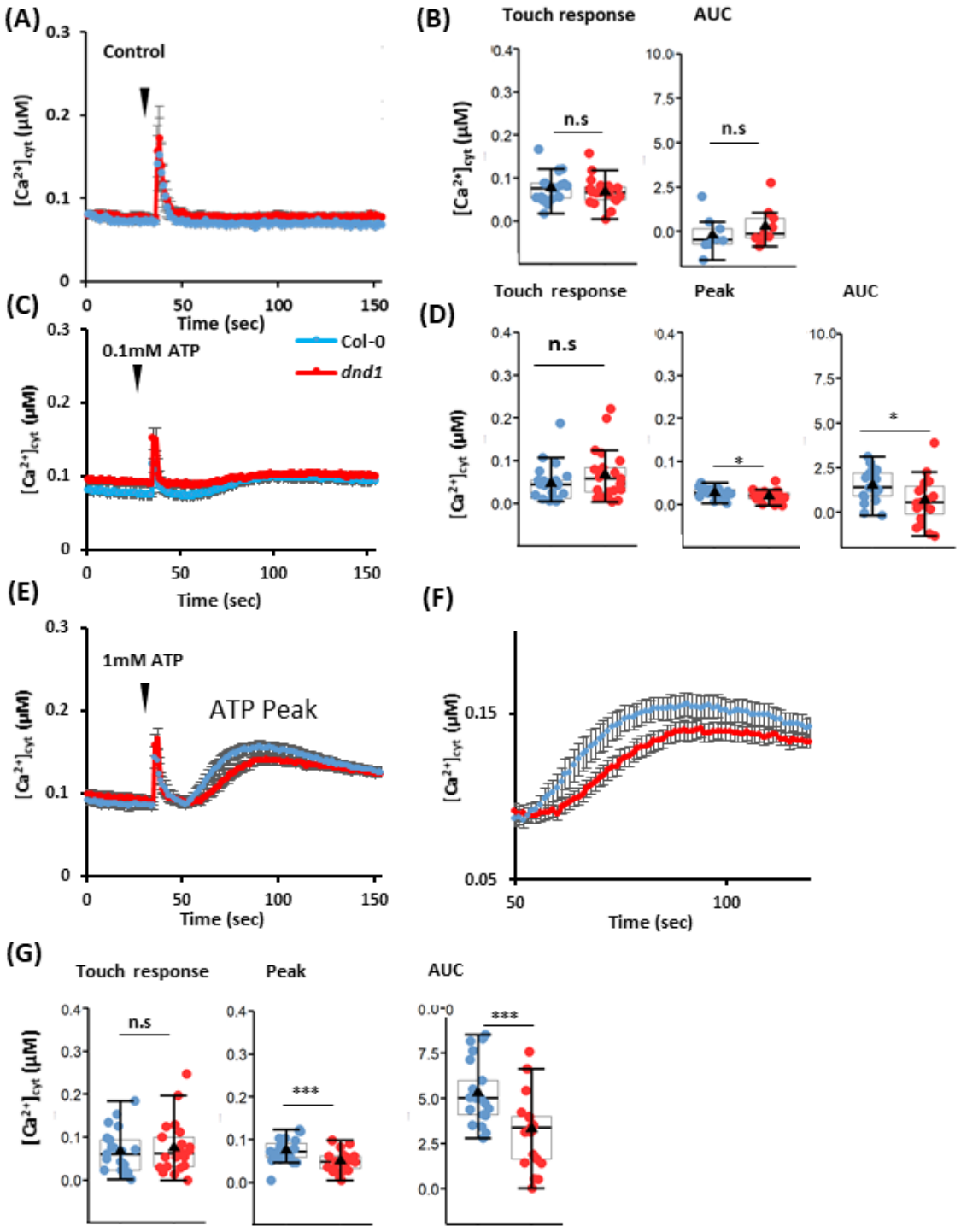
Figure 2


(A)
Control
$[Ca^{2+}]_{cyt}$ (µM)
Time (sec)
(B)
Touch response
AUC
n.s
n.s
(C)
0.1mM ATP
Col-0
dnd1
Time (sec)
(D)
Touch response
Peak
AUC
n.s
*
*
(E)
1mM ATP
ATP Peak
Time (sec)
(F)
Time (sec)
(G)
Touch response
Peak
AUC
n.s
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