Rapid report

Arabidopsis thaliana CYCLIC NUCLEOTIDE-GATED CHANNEL2 mediates extracellular ATP signal transduction in root epidermis

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Summary

- Damage can be signalled by extracellular ATP (eATP) using plasma membrane (PM) receptors to effect cytosolic free calcium ion ([Ca2+]cyt) increase as a second messenger. The downstream PM Ca2+ channels remain enigmatic. Here, the Arabidopsis thaliana Ca2+ channel subunit CYCLIC NUCLEOTIDE-GATED CHANNEL2 (CNGC2) was identified as a critical component linking eATP receptors to downstream [Ca2+]cyt signalling in roots.
- Extracellular ATP-induced changes in single epidermal cell PM voltage and conductance were measured electrophysiologically, changes in root [Ca2+]cyt were measured with aequorin, and root transcriptional changes were determined by quantitative real-time PCR. Two cngc2 loss-of-function mutants were used: cngc2-3 and defence not death1 (which expresses cytosolic aequorin).
- Extracellular ATP-induced transient depolarization of Arabidopsis root elongation zone epidermal PM voltage was Ca2+ dependent, requiring CNGC2 but not CNGC4 (its channel co-subunit in immunity signalling). Activation of PM Ca2+ influx currents also required CNGC2. The eATP-induced [Ca2+]cyt increase and transcriptional response in cngc2 roots were significantly impaired.
- CYCLIC NUCLEOTIDE-GATED CHANNEL2 is required for eATP-induced epidermal Ca2+ influx, causing depolarization leading to [Ca2+]cyt increase and damage-related transcriptional response.

Introduction

Extracellular ATP (eATP) has been shown to contribute to plant growth and development, stress responses, immunity, and damage (Matthus et al., 2019a). Two plasma membrane (PM) coreceptors for eATP, DOES NOT RESPOND TO NUCLEOTIDES1 (P2K1/DORN1) and P2K2, have been identified recently in Arabidopsis thaliana, with P2K1/DORN1 transphosphorylating P2K2 (Choi et al., 2014; Pham et al., 2020). P2K1/DORN1 commands an eATP-dependent transient increase of cytosolic free calcium ions ([Ca2+]cyt) as a second messenger (Choi et al., 2014). The root [Ca2+]cyt response to eATP (the ‘signature’) has a greater reliance on Ca2+ influx across the PM than the release of Ca2+ from intracellular stores (Demidchik et al., 2009; Rincón-Zachary et al., 2010). Lowering external Ca2+ from 10 to 0.1 mM causes an 85% decrease in the [Ca2+]cyt response (Demidchik et al., 2003). Ca2+...
influx across the PM helps explain the depolarizing effect that eATP has on root PM voltage (Lew & Deearlsey, 2000; Dindas et al., 2018), especially given that eATP causes instantaneous \([Ca^{2+}]_{\text{cyt}}\) increase and a cytosolic acidification consistent with PM H^+-ATPase inhibition in Arabidopsis roots (Waadt et al., 2020). Indeed, patch clamp electrophysiology has revealed eATP and P2K1/DORN1-dependent \(Ca^{2+}\)-permeable channel conductances in Arabidopsis root epidermal PM (Demidchik et al., 2009; Wang et al., 2018, 2019) that could contribute to PM depolarization and \([Ca^{2+}]_{\text{cyt}}\) increase. However, the identity of the channels remains unknown. Here, data support the involvement of a CYCLIC NUCLEOTIDE-GATED CHANNEL (CNGC).

Arabidopsis has a family of 20 CNGC subunits, with members contributing to \([Ca^{2+}]_{\text{cyt}}\) signatures evoked by abiotic stress, pathogen attack, and hormones (Jarratt-Barnham et al., 2021). Because eATP accumulates during pathogen infection and acts as a damage-associated molecular pattern (DAMP) that drives a transcriptional response through P2K1/DORN1 (Choi et al., 2014; Jewell et al., 2019; Kumar et al., 2020). CNGCs involved in pathogen sensing could also be acting in the eATP pathway. CYCLIC NUCLEOTIDE-GATED CHANNEL2 is a key candidate for testing, as it operates in root signalling (Chakraborty et al., 2021), it is involved in both DAMP and pathogen-associated molecular pattern (PAMP) signalling, and it generates a PM hyperpolarization-activated \(Ca^{2+}\)-permeable channel conductance (Qi et al., 2010; Tian et al., 2019). Cyclic Nucleotide-Gated Channel2’s closest parologue, CNGC4, can interact with CNGC2, and these two subunits are hypothesized to form a heteromeric channel in PAMP signalling (Chin et al., 2013; Tian et al., 2019). Cyclic Nucleotide-Gated Channel2 and CNGC4 could potentially work together in the eATP pathway.

Here, two Arabidopsis cngc2 loss of function mutants were used: cngc2-3 and defence not death1 (dnd1; which expresses cytosolic aequorin). Extracellular ATP-induced depolarization of PM voltage has been used as a diagnostic of PM \(Ca^{2+}\) channel activity in single epidermal and cortical root cells. Results show an absolute requirement for CNGC2 but not CNGC4 in the epidermis. Patch clamp electrophysiological analysis of eATP-induced PM \(Ca^{2+}\) influx conductance of epidermal cells confirmed an absolute requirement for CNGC2. Both root eATP-induced \([Ca^{2+}]_{\text{cyt}}\) signature and transcriptional response were impaired by loss of CNGC2 function.

### Materials and Methods

#### Plant material

Arabidopsis lines were in the Columbia (Col-0) ecotype. dorn1-1, dorn1-3, p2k2, and p2k1p2k2 mutants were as described previously (Choi et al., 2014; Pham et al., 2020). cngc2-3 (transfer DNA (T-DNA) insertion line Salk-066908) was described previously by Chin et al., 2013. Complemented cngc2-3 was generated with the CNGC2 coding sequence under the control of its endogenous promoter (Supporting Information Methods S1). dnd1 cngc2 loss-of-function mutant constitutively expressing cytosolic (apo)aequorin was described by Qi et al., 2010. cngc4-5 (SALK_081369; Tian et al., 2019) was obtained from the Nottingham Arabidopsis Stock Centre. Genotyping of insertional and complemented mutants is described in Methods S1. Primers are listed in Table S1. Growth conditions are described in Methods S2. Plants at 7–14 d old were used unless stated otherwise.

#### Membrane potential measurements

Plasma membrane potential \(E_{m}\) of root elongation zone cells was measured using a glass microelectrode. A plant was fixed in a plexiglass chamber and immersed in assay solution (10 ml) containing 2 mM calcium chloride (CaCl2; with or without 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) (EGTA) or with or without 0.5 mM lanthanum chloride (LaCl3), 0.1 mM potassium chloride (KCl), 1 mM MES–Tris (pH 6.0) for at least 30 min before impalement. Microelectrode construction, recording circuitry, and impalement are described in Methods S3. After observing a stable \(E_{m}\) (> 6 min), eATP (ATP magnesium salt (MgATP) or ATP disodium salt (Na2ATP); Sigma) was added to the chamber (final concentration 300 µM in the assay medium, pH 6.0). In controls, magnesium sulphate (MgSO4) or sodium sulphate (Na2SO4) was added.

#### Patch clamp recordings

Protoplasts were isolated from root elongation zone epidermis, with origin confirmed using the N9093 epidermal-specific green fluorescent protein reporter line as described by Wang et al. (2019). Details of isolation, patch clamp solutions, and protocols are in Methods S4.

#### Cytosolic free calcium ion measurement

Excised primary roots of Col-0 and dnd1 expressing cytosolic (apo)aequorin were used for luminescence-based quantification of \([Ca^{2+}]_{\text{cyt}}\). Roots were placed individually into a 96-well plate (one root per well) and incubated overnight at room temperature in darkness with 10 µM coelenterazine in 100 µl of buffer: 2 mM CaCl2, 0.1 mM KCl, 1 mM MES–Tris (pH 5.6). CaCl2 was included to maintain a similar level to that of the growth medium. Samples were washed with coelenterazine-free buffer and left to recover for at least 20 min in darkness. A FLUOstar Optima plate reader (BMG Labtech, Ortenberg, Germany) was used to record luminescence as described in Matthys et al. (2019b). \([Ca^{2+}]_{\text{cyt}}\) was calculated as described by Knight et al., 1997.

#### Analysis of gene expression

Total RNA was extracted from roots (frozen in liquid nitrogen) using the RNAeasy Plant Mini Kit (Qiagen) and subjected to DNase I treatment (RNase-free DNase kit; Qiagen). Complementary DNA (cDNA) was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time (qRT)-PCR was performed in aRotor-Gene 3000 thermocycler with the Rotor-Gene™ SYBR® Green PCR Kit (Qiagen). UBQ10 and
TUB4 acted as internal controls. Primers are listed in Table S1. Further details are in Methods S5.

Statistical analysis

Data normality was first analysed with the Shapiro–Wilk test in R. Student’s t-test or Tukey’s honestly significant difference was used for parametric data comparison, whereas the Mann–Whitney U test was used to compare the nonparametric data.

Results

AtCNGC2 mediates the extracellular-ATP-induced depolarization of root epidermal plasma membrane voltage and does not require AtCNGC4

The stable resting membrane voltage \( E_m \) of a single Col-0 root elongation zone epidermal cell (Fig. 1a) was significantly but transiently depolarized by 300 \( \mu M \) eATP (Fig. 1b). This concentration of eATP was found previously to activate a PM \( Ca^{2+} \) influx conductance in this cell type (Wang et al., 2019). Mean maximal depolarization from \(-118.9 \pm 4.8 \) to \(-69.2 \pm 7.6 \) mV (Fig. 1c,d; Table S2) occurred 1.8 \pm 0.3 min after eATP application (MgATP or Na2ATP), and \( E_m \) recovered fully after 14.7 \pm 2.2 min (Fig. 1e,f) in the continued presence of eATP. In controls, neither 300 \( \mu M \) MgSO4 nor 300 \( \mu M \) Na2SO4 (Figs 1g,h, S1a,b) affected \( E_m \), confirming that the response was due to eATP. Incubation with 5 mM EGTA (to chelate extracellular \( Ca^{2+} \)) abolished the response to 300 \( \mu M \) eATP (Figs 1g,h, S1c), showing that depolarization required \( Ca^{2+} \) influx. However, as EGTA treatment resulted in a less negative \( E_m \) that could have compromised depolarization, a further test of \( Ca^{2+} \) influx was conducted. Addition of 0.1 mM LaCl3 as a blocker of PM \( Ca^{2+} \)-permeable channels prevented significant depolarization by eATP (Figs 1g,h, S1d). The loss-of-function cngc2-3 mutant (T-DNA insert in second exon) and the complemented cngc2-3,CNGC2::CNGC2 mutant (Fig. S2a–c) were then analysed. Expression levels of \( P2K1/DORN1 \) and the coreceptor \( P2K2 \) were normal in cngc2-3 roots, indicating that eATP perception itself would be unimpaired (Fig. S2d). There were no significant differences in resting \( E_m \) between genotypes (Table S2). In contrast to Col-0, 300 \( \mu M \) eATP failed to depolarize cngc2-3 \( E_m \) (Fig. 1b–d; Table S2). Complementation fully restored the mutant’s \( E_m \) response to eATP (depolarization and recovery time) (Fig. 1b–e), but maximum \( E_m \) depolarization occurred sooner than in Col-0 (Fig. 1f). This may reflect the approximately doubled abundance of CNGC2 transcript in the complemented mutant, although this was not statistically significant (Fig. S2e). To verify the cngc2-3 results, the CNGC2 dnd1 mutant (Fig. S3a–c) was also tested. This has a single point mutation causing a stop codon in the third exon and expresses cytosolic aequorin (Qi et al., 2010). Resting dnd1 \( E_m \) was not significantly different to those of other genotypes and was unaffected by eATP treatment (Fig. S3d–f; Table S2). These results show that the eATP-induced and \( Ca^{2+} \)-dependent PM \( E_m \) response is reliant on CNGC2.

Elongation zone epidermal cells of the dorn1-3 loss-of-function mutant, the dorn1-1 kinase mutant, and the p2k2 mutant all retained a small but significant depolarization of \( E_m \) when challenged with 300 \( \mu M \) eATP (Fig. S4a–d; Table S2). CNGC2 transcript levels were normal in both dorn1-3 and p2k2 mutant roots, so their lowered response is most likely due to loss of receptor function rather than channel function (Fig. S4e). The dorn1-3,p2k2 double mutant (p2k1,p2k2) also sustained a small but significant depolarization of \( E_m \) when challenged with 300 \( \mu M \) eATP, but this was not significantly different to that caused by the Na2SO4 control (Fig. S5a–c; Table S2; \( P = 0.74 \)). Under control conditions, the p2k1,p2k2 mutant had a significantly more negative \( E_m \) \((-143.9 \pm 4.3 \) mV; \( n = 10 \)) than its paired Col-0 wild-type \((-129.9 \pm 4.6 \) mV; \( n = 5 \); \( P = 0.005 \)), and this may help explain why sodium ions (\( Na^+ \)) caused a depolarization in this mutant but not in Col-0. Overall, the results suggest that the two receptors working together are sufficient to initiate the eATP-activated depolarization of \( E_m \) and that CNGC2 is an absolute requirement in this cell type.

Cyclic Nucleotide-Gated Channel2 has been shown to interact with CNGC4 in immune signalling (Chin et al., 2013; Tian et al., 2019). Here, the root elongation zone epidermis of the cngc4-5 loss-of-function mutant (Fig. S6a–d) was impaled and tested with 300 \( \mu M \) eATP. The eATP caused a significant depolarization of \( E_m \) to \(-69.4 \pm 10.9 \) mV, similar to Col-0 wild-type (\( P = 0.05 \); Fig. S6e–g; Table S2). These results show that CNGC2 controls the PM \( E_m \) response to eATP without the need for CNGC4.

Plasma membrane calcium-ion currents induced by extracellular ATP in Col-0 root epidermal protoplasts require CNGC2

Whole-cell currents across the PM of root elongation zone epidermal protoplasts Wang et al. (2019) of Col-0 and cngc2-3 were recorded. No significant differences in control currents or reversal potential were found between genotypes (mean \pm SE reversal potential: Col-0 \(-59 \pm 16.3 \) mV, \( n = 4 \); cngc2-3 \(-35 \pm 8.9 \) mV, \( n = 4 \)). For Col-0, 300 \( \mu M \) eATP activated whole-cell inward current upon membrane hyperpolarization, but not outward current upon membrane depolarization (Fig. 2a). No effect of \( Na^+ \) as the salt control was found in previous trials (Wang et al., 2018, 2019). Analysis of the reversal potential of eATP-activated currents (average control (no ATP) currents were subtracted from average eATP-activated currents (Wang et al., 2013)) revealed an approximate value of \(+22 \) mV (\( n = 4 \)), far from the equilibrium potentials of potassium ions (\( K^+ \); \(-79 \) mV) and chloride ions (\(-28 \) mV) and indicating \( Ca^{2+} \) permeability. Extracellular ATP-activated inward current was significantly inhibited by 100 \( \mu M \) gadolinium ions (Gd3\(+\)), a plant \( Ca^{2+} \) channel blocker that is effective against CNGC2 (Demidchik et al., 2009; Wang et al., 2018, 2019; Tian et al., 2019; Fig. 2a). These results suggest that \( Ca^{2+} \) influx across the PM contributed to the eATP-activated current in Col-0. As Gd3\(+\) is an effective blocker of a variety of PM \( Ca^{2+} \)-permeable channels (Demidchik et al., 2002, 2009; Wang et al., 2018, 2019) it is likely that it also blocked \( Ca^{2+} \)-permeable channels that were not activated by eATP, causing the significant reduction in inward current in the presence of both eATP and Gd3\(+\) to below the control value. The eATP-activated \( Ca^{2+} \) inward current was absent from dorn1-3 PM (Fig. S7). At resting \( E_m \) of...
Col-0 epidermal cells ($-120$ mV) the eATP-activated current would deliver Ca$^{2+}$ to the cytosol, which would both elevate [Ca$^{2+}$]$_{cyt}$ and initiate depolarization. It can be inferred that some eATP-activated Ca$^{2+}$ influx should have occurred in membrane voltage trials at the less negative $E_m$ caused by EGTA ($-85.2 \pm -5.4$ mV; Figs 1(g,h), S1c) but this was not observed, further supporting the role of Ca$^{2+}$ influx in eATP-induced depolarization of $E_m$. In contrast to Col-0, PM whole-cell currents of cngc2–3
Extracellular ATP (eATP) activates inward currents in Arabidopsis Col-0 but not cngc2-3 root elongation zone epidermal protoplasts. (a) Left panel: typical whole-cell plasma membrane currents in Col-0 protoplasts before (−); black) and after (+; light blue) application of 300 µM eATP. Extracellular ATP effects were observed 30 s to 2 min after addition. Bath solution contained 50 mM calcium chloride, 1 mM potassium chloride (KCl), and 10 mM MES-Tris (pH 5.6). Pipette solution comprised 5 mM barium chloride, 20 mM KCl, and 10 mM HEPES-Tris (pH 7.5). Centre panel: mean ± SE current-voltage (I–V) relationships of Col-0 before (−), after (+) ATP and in 100 µM gadolinium ions (Gd³⁺; dark blue; the calcium channel blocker was applied after eATP treatment) (n = 4). Right panel: comparison of the inward currents at −190 mV (solid bars) and the outward currents at +50 mV (hollow bars) before and after eATP addition and in the presence of Gd³⁺. Gd³⁺ block of control inward currents is also evident. (b) As (a), but for cngc2-3 protoplasts. The mutant did not respond to eATP even with an extended observation period (10 min). Data are means ± SE (n = 4; * P < 0.05; ns, not significant).

Extracellular-ATP-induced cytosolic free calcium ion increase in roots is impaired in dnd1
The requirement for CNGC2 in eATP-activated epidermal PM depolarization and Ca²⁺ influx conductance should manifest in impaired eATP-induced [Ca²⁺]cyt elevation in the dnd1 mutant, which expresses cytosolic (apo)aequorin as a bioluminescent [Ca²⁺]cyt reporter. The typical monophasic [Ca²⁺]cyt increase (‘touch response’) after sodium chloride (NaCl) addition (control for mechanostimulation and cation effect of Na₂ATP) was observed in individual roots of Col-0 and dnd1. The amplitude of the touch peak and total [Ca²⁺]cyt mobilized did not differ significantly between genotypes (Fig. 3a). By contrast, 300 µM eATP caused a biphasic [Ca²⁺]cyt increase (after the touch response) in both Col-0 and dnd1 roots (Fig. 3b), confirming that this part of the [Ca²⁺]cyt signature was caused by eATP. This biphasic signature (‘peak 1’ and ‘peak 2’) was observed in previous studies on Arabidopsis roots and seedlings using aequorin (Demidchik et al., 2003; Tanaka et al., 2010; Mathus et al., 2019a,b; Mohammad-Sidik et al., 2021) and also root tips using YC3.6 (Tanaka et al., 2010). dnd1 roots were significantly impaired in the amplitude of both the eATP-induced [Ca²⁺]cyt peaks and also total [Ca²⁺]cyt mobilized (Fig. 3d). Significant impairment was also observed at 100 µM and 1 mM eATP (Fig. S8). Since P2K1/DORN1 governs the eATP-induced [Ca²⁺]cyt signature in Arabidopsis roots (Mathus et al., 2019a), impairment of the [Ca²⁺]cyt response in dnd1 helps place CNGC2 downstream of that eATP receptor, consistent with the electrophysiological data presented here.

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Root cortical plasma membrane depolarization does not require CNGC2 but may require CNGC4

The residual eATP-induced [Ca^{2+}]_{cyt} increase seen in dnd1 roots suggests CNGC2-independent Ca^{2+} influx pathways in other cells, such as the cortex. Cortical cells also increase [Ca^{2+}]_{cyt} in response to eATP (Krogman et al., 2020). Cyclic Nucleotide-Gated Channel2 redundancy was investigated by measuring elongation zone cortical cell $E_m$. Resting Col-0 cortical cell $E_m$ was $-131.6 \pm 9.1$ mV (Fig. S9a; Table S2), which was not significantly different to the epidermis. Application of eATP (300 µM) to the root transiently and significantly depolarized the cortical PM (Fig. S9a; Table S2). There was no significant difference between cortex and epidermis in the terms of the maximum depolarization amplitude, the time to reach the maximum depolarization, or recovery time. The $E_m$ of elongation zone cortical cells in the two CNGC2 mutants was then investigated. Unlike the null response of epidermal cells of cngc2-3 and dnd1, addition of eATP to the root triggered cortical $E_m$ depolarization in both mutants (Fig. S9b,c; Table S2). No significant difference in the PM $E_m$ before (no ATP added) or after ATP (ATP added) was observed between Col-0 and these two mutants (Fig. S9d,e, Table S2), but this was significantly smaller than that found previously in its epidermal cells (cortex, $21.6 \pm 6.8$ mV; epidermis, $62.2 \pm 8.8$ mV; $P=0.012$). This indicates a CNGC4-dependent pathway in the cortex. The residual
Depolarization in the cngc4-5 implies involvement of other CNGCs (but not CNGC2) or other transport systems (Fig. S9f). Together, the results help explain the residual eATP-induced \([Ca^{2+}]_{\text{cyt}}\) increase in dnd1 roots; CNGC2 does not operate in all other cells.

CNGC2 is implicated in extracellular-ATP-responsive gene expression

The eATP-responsive transcriptome is highly enriched in defence-related and wound-response genes, including MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3), WRKY DNA-BINDING PROTEIN 40 (WRKY40), CALCIUM-DEPENDENT PROTEIN KINASE 28 (CPK28), and the cysteine protease METACASPASE 7 (MC7) (Choi et al., 2014; Jewell et al., 2019). Transcriptional upregulation of those genes by eATP is P2K1/DORN1 dependent (Choi et al., 2014; Jewell et al., 2019), and their response to eATP was examined here in Col-0, cngc2-3, and cngc2-3::CNGC2 roots by qRT-PCR. Extracellular ATP (300 \(\mu\)M for 30 min) significantly upregulated expression of all four genes in Col-0, with no significant difference between Col-0 and cngc2-3::CNGC2 (Fig. 4). However, transcript levels of MPK3, WRKY40, CPK28, and MC7 were all significantly lower in cngc2-3 compared with Col-0 or (with the exceptions of CPK28 and MC7) compared with cngc2-3::CNGC2.

**Fig. 4** CYCLIC NUCLEOTIDE-GATED CHANNEL2 (CNGC2) is implicated in the extracellular ATP (eATP)-induced transcriptional response in Arabidopsis roots. Col-0, cngc2-3, and cngc2-3::CNGC2 whole roots were treated with control (sodium chloride) buffer (Mock) or 300 \(\mu\)M eATP for 5 min (ATP-5) or 30 min (ATP-30). Two housekeeping genes, AtUBQ10 and AtTUB4, were used for data normalization. Data are mean ± SE from three independent trials with \(n≥4\) biological replicates. (a) Results for MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3). (b) Results for WRKY DNA-BINDING PROTEIN 40 (WRKY40). (c) Results for CALCIUM-DEPENDENT PROTEIN KINASE 28 (CPK28). (d) Results for METACASPASE 7 (MC7). Significant differences between cngc2-3 and the other two genotypes were found at ATP-30, and \(P\) values are shown. No significant differences were observed between Col-0 and cngc2-3::CNGC2 at ATP-30. Asterisks indicate the statistical significance relative to the mock treatment (*, \(P<0.05\); **, \(P<0.01\); ***, \(P<0.001\)). (e) Summary of possible signalling events at epidermis and cortex. DOES NOT RESPOND TO NUCLEOTIDES1 (DORN1/P2K1) and P2K2 (P2) together promote CNGC2 channel opening to mediate calcium ion (\(Ca^{2+}\)) influx, plasma membrane potential \(E_m\) depolarization, and cytosolic free \(Ca^{2+}\) (\([Ca^{2+}]_{\text{cyt}}\)) increase. The mechanism is unknown, but it could include phosphorylation or direct production of cyclic nucleotide monophosphates by cryptic catalytic centres (Al-Younis et al., 2021). Extracellular ATP could follow the apoplastic pathway to initiate events in cortical cells, potentially through the P2 receptor complex and with CNGC4 as a component of \(Ca^{2+}\) influx, \(E_m\) depolarization, and \([Ca^{2+}]_{\text{cyt}}\) increase. Other stimuli could be transmitted from the epidermis to the cortex in a CNGC2-independent pathway.
Research that Arabidopsis pollen grain PM has an eATP-activated Ca\textsuperscript{2+} kinase (Tian CNGC4’s pivotal role in the PAMP signalling CNGC2/4 heterotetramer (Choi et al., 2014). Here, eATP’s ability to depolarize root PM $E_{m}$ (Lew & Dearnley, 2000) was used in a targeted gene approach. Depolarization can arise from Ca\textsuperscript{2+} influx across the PM (Dindas et al., 2018), and eATP causes a rapid [Ca\textsuperscript{2+}]\textsubscript{cyt} increase in roots that could initiate depolarization (Waadt et al., 2020) as a multiconductance process (Wang et al., 2019). Here, eATP-induced depolarization required extracellular Ca\textsuperscript{2+} (Figs 1g,h, S1c,d), showing its reliance on Ca\textsuperscript{2+} influx. Thus, the unresponsiveness of cngc2 mutant root elongation zone epidermal PM to eATP (Fig. 1) is consistent with its lack of eATP-induced PM Ca\textsuperscript{2+} influx currents (Fig. 2) and reveals CNGC2 as a necessary component for initiating depolarization downstream of P2K1/DORN1/P2K2 in young epidermal root cells (Fig. 4e).

Cyclic Nucleotide-Gated Channel2 works together with CNGC4 in PAMP signalling, acting as a heterotrimeric Ca\textsuperscript{2+} channel in the flagellin 22 pathway (Chin et al., 2013; Tian et al., 2019). During the course of this study, Wu et al. (2021) reported that Arabidopsis pollen grain PM has an eATP-activated Ca\textsuperscript{2+} influx conductance, measured using whole-cell patch clamp electrophysiology. This conductance was impaired in both a single mutant of CNGC2 and a single mutant of CNGC4, suggesting that these two channel subunits might work together to facilitate germination. Whether CNGC2 and CNGC4 underpin eATP-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation and transcription in pollen remains untested. Here, with eATP as a potential DAMP, CNGC2 could be acting either as a homotetramer or a heterotetramer (that includes CNGC4) in the root epidermis, but in either event it is the obligate component of the depolarization response given CNGC4’s redundancy (Fig. 5e–g; Table S2). If a heterotetramer included CNGC4 (which is expressed at almost half the level of CNGC2 in the epidermis; Dinneny et al., 2008), that CNGC4 subunit could be replaced. This is in contrast to CNGC4’s pivotal role in the PAMP signalling CNGC2/4 heterotetramer, where CNGC4 is the phosphorylation target of the BIK1 kinase (Tian et al., 2019).

A residual [Ca\textsuperscript{2+}]\textsubscript{cyt} signature and a transcriptional response were still observed in CNGC2 mutants, showing that other channels are involved in the root’s overall response to eATP that now need to be identified. The results here from the cortex implicate a role for CNGC4 (Figs 4e, S9f). Annexin1 is implicated at whole root level, involved in the root’s overall response to eATP that now need to be identified. The results here from the cortex implicate a role for CNGC4 (Figs 4e, S9f). Annexin1 is implicated at whole root level, showing its reliance on Ca\textsuperscript{2+} influx. Thus, the unresponsiveness of cngc2 mutant root elongation zone epidermal PM to eATP (Fig. 1) is consistent with its lack of eATP-induced PM Ca\textsuperscript{2+} influx currents (Fig. 2) and reveals CNGC2 as a necessary component for initiating depolarization downstream of P2K1/DORN1/P2K2 in young epidermal root cells (Fig. 4e).

**Discussion**

Effects of eATP on plants were reported almost half a century ago (Jaffe, 1973), yet relatively few components of eATP signalling pathways have been identified. A forward genetic screen based on eATP’s ability to increase [Ca\textsuperscript{2+}]\textsubscript{cyt} led to the identification of the first angiosperm eATP receptor, P2K1/DORN1 (Choi et al., 2014). Here, eATP’s ability to depolarize root PM $E_{m}$ (Lew & Dearnley, 2000) was used in a targeted gene approach. Depolarization can arise from Ca\textsuperscript{2+} influx across the PM (Dindas et al., 2018), and eATP causes a rapid [Ca\textsuperscript{2+}]\textsubscript{cyt} increase in roots that could initiate depolarization (Waadt et al., 2020) as a multiconductance process (Wang et al., 2019). Here, eATP-induced depolarization required extracellular Ca\textsuperscript{2+} (Figs 1g,h, S1c,d), showing its reliance on Ca\textsuperscript{2+} influx. Thus, the unresponsiveness of cngc2 mutant root elongation zone epidermal PM to eATP (Fig. 1) is consistent with its lack of eATP-induced PM Ca\textsuperscript{2+} influx currents (Fig. 2) and reveals CNGC2 as a necessary component for initiating depolarization downstream of P2K1/DORN1/P2K2 in young epidermal root cells (Fig. 4e).

Cyclic Nucleotide-Gated Channel2 works together with CNGC4 in PAMP signalling, acting as a heterotrimeric Ca\textsuperscript{2+} channel in the flagellin 22 pathway (Chin et al., 2013; Tian et al., 2019). During the course of this study, Wu et al. (2021) reported that Arabidopsis pollen grain PM has an eATP-activated Ca\textsuperscript{2+} influx conductance, measured using whole-cell patch clamp electrophysiology. This conductance was impaired in both a single mutant of CNGC2 and a single mutant of CNGC4, suggesting that these two channel subunits might work together to facilitate germination. Whether CNGC2 and CNGC4 underpin eATP-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation and transcription in pollen remains untested. Here, with eATP as a potential DAMP, CNGC2 could be acting either as a homotetramer or a heterotetramer (that includes CNGC4) in the root epidermis, but in either event it is the obligate component of the depolarization response given CNGC4’s redundancy (Fig. 5e–g; Table S2). If a heterotetramer included CNGC4 (which is expressed at almost half the level of CNGC2 in the epidermis; Dinneny et al., 2008), that CNGC4 subunit could be replaced. This is in contrast to CNGC4’s pivotal role in the PAMP signalling CNGC2/4 heterotetramer, where CNGC4 is the phosphorylation target of the BIK1 kinase (Tian et al., 2019).

A residual [Ca\textsuperscript{2+}]\textsubscript{cyt} signature and a transcriptional response were still observed in CNGC2 mutants, showing that other channels are involved in the root’s overall response to eATP that now need to be identified. The results here from the cortex implicate a role for CNGC4 (Figs 4e, S9f). Annexin1 is implicated at whole root level, but its mode of action is not yet determined (Mohammad-Sidik et al., 2014; Jewell et al., 2013; Tian et al., 2019). Metacaspase 7 expression can be upregulated by the necrotrophic fungus Alternaria brassicicola (Kwon & Hwang, 2013). Its CNGC2-dependent upregulation by eATP may relate specifically to DAMP signalling following ATP release by damaged cells. Wounded root cells not only release ATP (Dark et al., 2011) that could act as a DAMP for their neighbours but also release another DAMP, the peptide PLANT ELICITOR PEPTIDE 1 (PEP1; Hander et al., 2019). This is perceived in neighbouring cells by the cognate PM receptors PEP1 RECEPTOR 1 (PEPR1) and PEPR2 that relay to CNGC2 to cause [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation (Qi et al., 2010). PEPR2 is coexpressed with P2K1/DORN1 (Tripathi et al., 2017). Extracellular ATP also upregulates PEPR1 and PEPR2 transcription (Jewell et al., 2019), so CNGC2 could be a common component in these DAMP pathways to facilitate the adaptive response.

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**Author contributions**

JS, BM, NL-F, VL, GS, JMD: Project conception. LW, YN, JS, REM, EM, KAW, AD, A-AV, LR, KY, WM, JMD: Experimental design, execution, and analyses. All authors contributed to writing.

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**Data availability**

All lines and data will be made available in a timely manner upon request.
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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Controls for depolarization of elongation zone epidermis and effect of extracellular Ca2+-chelation or channel block.

Fig. S2 Growth of *cnge2-3* and receptor expression.

Fig. S3 Extracellular ATP (eATP) did not depolarize *dnd1* elongation zone epidermis.

Fig. S4 Single receptor mutants supported a small but significant extracellular ATP (eATP)-induced depolarization of elongation zone epidermal $E_{in}$. 

Fig. S5 The p2k1p2k2 double receptor mutant lacked the extracellular ATP (eATP)-induced depolarization of elongation zone epidermal $E_{in}$. 

**Fig. S6** cngc4-5 supported a significant extracellular ATP (eATP)-induced depolarization of elongation zone epidermal $E_m$.

**Fig. S7** Extracellular ATP (eATP) did not activate inward currents in *dorn1-3* root elongation zone epidermal protoplasts.

**Fig. S8** Cyclic Nucleotide-Gated Channel2 (CNGC2) contributed to the extracellular ATP (eATP)-induced $[Ca^{2+}]_{cyt}$ increase in roots.

**Fig. S9** Cyclic Nucleotide-Gated Channel2 (CNGC2) is not required for extracellular ATP (eATP)-induced depolarization of primary root elongation zone cortical plasma membrane potential but CNGC4 is involved.

**Methods S1** Genotyping *enge* insertional and complemented mutants.

**Methods S2** Growth conditions.

**Methods S3** Membrane voltage measurement.

**Methods S4** Patch clamp recordings.

**Methods S5** Quantitative real-time PCR analysis of gene expression.

**Table S1** Primers used for genotyping transfer DNA mutant lines and quantitative real-time PCR.

**Table S2** Mean ± SE membrane voltage $E_m$ measurements.

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