Extracellular diadenosine tetraphosphate (Ap₄A) is recognized by the plasma membrane purinoreceptor P2K1/DORN1 and closes stomata in *Arabidopsis thaliana*

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Summary:

- Dinucleoside polyphosphates (N_pNs) are considered novel signalling molecules involved in the induction of plant defence mechanisms. However, the N_pNs signal recognition and transduction are still enigmatic. Here we report, for the first time, that diadenosine tetraphosphate (Ap_4A) is recognized by the Arabidopsis thaliana purinoreceptor P2K1/DORN1 (Does Not Respond to Nucleotides 1) and causes stomatal closure.

- Extracellular Ap_4A- and dicytosine tetraphosphate (Cp_4C)-induced stomatal closure was observed using a microscope. Reactive oxygen species (ROS) accumulation was determined by staining with nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine tetrahydrochloride (DAB). Transcriptional changes were determined by quantitative real-time PCR. Wild-type Col-0 and the dorn1-3 A. thaliana knockout mutant were used.

- Examination of the leaf epidermis dorn1-3 mutant provided evidence that P2K1/DORN1 recognizes extracellular Ap_4A but not Cp_4C. ROS are involved in signal transduction caused by Ap_4A and Cp_4C, leading to stomatal closure. Ap_4A induced and Cp_4C suppressed the transcriptional response in wild-type plants. Moreover, in dorn1-3 leaves, the effect of Ap_4A on gene expression was impaired.

- Our research demonstrated, for the first time, that P2K1/DORN1 is a plant purinoreceptor for Ap_4A. This interaction leads to changes in the transcription of signalling hubs in signal transduction pathways.

Keywords:
Abscisic acid, diadenosine tetraphosphate (Ap_4A), dicytosine tetraphosphate (Cp_4C), dinucleoside polyphosphates (N_pNs), extracellular ATP (eATP), plant signalling, reactive oxygen species (ROS), uncommon nucleotides.

Introduction
Regulation of plant metabolic processes takes place at a molecular level. The defence reactions are among the processes in which signal transduction plays a key role. Based on the criterion of the distance that a given signal molecule can cover, short-distance molecules cause local intercellular responses, and long-distance molecules trigger systemic responses. Signalling molecules regulate many processes throughout various signal transduction pathways and specific or unspecific
receptors (Sun & Zhang, 2021). Unlike animals, the ability of extracellular nucleotides to initiate diverse signalling responses in plants remained enigmatic for years. A growing number of nucleotides classified as signalling molecules have been identified in plants (Pietrowska-Borek et al., 2020a). Among them, extracellular ATP (eATP) plays an essential role in plant growth (Kim et al., 2006; Wu et al., 2007; Riewe et al., 2008; Tonón et al., 2010; Clark et al., 2010; Zhu et al., 2020) and development (Reichler et al., 2009; Wu et al., 2018). Extracellular ATP regulates responses to biotic stress (Chivasa et al., 2005; Chen et al., 2017; Tripathi et al., 2018; Goodman et al., 2022) and abiotic stress (Thomas et al., 2000; Kim et al., 2009; Sun et al., 2012; Hou et al., 2018). One of the reactions that eATP can control is stomatal movements (Chen et al., 2017; Duong et al., 2021; Wang et al., 2022). In this reaction, the cytoplasmic Ca$^{2+}$ ions ([Ca$^{2+}]_{\text{cyt}}$) and the complex signalling cross-talk between second messengers, such as nitric oxide (NO) (Foresi et al., 2007; Wu & Wu, 2008; Clark et al., 2010), and reactive oxygen species (ROS) (Song et al., 2006; Wu et al., 2008; Demidchik et al., 2009; Chen et al., 2017) plays a crucial role, as a mediator in the signal transduction pathway. Consequently, these messenger agents affect the phosphorylation of mitogen-activated protein kinase (MAPK) and the expression of defence-related genes (Choi et al., 2014; Chen et al., 2017; Li et al., 2021).

We have a longstanding interest in the function of dinucleoside polyphosphates (Np$_n$Ns) in plant cells. Our papers describe changes in gene expression profile and metabolism in Arabidopsis thaliana and Vitis vinifera treated with a broad spectrum of Np$_n$Ns. We postulated the participation of Np$_n$Ns in the plant defence responses since they induce synthesis of the phenylpropanoid pathway-delivered secondary metabolites (Pietrowska-Borek et al., 2011, 2014, 2020b). The phenylpropanoid pathway participates in plant defence responses (Dixon & Paiva, 1995; Sharma et al., 2019). Identification of Ap$_4$A and other Np$_n$Ns across prokaryotic and eukaryotic cells testifies to their universality (Ferguson et al., 2020). Due to the dramatic increase in levels of various Np$_n$Ns observed in cells subjected to abiotic stress factors (Lee et al., 1983; Bochner et al., 1984; Baltzinger et al., 1986; Coste et al., 1987; Pálfi et al., 1991), these compounds have been termed "alarmones", triggering stress adaptive processes. Our latest findings confirmed the induction of the phenylpropanoid pathway by purine, pyrimidine, and purine-pyrimidine hybrids of Np$_n$Ns. Moreover, we observed that diadenosine polyphosphates (Ap$_4$A) induced stilbene biosynthesis. In contrast, dicytidine polyphosphates (Cp$_n$C) strongly inhibited this reaction but markedly induced expression of the cinnamoyl-CoA reductase gene that controls lignin
biosynthesis (Pietrowska-Borek et al., 2020b). Nonetheless, the underlying mechanism of Np₄Ns signal recognition and transduction in plants remains elusive. The growing number of plant enzymes found to be involved in Np₄Ns biosynthesis and degradation strengthens the hypothesis of their signalling function (Pietrowska-Borek et al., 2020a; Ferguson et al., 2020).

Plants can respond to extracellular purine nucleotides, such as eATP, through plasma membrane receptors. So far, two plant receptors with an eATP binding domain have been identified. They are P2K1/DORN1 (Does Not Respond to Nucleotides 1) (Choi et al., 2014) and P2K2/DORN2, which belong to the L-type lectin receptor kinase (LecRK) protein family (Pham et al., 2020; Cho et al., 2023). LecRK proteins activate the processes controlling stress responses, development, growth, and disease resistance (Jose et al., 2020). Although eATP sensing and action in plants have been elucidated, the mechanisms of signal perception and transduction evoked by Np₄Ns, such as Ap₄A and Cp₄C, remain enigmatic. In animal cells, among different nucleotides and nucleosides, eATP, together with Ap₄A, shares access to the same receptors that belong to the P2 group, which is divided into two classes, namely ligand-gated ion channels (P2Xs) and G protein-coupled (P2Ys) receptors (Vigne et al., 2000; McDonald et al., 2002; Wang et al., 2003; Verspohl et al., 2010; Burnstock, 2018). Therefore we hypothesise that the purinoreceptor P2K1/DORN1, a receptor of eATP, is also necessary for sensing Ap₄A in plant cells. Moreover, we wondered whether P2K1/DORN1 can also recognise the pyrimidine nucleotide Cp₄C.

Here we present, for the first time, evidence for the involvement of the P2K1/DORN1 receptor in the sensing of Ap₄A in plants. All experiments were conducted on 4-week-old Arabidopsis thaliana wild-type Col-0 and dorn1-3 knockout mutant leaves. Our research showed that extracellular Ap₄A and Cp₄C evoked stomatal closure in Col-0 plants. This effect was abolished in the dorn1-3 mutant by Ap₄A but not Cp₄C. This result confirms the requirement of P2K1/DORN1 for Ap₄A-induced stomatal closure. Nevertheless, our research indicates the involvement of superoxide (\(\text{O}_2^−\)) and hydrogen peroxide (H₂O₂) in the signal transduction evoked by Ap₄A and Cp₄C, leading to stomatal closure. Furthermore, we analysed the expression of genes encoding selected proteins integrated within the signalling hubs. It concerns NADPH oxidases (RBOHD and RBOHF), MAPK cascades, SNF1/AMPK-related protein kinases (SnRKs) and transcriptional factors such as ZAT6 and ZAT12. Notably, Ap₄A induced expression of the tested genes. Moreover, the gene expression in dorn1-3 was almost abolished by the Ap₄A effect.
Materials and Methods

Nucleotides

Ap₄A and Cp₄C were synthesized following previously reported procedures, purified by reversed phase HPLC, and isolated as ammonium (NH₄⁺) salts. The purities (>95%) were confirmed by analytical HPLC, ¹H NMR and ³¹P NMR (Pietrowska-Borek et al., 2020b).

Plant material

Arabidopsis thaliana lines were in the Columbia (Col-0) ecotype. A T-DNA insertion line of LecRK-I.9 (Salk_042209; dorn1-3) was obtained from NASC (Nottingham Arabidopsis Stock Centre, Nottingham, UK). Surface-sterilised seeds were stratified in darkness at 4°C for 48 h and transferred to a growth chamber. Plants were grown for four weeks on the soil at 21-23°C, 60-70% humidity, under a long-day photoperiod (16 h light and 8 h dark), 120 µmol m⁻² s⁻¹ light intensity. Genotyping of insertional mutants is described in Methods S1. Primers are listed in Table S1.

Stomatal aperture measurement

To ensure fully open stomata, plants were placed for 3 h under light intensity 120 µmol m⁻² s⁻¹. Samples of leaf epidermis were obtained from the abaxial side. They were placed on a microscope slide for 2 h of incubation in (i) MOCK solution MES/KOH opening buffer containing 10 mM MES pH 6.15, 10 mM KCl, 10 µM CaCl₂ (control), (ii) 10 µM abscisic acid (ABA, Sigma Aldrich, A1049) dissolved in the MOCK solution MES/KOH buffer, and (iii) 2 mM ADP (Sigma, A2754), ATP (Sigma, AA8937), Ap₃A, Ap₄A, and CDP (Sigma, C9755), CTP, Cp₃C, Cp₄C dissolved in the MOCK solution MES/KOH buffer. CTP and Np₃Ns were synthesised as described previously (Pietrowska-Borek et al., 2020b). Stomata were observed using the ZOE Fluorescent Cell Imager (Bio-Rad1450031EDU). Measurements, including stomatal aperture width and length, were performed with the ImageJ software. The involvement of ROS in stomatal movement under nucleotide treatment was examined by the simultaneous addition of ROS enzyme scavengers to the nucleotide solutions. Catalase (CAT) (Sigma Aldrich, C100) and superoxide dismutase (SOD)
(Sigma Aldrich, S9697), in a concentration of 100 units ml\(^{-1}\) and 500 units ml\(^{-1}\), respectively, were used together in an incubation mixture.

**Detection of intracellular ROS burst in leaves**

Two leaves were incubated in 3 ml of MOCK solution MES/KOH opening buffer or the buffer enriched in 2 mM concentrations of tested nucleotides. After 2 h the incubating buffers were gently replaced with 3 ml of staining solutions and submerged leaves were vacuum infiltrated three times (1 min each time). The staining solution for \(\cdot O_2^-\) detection was composed of 0.5% nitroblue tetrazolium (NBT) dissolved in 10 mM potassium phosphate buffer, pH 7.8 (Müller *et al.*, 2009), and the staining solution for H\(_2\)O\(_2\) synthesis was composed of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (1 mg ml\(^{-1}\) DAB) dissolved in 10 mM potassium phosphate buffer, pH 7.4, and 0.05% Tween (Daudi & O’Brien, 2012). Samples were incubated at room temperature for the next 2 h in the dark with continuous shaking. Then, leaves were incubated in 96% ethanol overnight for bleaching, and the photographs were taken with an Epson Perfection V700 scanner.

**Gene expression analyses**

According to the manufacturer's instructions, total RNA was extracted from leaves using the RNeasy Plant Mini Kit (Qiagen). Evaluation of RNA purity, cDNA synthesis, reverse transcription, and RT-qPCR were performed as described previously by Pietrowska-Borek and co-workers (Pietrowska-Borek *et al.*, 2011, 2015; Pietrowska-Borek & Nuc, 2013). The qRT-PCR reactions were performed using a CFX96 Real-Time PCR Detection System (Bio-Rad). The specific primers for *Arabidopsis thaliana* genes are listed in Table S1. The 2\(^{-\Delta\Delta Ct}\) method (Schmittgen & Livak, 2008) was applied to calculate the relative gene expression. The data were normalised against the reference gene, *ACTIN2* (*ACT2*). For statistical analysis, the gene expression data were Log\(_2\)-transformed to meet distribution and variance assumptions.

**Statistical analysis**

All experiments were performed at least three times. The results are shown as the mean ± SD. The statistical significance of the differences among the means was analysed using Statistica, Version 13 (TIBCO Software Inc., Palo Alto, CA, USA, [http://statistica.io](http://statistica.io)).
Results

Ap₄A and Cp₄C induce stomatal closure

Our previous research showed that exogenous N₃N₉ induce biosynthesis of secondary metabolites that play an essential role in the plant defence strategy (Pietrowska-Borek et al., 2011, 2014, 2020b). We wondered how the signal evoked by N₃N₉ could be sensed and transduced in plant cells and whether plants contain cell membrane receptor(s) for these molecules. It is known that eATP, one of the exogenous purine nucleotides, evokes stomatal closure with involvement of the purinoreceptor P2K1/DORN1 in Arabidopsis thaliana (Choi et al., 2014; Chen et al., 2017). Therefore based on similarities in ATP and Ap₄A structure, we tested the effect of these nucleotides on stomatal movements. Moreover, we also included cytosine nucleotides in our research because of the different effects of purine and pyrimidine N₃N₉ on the phenylpropanoid pathway in Vitis vinifera cells (Pietrowska-Borek et al., 2020b). To trace stomatal movement under the nucleotide treatment, we examined the ability of purine N₃N₉ such as Ap₃A and Ap₄A to stimulate stomatal closure. Additionally, for the positive control, we tested the effects of ADP and ATP as described earlier (Choi et al., 2014; Chen et al., 2017) as well as ABA – a well-known molecule controlling stomatal movements (Danquah et al., 2014). Exogenous Ap₄A significantly reduced the stomatal aperture in the light. It was at a similar level compared to the effect evoked by ATP and ADP. However, Ap₃A did not evoke such an effect (Fig. 1). We also examined stomatal movement under the treatment of cytidine mono- and dinucleotides (CDP, CTP, Cp₃C, Cp₄C). Interestingly, only Cp₄C triggered significant stomatal closure among tested cytidine nucleotides. As expected, ABA closed stomata (Bharath et al., 2021) (Fig. 1).
**Fig. 1** Ap₄A and Cp₄C, similarly to ADP and ATP, induce stomatal closure in *Arabidopsis thaliana* Col-0 plants. Images represent stomata in the abaxial epidermis of a leaf treated for 2 h with the MOCK solution MES/KOH opening buffer, 10 μM ABA, and 2 mM purine and pyrimidine nucleotides. White bar = 25 μm. Bars represent mean values ± SD, n ≥ 20, three biological replicates. Different letters above the error bars indicate statistically significant differences according to the ANOVA analysis with the post-hoc Tukey’s HSD multiple comparisons test (p < 0.05).

In plant cells there are enzymes degrading NpₙNs to mononucleotides (Guranowski, 2004). To confirm that Ap₄A and Cp₄C evoke stomatal closure but not by the products of their degradation (AMP, ADP, ATP, and CMP, CDP, CTP, respectively), we collected samples of leaf epidermis from the microscope slides after incubation of nucleotides, and application of the HPLC assay (Method S2) proved that Ap₄A was not degraded to the corresponding mononucleotide. Only a trace amount of CTP was detected in a solution of Cp₄C after the investigation (Fig. S1a,b).
P2K1/DORN1 is involved in signal perception evoked by Ap₄A but not Cp₄C

Plants respond to eATP by induction of a complex signalling network after signal recognition by the P2K1/DORN1 and P2K2 receptors (Choi et al., 2014; Pham et al., 2020). Similarities in stomatal movements evoked by eATP, Ap₄A, and Cp₄C led us to hypothesise that those nucleotides could interact with P2K1/DORN1. Based on the results presented in Fig. 1, Ap₄A and Cp₄C were chosen for further experiments. The dorn1-3 mutant, having a T-DNA insertion in the extracellular legume-type lectin domain, was selected based on literature data (Choi et al., 2014; Chen et al., 2017). We found that Ap₄A and eATP did not close stomata in dorn1-3 mutant leaves. Contrary to this, Cp₄C significantly closed stomata in dorn1-3 mutant leaves. As expected, ABA-treated mutant leaves also showed closed stomata (Chen et al., 2017) (Fig. 2). Thus, the results strongly suggest that besides eATP, P2K1/DORN1 may also be involved in signal perception elicited by Ap₄A but not Cp₄C.

**Fig. 2** Ap₄A, similarly to eATP, did not induce stomatal closure in the *dorn1-3 Arabidopsis thaliana* mutant. However, Cp₄C and ABA evoked stomatal closing. Images represent stomata in the abaxial epidermis of *dorn1-3* leaf treated for 2 h with MOCK solution opening buffer, 10 µM ABA and 2 mM ATP, Ap₄A, and Cp₄C. White bar = 25 μm. Bars represent mean values ± SD, n ≥ 20, three biological replicates. Different letters above the error bars indicate statistically significant differences according to the ANOVA analysis with the post-hoc Tukey’s HSD multiple comparisons test (p < 0.05).
ROS are produced in leaves under nucleotide treatment

It was previously found that elevated production of ROS and stomatal closure are mediated by eATP recognition by the receptor P2K1/DORN1, followed by direct phosphorylation of the NADPH oxidase RBOHD (Chen et al., 2017). This phosphorylation causes an increase in generation of extracellular ROS, such as 'O_2^−', which is then converted into H_2O_2 in the extracellular environment (Waszczak et al., 2018; Smirnoff & Arnaud, 2019). Notably, the apoplastic production of ROS is one of the fastest physiologically common responses to external stimuli observed in plants (Macho & Zipfel, 2014; Mittler et al., 2022). Considering all the above-described information, we decided to investigate the accumulation of 'O_2^−' and H_2O_2 in Arabidopsis thaliana leaves in response to 2 mM ATP, CTP, Ap_4A, and Cp_4C. Our experiments revealed that blue staining of leaves, indicating 'O_2^−' accumulation, was increased in Col-0 leaves treated with CTP, Ap_4A, and Cp_4C but not by eATP, while in the dorn1-3 mutant, only Cp_4C evoked accumulation of 'O_2^−' (Fig. 3a). Brown staining representing the concentration of H_2O_2 in leaves was increased in Col-0 leaves under eATP, Ap_4A, and Cp_4C, while CTP caused only slightly brown staining. In the dorn1-3 mutant, only CTP and Cp_4C evoked an accumulation of H_2O_2 in the leaves. Nevertheless, only weak brown staining was caused by CTP (Fig. 3b).

![Fig. 3 Histochemical detection of (a) 'O_2^−' and (b) H_2O_2 in leaves of Arabidopsis thaliana Col-0 and the dorn1-3 mutant triggered by 2 mM ATP, CTP, Ap_4A, and Cp_4C after 2 h treatment. Leaves were stained with NBT and DAB for 'O_2^−' and H_2O_2 detection, respectively. The experiment was repeated six times, and representative leaves were chosen.](https://doi.org/10.1101/2023.05.10.537060)
ROS are involved in signal transduction evoked by eATP, Ap₄A and Cp₄C, leading to stomatal closure

Based on the results indicating that Ap₄A and Cp₄C induced the production of ROS (Fig. 3a,b), we wondered whether these key signalling molecules are components of signal transduction pathways evoked by N₆Ns leading to stomatal closure. We simultaneously applied superoxide dismutase (SOD) and catalase (CAT), enzymes scavenging ROS (Khokon et al., 2011; Mittler et al., 2022), and thereby sought to confirm the role of O₂⁻ and H₂O₂ in the transduction pathway of the signal generated by Ap₄A and Cp₄C. Interestingly, CAT and SOD eliminated the effect of stomatal closure under simultaneous nucleotide treatment, so our observations showed direct involvement of O₂⁻ and H₂O₂ in stomatal closure evoked by eATP, Ap₄A, and Cp₄C. However, the plants did close their stomata upon adding ABA (Fig. 4).

Fig. 4 ROS enzyme scavengers, CAT and SOD, eliminate the effect of stomatal closure after the 2 mM ATP, Ap₄A, and Cp₄C treatment in Arabidopsis thaliana Col-0 leaves. White bar = 25 μm. Bars represent mean values ± SD, n ≥ 20, three biological replicates. Different letters above the error bars indicate statistically significant differences according to the ANOVA analysis with the post-hoc Tukey’s HSD multiple comparisons test (p < 0.05).

P2K1/DORN1 is implicated in Ap₄A- and eATP-responsive gene expression

It is known that transcriptional upregulation of defence-related and wound-response genes by eATP is P2K1/DORN1-dependent (Choi et al., 2014; Jewell et al., 2019). Thus we decided to
investigate whether Ap₄A also changes the expression of the defence-related genes and whether the plasma membrane receptor P2K1/DORN1 is engaged in this regulation. To understand the signal transduction pathway evoked by Ap₄A, we tested the gene expression coding for proteins as a component of signalling hubs known as key points in response to stresses. First, we studied the NADPH oxidases respiratory burst oxidase homologs (RBOHs), RBOHD, and RBOHF, which generate ROS (Mittler et al., 2022). We found that Ap₄A up-regulated RBOHF but not by eATP in Col-0 plants. Interestingly, both eATP and Ap₄A downregulated RBOHF expression in the dorn1-3 mutant (Fig. 5a). The expression of RBOHD was drastically induced (the most among all studied genes) by eATP but only in Col-0 plants. In contrast, in the dorn1-3 plants this effect was weak. Ap₄A evoked slight changes in expression levels of RBOHD in Col-0 and dorn1-3 plants (Fig. 5a).
**Fig. 5** The purinoceptor P2K1/DORN1 is involved in the Ap₄A-induced transcriptional response in Arabidopsis thaliana Col-0 leaves. Graphs present the changes in the gene expression level for (a) NADPH oxidase respiratory burst homologs (*RBOHD* and *RBOHF*), (b) cyclic nucleotide-gated channel 2 (*CNGC2*), (c) SNF1/AMPK-related protein kinases (*SnRKs*), (d) mitogen-activated protein kinase 6 (*MAPK6*), (e) transcription factors (*ZAT6* and *ZAT12*). Leaves taken from Col-0 and the *dorn1-3* mutant were treated for 2 h with 2 mM ATP and Ap₄A. Transcript levels are represented as Log₂(2⁻ΔΔCt) compared to the MOCK-treated (control) plants. The housekeeping gene *AtACT2* was used for data normalisation as an endogenous control. Data are
mean ± SD from 3 biological replicates. Different letters above the error bars indicate statistically significant differences according to the ANOVA analysis with the post-hoc Tukey’s HSD multiple comparisons test (p < 0.05).

Other components involved in a variety of signalling pathways, ranging from development to stress responses, are cyclic nucleotide-gated channels (CNGCs) (Duszyn et al., 2019; Jarratt-Barnham et al., 2021). Moreover, AtCNGC2 mediates eATP signal transduction in cells of the root epidermis (Wang et al., 2022). We found that Ap₄A induced CNGC2 expression in Col-0 plants and decreased the expression in the dorn1-3 mutant. Extracellular ATP decreased the expression of CNGC2 in both Col-0 and dorn1-3 mutant plants (Fig. 5b). We also focused on essential protein kinases, such as SnRKs, that regulate cellular energy homeostasis, stress response, and growth (Zhang et al., 2020). Thus, we checked the changes in the expression of SnRK1.1, SnRK1.2, SnRK2.1, SnRK2.2, and SnRK2.6. We also tested the expression of PV42a encoding cystathionine-β-synthase (CBS) domain-containing protein belonging to the PV42 class of γ-type subunits of the plant SnRK1 complexes. It is known that CBS domains generally act as regulatory domains of protein activity through adenosyl ligand binding (Baudry et al., 2022). Our experiments showed that eATP strongly induced the expression of SnRK1.1, SnRK1.2, and PV42a in Col-0 plants. Although Ap₄A causes a lower effect than eATP, the elevation in the expression of SnRK1.1 and SnRK1.2 was statistically significant. Interestingly, in Col-0 plants, only eATP up-regulates the transcription of PV42a. Still, in the dorn1-3 mutant compared to Col-0, only Ap₄A treatment caused induction of the expression (Fig. 5c). Extracellular ATP and Ap₄A increased the expression of SnRK2.2, SnRK2.3, and SnRK2.6 in Col-0 plants. In the dorn1-3 mutant plants, Ap₄A down-regulated SnRK2.2, SnRK2.3, and SnRK2.6. Still, the effect of eATP in the mutant was not the same for the expression of the three SnRK2 genes; namely, the expression of SnRK2.2 was decreased, SnRK2.3 was slightly increased, and there was no effect on SnRK2.6 expression (Fig. 5c). The strong relationships between secondary messengers, such as ROS and MAPKs, are often highlighted in the literature (Matsushita et al., 2020; Byrne et al., 2020). MAPK6, among its roles in various metabolic processes in plants, can regulate the activities of diverse targets, including transcription factors (Smékalová et al., 2014). We observed up-regulation of MAPK6 expression by both eATP and Ap₄A in Col-0 plants and down-regulation in the dorn1-3 mutant (Fig. 5d). Among transcription factors that MAPKs regulate, we tested the regulation of expression of the
zinc-finger transcription factors (ZAT6 and ZAT12) and we found that eATP and Ap₄A upregulated the expression of both genes, as mentioned above in Col-0 plants. Extracellular ATP increased the expression of ZAT6 and ZAT12 also in the dorn1-3 mutant, but Ap₄A downregulated the expression of both genes in the mutant plants (Fig. 5e).

**Discussion**

Plants are exposed to continuous changes in environmental conditions that lead to an imbalance in cellular homeostasis. It is known that in response to various stresses in prokaryotic and eukaryotic cells, Np₄Ns accumulate. The accumulation of such uncommon nucleotides can be considered in the context of the "friend hypothesis" (alarmone) and "foe hypothesis" regarding critically damaged cells as a result of internal and external stresses (McLennan, 2000; Ferguson *et al.*, 2020). Although there are identified Ap₄A-binding protein targets in cells (Ferguson *et al.*, 2020), the signalling pathways are still unclear. We reported previously that extracellular Np₄Ns regulate the phenylpropanoid pathway producing secondary metabolites – key molecules in response to abiotic stress in *Arabidopsis thaliana* and *Vitis vinifera* (Pietrowska-Borek *et al.*, 2011, 2014, 2020b). Notably, one of the phenylpropanoid pathway enzymes, 4-coumarate:CoA ligase, is known to catalyse synthesis of Ap₄A (Pietrowska-Borek *et al.*, 2003), and its activity was increased by Ap₄A (Pietrowska-Borek *et al.*, 2011). It is known that some extracellular Np₄N may become internalised and operate intracellularly (Ferguson *et al.*, 2020). Despite this obvious evidence of the signalling function of uncommon nucleotides in regulating phenylpropanoid synthesis, no receptors or signalling pathways have been identified in plants until now. Here we demonstrated, for the first time, that Ap₄A evoked stomatal closure in *Arabidopsis thaliana* leaves (Fig. 1). We did not observe such an effect in dorn1-3 plants (Fig. 2). So we can conclude that plasma membrane purinoreceptor P2K1/DORN1 recognises Ap₄A. However, our research also indicates that P2K1/DORN1 is not involved in signal perception elicited by Cp₄C (Fig. 2). Such results suggest that in plants, there is not or is other Cp₄C-binding protein(s). After Ap₄A signal recognition, P2K1/DORN1 stimulates ROS burst and the defence-related response. Our data indicating ROS involvement in the plant response to Ap₄A and Cp₄C support the hypothesis concerning the signalling function of Np₄Ns (Fig. 3 and Fig. 4). Moreover, the HPLC assay proved that Ap₄A was not degraded to corresponding mononucleotides, which could evoke stomatal closure during the experiment (Fig. S1). Only a tiny amount of CTP was detected in a solution of Cp₄C after the
investigation. Still, as we proved, CTP did not evoke stomatal closure (Fig. 1). Therefore, it confirms that the observed stomatal closure and ROS accumulation were caused by Ap4A and Cp4C but not by their decomposition products.

The upregulation of defence-related genes encoding proteins involved in signalling hubs was reported (Zhang et al., 2020). The expression of the genes described in this research was mostly abolished or down-regulated in the dornl-3 mutant (Fig. 5). Recent studies consider cross-talk between diverse plant defence response markers such as ROS, hormones, and kinase cascades, leading to transcriptional, translational, and metabolic reprogramming (Mittler et al., 2022). Our transcriptional analysis focused on elements that integrate various signals and included cyclic nucleotide-gated channels (CNGC) and NADPH oxidases – respiratory burst oxidase homologs (RBOHD, and RBOHF) that generate ROS. Moreover, our studies are focused on SNF1-related protein kinases (SnRKs) and PV42a, a cystathionine-β-synthase (CBS) domain-containing protein belongs to the PV42 class of γ-type subunits of the plant SnRK1 complexes. The next elements of signal transduction pathways that we tested concern MAPK6, and transcription factors, ZATs (Fig. 5). The transcript level of CGNC2 increased only under Ap4A in Col-0 plant leaves (Fig. 5b). Involving CGNC2 in another purine nucleotide, eATP, signal transduction in the root epidermis and eATP-induced Ca2+ influx were described by Wang (Wang et al., 2022). This result suggests that CNGC channels can be a part of signal transduction evoked by Ap4A.

Rapid systemic signalling in response to stress can be stimulated by RBOHD and RBOHF, producing apoplastic ROS (Choi et al., 2016). It is known that the elevated production of ROS and stomatal closure are mediated by eATP recognition by the receptor P2K1/DORN1, followed by direct phosphorylation of RBOHD (Chen et al., 2017), while RBOHD expression was significantly reduced in dornl-3 mutant plants. Our studies showed that transcriptomic changes in both RBOHD and RBOHF evoked by Ap4A are similar, but in the dornl-3 plants, expression of RBOHF also was strongly inhibited (Fig. 5a). This observation correlated with the accumulation of ROS in Arabidopsis thaliana leaves (Fig. 3). Stress signalling in plants also involves different families of kinases, including the MAPK module, that can be activated by ROS (Meng & Zhang, 2013). Moreover, it was previously shown that MAPKs are activated by eATP (Choi et al., 2014; Medina-Castellanos et al., 2014; Chen et al., 2021; Cho et al., 2022). We observed the induction of MAPK6 expression evoked by eATP and Ap4A (Fig. 5d), and it is known that MPK6 modulates actin remodelling to activate stomatal defence in Arabidopsis thaliana (Zou et al., 2021). MAPK
pathways are necessary for several ABA responses in many plant species, including antioxidant defence and guard cell signalling (Danquah et al., 2014). A complex protein complex, SNF1-related protein kinase 1s (SnRK1s) and SnRK2s, plays a prominent role in ABA signalling (Jossier et al., 2009; Ou et al., 2022). Numerous studies indicate SnRK1s and SnRK2s as regulators of the target of rapamycin (TOR) kinase activity in controlling autophagy (Signorelli et al., 2019; Belda-Palazón et al., 2020). We observed that Ap₄A induced the expression of both SnRK1s and SnRK2s at a similar level in Col-0 plants. However, induction evoked by eATP was much higher for SnRK1s than SnRK2s in wild-type plants. In the dorn1-3 mutant, the expression of SnRK1s and SnRK2s was decreased (Fig. 5c). Also both tested pyrimidine nucleotides, CTP and Cp₄C, did not affect expression of SnRKs in Col-0 plants (Fig. S2). It is known that SnRKs can regulate RBOH, which is engaged in ROS production (Mittler et al., 2022). The SnRK1s and SnRK2s were identified as critical nodes for stress and growth signalling pathways (Zhang et al., 2020). Moreover, it was suggested that under normal conditions, cytosol-localised SnRK1.1, in response to high-ammonium or low-pH stress, migrates to the nucleus and promotes the phosphorylation of the transcription factors regulating the expression of responsive genes (Sun et al., 2021). Studies on AKINβ1, subunit SnRK1, showed its regulatory effect on secondary metabolic processes (e.g. phospholipid and flavonoid metabolism) (Wang et al., 2020). Another SnRK1 subunit is PV42a, which is the CBS domain protein. Ap₄A did not change the expression of the gene encoding AtPV42a in Col-0 plants (Fig. 5c). It is known that enzymes containing CBS domains can be regulated by Ap₄A binding (Ferguson et al., 2020). Therefore we postulate that AtPV42a regulates SnRK1s in response to Ap₄A. Moreover, SnRK1, SnRK2, and MAPK interact with transcriptional factors (Zhang et al., 2022; Son et al., 2023). The induction of ZAT12 and ZAT6 transcription factors in which MAPK6 is involved in an abiotic stress marker was described (Smékalová et al., 2014). In the present research, we found that Ap₄A and eATP induced both ZAT6 and ZAT12 gene expression in Col-0 plants, and lack of the P2K1/DORN1 receptor in the dorn1-3 mutants diminished this effect (Fig. 5e). It is known that the transcript level of ZAT6 positively affected the concentrations of phenylpropanoids, including anthocyanin and total flavonoids (Shi et al., 2018). Moreover, it was proved that ZAT6 and ZAT12 are involved in the response to cadmium stress and abiotic stress in plants (Opdenakker et al., 2012; Shi et al., 2014; Chen et al., 2016; Dang et al., 2022) and the expression of ZAT12 was strictly dependent on the ROS wave (Brumbarova et al., 2016; Myers et al., 2022).
The results of our research presented here shed more light on the signalling function of
Ap4A, its perception and signal transduction pathway in plants. We had previously proposed a
hypothetical NpN signalling network in a plant cell. Then we strongly suggested the existence
of some receptor and signalling transduction pathways involving signalling hubs and transcription
factors resulting in gene expression changes, including genes coding for enzymes catalysing the
phenylpropanoid pathway (Pietrowska-Borek et al., 2011, 2014, 2020b, a). Here, we fill a few gaps
in this network (Fig. 6). Nevertheless, further studies are required to fully describe the role of
NpN in signalling hubs and better understand the function of uncommon nucleotides in plants.
Fig. 6 Hypothetical working model of Ap₄A signalling network in a plant cell. Ap₄A, similarly to eATP (Choi et al., 2014), can be recognized by the purinoreceptor P2K1/DORN1, and lead to stomatal closure. As our study showed, Ap₄A triggered the ROS wave, which evoked changes in the expression of the defence-related genes encoding proteins involved in signalling hubs, such as CNGC2; RBOHD and RBOHF generate ROS; SnRKs; AtPV42a, γ-type subunits of the plant...
SnRK1 complexes; MAPK cascades, and transcription factors, ZATs. The wounded cell membrane and transporters can release ATP to the extracellular space matrix: PGP1, p-glycoprotein belonging to ATP-binding cassette ABC transporters, and PM-ANT1, plasma membrane-localized nucleotide transporters (Thomas et al., 2000; Rieder & Neuhaus, 2011). Extracellular ATP recognition by P2K1/DORN1 evoked phosphorylation of RBOHD (Chen et al., 2017). Also, CNGC2 (Wang et al., 2022) and MAPK cascades are involved in eATP signal transduction (Choi et al., 2014; Medina-Castellanos et al., 2014; Chen et al., 2021; Cho et al., 2022). We previously described that 4-coumarate:CoA ligase (4CL), the branch point of the phenylpropanoid pathway, can synthesize Ap4A (Pietrowska-Borek et al., 2003), and its activity is induced by Ap4A (Pietrowska-Borek et al., 2011). As yet, no channel or transporter for Ap4A in plants is known. PM, plasma membrane; P, phosphate.

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Competing interests

None declared.

Author contributions

JD co-designed the studies, carried out experiments, analysed results, and was involved in statistical analysis, visualisation, and writing of the original draft of the manuscript; VHN and JK synthesized the dinucleoside polyphosphates and participated in reviewing and editing of the manuscript; SB participated in writing and critically reviewing the manuscript, and co-designed and prepared Fig. 6; MP-B conceived the topic of the research, planned and supervised all experiments, analysed all results, performed the statistical analysis, participated in writing the draft.
of the manuscript, co-designed and co-created all figures, and prepared the final version of the manuscript. All authors read and approved the manuscript.

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Data availability
The original data obtained during this research are available from the corresponding authors on reasonable request, and some of them are also accessible in the Supplementary materials.

References


**Support Information**

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

**Fig. S1** High-performance liquid chromatography analysis of Ap\(_4\)A and Cp\(_4\)C solutions after 2 h of leaf epidermal peel treatment.

**Fig. S2** CTP and Cp\(_4\)C do not up-regulate expression of SnRK genes.

**Methods S1** Genotyping *dorn1-3* insertional mutant.

Table S1  List of primers used for genotyping T-DNA mutant and qPCR.

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