Wydział Rolnictwa, Ogrodnictwa i Biotechnologii
Katedra Biochemii i Biotechnologii

Jędrzej Dobrogojski

Rola dinukleozydopolifosforanów (Np_nN') i nukleozydo 5′-fosforamidów (NH_2-pN) w transdukcji sygnału i regulacji szlaku fenylopropanoidowego u winorośli właściwej (*Vitis vinifera* L.) i rzodkiewnika pospolitego (*Arabidopsis thaliana* (L.) Heynh.)

The role of dinucleoside polyphosphates ($Np_nN's$) and nucleoside 5′-phosphoramidates (NH_2-pNs) in signal transduction and regulation of the phenylpropanoid pathway in grapevine (*Vitis vinifera* L.) and thale cress (*Arabidopsis thaliana* (L.) Heynh.)

PRACA DOKTORSKA

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Promotor: dr hab. Małgorzata Pietrowska-Borek, prof. UPP

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Dziękuję Promotorowi pracy,
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Publikacja 1.
Pietrowska-Borek M., **Dobrogojski J**., Sobieszczuk-Nowicka E., Borek S. 2020. New insight into plant signaling: extracellular ATP and uncommon nucleotides. *Cells*, 9, 345. Doi.org/10.3390/cells9020345,
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Pietrowska-Borek M., Wojdyła-Mamoń A., **Dobrogojski J**., Młynarska-Cieślak A., Baranowski MR., Dąbrowski JM., Kowalska J., Jemielity J., Borek S., Pedreño MA., Guranowski A. 2020. Purine and pyrimidine dinucleoside polyphosphates differentially affect the phenylpropanoid pathway in *Vitis vinifera* L. cv. Monastrell suspension cultured cells. *Plant Physiology and Biochemistry*, 147, 125-132.
Doi.org/10.1016/j.plaphy.2019.12.015,
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Publikacja 3.
Pietrowska-Borek M., **Dobrogojski J**., Wojdyła-Mamoń AM., Romanowska J., Gołębiewska J., Borek S., Murata K., Ishihara A., Pedreño MÁ., Guranowski A. 2021. Nucleoside 5′-phosphoramidates control the phenylpropanoid pathway in *Vitis vinifera* suspension-cultured cells. *International Journal of Molecular Sciences*, 22, 13567.
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Publikacja 4.
Dobrogojski J., Nguyen V.H., Kowalska J., Borek S., Pietrowska-Borek M. 2023. The plasma membrane purinoreceptor P2K1/DORN1 is essential in stomatal closure evoked by extracellular diadenosine tetraphosphate (Ap_4A) in *Arabidopsis thaliana*. *International Journal of Molecular Sciences*, 24, 16688.
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Wykaz skrótów

$[Ca^{2+}]_{cyt}$ – cytozolowy wapń wolny

$^{\bullet}O_2^-$ – anionorodnik ponadtlenkowy

4CL – ligaza 4-kumarylo-CoA

ABA – kwas abscysynowy

ABCG44 – białka błonowe z kasetą wiążącą ATP z podrodziny G (ang. *ATP-Binding Cassette, subfamily G*)

ADP – adenozyno-5′-difosforan

AMP – adenozyno-5′-monofosforan

Ap_3A – diadenozyno 5′,5‴-P^1,P^3-trifosforan

Ap_3C – P^1-(5′-adenozyno)- P^3-(5′-cytydyno)-trifosforan

Ap_3U – P^1-(5′-adenozyno)- P^3-(5′-urydyno)-trifosforan

Ap_4A – diadenozyno 5′,5‴-P^1,P^4-tetrafosforan

Ap_4C – P^1-(5′-adenozyno)- P^4-(5′-cytydyno)-tetrafosforan

ATP – adenozyno-5′-trifosforan

BenPut – N-benzoiloputrescyna

C4H – 4-hydroksylaza kwasu cynamonowego

CAD – dehydrogenaza alkoholu cynamylowego

cAMP – cykliczny 3′,5′-adenozynomonofosforan

CAT – katalaza

CCR – reduktaza cynamylo-CoA

CDP – cytydyno-5′-difosforan

cGMP – cykliczny 3′,5′-guanozynomonofosforan

CHS – syntaza chalkonowa

CMP – cytydyno-5′-monofosforan

CNGC – kanały bramkowane cyklicznymi nukleotydami

Cp_3C – dicytydyno 5′,5‴-P^1,P^3-trifosforan

Cp_4C – dicytydyno 5′,5‴-P^1,P^4-tetrafosforan

CTP - cytydyno-5′-trifosforan

DW – sucha masa (ang. *dry weight*)

eATP – zewnątrzkomórkowy ATP

GMP – guanozyno-5′-monofosforan

Gp_3G – diguanozyno 5′,5‴-P^1,P^3-trifosforan

Gp_4G – diguanozyno 5′,5‴-P^1,P^4-tetrafosforan

H_2O_2 – nadtlenek wodoru

MAP – białkowe kinazy aktywowane mitogenami

NAD^+ – dinukleotyd nikotynoamidoadeninowy

$NADP^+$ – fosforan NAD^+

NADPH – zredukowany fosforan dinukleotydu nikotynoamidoadeninowego

NH_2-pA – adenozyno 5′-fosforamid

NH_2-pC – cytydyno 5′-fosforamid

NH_2-pG – guanozyno 5′-fosforamid

NH_2-pN – nukleozydo 5′-fosforamid

NH_2-pU – urydyno 5′-fosforamid

NO – tlenek azotu

Np_nN' – dinukleozydopolifosforany

P2K1/DORN1 – błonowa receptorowa kinaza białkowa LecRK-I.9

P2K2/DORN2 – błonowa receptorowa kinaza białkowa LecRK-I.5

PAL – amoniakoliaza fenyloalaniny

RBOHD – homolog D oksydazy NADPH wybuchu tlenowego (ang. *NADPH Respiratory Burst Oxidase Homolog Protein D*)

RBOHF – homolog F oksydazy NADPH wybuchu tlenowego (ang. *NADPH Respiratory Burst Oxidase Homolog Protein F*)

RFT – reaktywne formy tlenu

SnRK – kinazy serynowo-treoninowe spokrewnione z kinazami SNF-1 (ang. *SNF1 (sucrose non fermentation 1)– Related Kinases*)

SOD – dysmutaza ponadtlenkowa

STS – syntaza stilbenowa

UMP – urydyno-5′-monofosforan

Up_3U – diurydyno 5′,5‴-P^1,P^3-trifosforan

Up_4U – diurydyno 5′,5‴-P^1,P^4-tetrafosforan

ZAT – palec cynkowy *Arabidopsis thaliana* (ang. *ZINC FINGER of Arabidopsis thaliana*)

Streszczenie rozprawy doktorskiej

Ze względu na brak mobilności rośliny wykształciły unikalne mechanizmy obronne uruchamiane w odpowiedzi na niekorzystne warunki środowiskowe. Reakcje na czynniki biotyczne jak i abiotyczne odbywają się m.in. na poziomie molekularnym, w którym to kluczową rolę pełni odbiór, a następnie transdukcja sygnału. Aktywacja szlaku fenylopropanoidowego, a co za tym idzie indukcja syntezy produktów z niego pochodzących oraz zamykanie aparatów szparkowych stanowią elementy strategii obronnej u roślin wyższych. Do molekuł pełniących funkcje sygnalne w komórkach roślinnych zaliczane są niektóre nukleotydy. W ich obrębie wyróżnić można nukleotydy zwane nietypowymi, do których należą dinukleozydopolifosforany (Np_nN') oraz nukleozydo 5′-fosforamidy (NH_2-pN).

Nadrzędnym celem pracy było określenie roli Np_nN' i NH_2-pN w transdukcji sygnału i regulacji szlaku fenylopropanoidowego u winorośli właściwej (*Vitis vinifera* L.) i rzodkiewnika pospolitego (*Arabidopsis thaliana* (L.) Heynh.). Potwierdzono, że egzogenne Np_nN' regulują szlak fenylopropanoidowy w zawiesinowej kulturze komórkowej *V. vinifera* cv. Monastrell zależnie od typu nukleozydu. Odnotowano istotne różnice akumulacji dwóch stilbenów: *trans*-resweratrolu oraz *trans*-piceidu w pożywce, zarówno pod wpływem Np_nN' purynowych (Ap_3A i Gp_3G), pirymidynowych (Up_3U i Up_4U) oraz puryno-pirymidynowej hybrydy (Ap_3U). Co więcej, wykazano wpływ badanych nukleotydów na poziom transkryptów genów kodujących enzymy zaangażowane w szlaku fenylopropanoidowym. Wzrost akumulacji stilbenów pod wpływem Ap_3A, Gp_3G, Up_3U i Up_4U był skorelowany z indukcją ekspresji genu *STS1* kodującego syntazę stilbenową oraz genu *C4H1* kodującego hydroksylazę kwasu cynamonowego. Te same nukleotydy indukowały ekspresję genu *ABCG44* kodującego białko ABCG44 transportujące *trans*-resweratrol na zewnątrz komórki. Pirymidynowe Np_nN' takie jak Cp_3C, Cp_4C i purynowo-pirymidynowa hybrydy Ap_4C, silnie hamowały biosyntezę stilbenów co związane było z obniżeniem ekspresji genów *STS1* i *ABCG44* przy jednoczesnej indukcji ekspresji genu *CCR2* kodującego reduktazę cynamylo-CoA kontrolującą biosyntezę lignin. Podobnie do badań nad wpływem Np_nN', wykazano że NH_2-pN również regulują szlak fenylopropanoidowy w komórkach zawiesinowej kultury *V. vinifera* cv. Monastrell zależnie od typu nukleozydu. Odnotowano istotne różnice akumulacji *trans*-resweratrolu oraz *trans*-piceidu w komórkach oraz pożywce zarówno pod wpływem

NH_2-pN purynowych (NH_2-pA, NH_2-pG) jak i pirymidynowych (NH_2-pU, NH_2-pC). Wykazano, że badane nukleotydy modyfikowały ekspresję genów kontrolujących biosyntezę stilbenów i lignin przy czym najistotniejsze różnice ekspresji genów odnotowano pod wpływem NH_2-pC. Ponadto nukleotyd ten indukował szybki wzrost poziomu N-benzoiloputrescyny.

Kluczowym elementem percepcji sygnału są oddziaływania pomiędzy molekułą sygnalną a receptorem. Poznanym i dobrze opisanym receptorem u roślin dla zewnątrzkomórkowego nukleotydu purynowego, eATP, jest błonowa receptorowa kinaza białkowa LecRK-I.9 (P2K1/DORN1). Pomimo wielu przesłanek wskazujących na działanie egzogennych Np_nN' jako molekuł sygnalnych, mechanizmy percepcji i transdukcji sygnału wywołanych przez Np_nN' u roślin pozostawały nieznane. W badaniach przeprowadzonych w ramach realizacji niniejszej rozprawy doktorskiej wykazano, że P2K1/DORN1 jest kluczowym elementem w percepcji i transdukcji sygnału wywołanego przez Ap_4A. Co więcej, egzogenne Ap_4A i Cp_4C istotnie zmniejszały stopień rozwarcia aparatów szparkowych u *A. thaliana*, jednakże proces ten pod wpływem Cp_4C zachodził niezależnie od P2K1/DORN1. Udowodniono, że molekułami sygnalnymi pośredniczącymi w zamykaniu aparatów szparkowych przez Ap_4A i Cp_4C są reaktywne formy tlenu (RFT) w postaci anionorodnika ponadtlenkowego ($^{\bullet}O_2^-$), i nadtlenku wodoru (H_2O_2). Ponadto u *A. thaliana* typu dzikiego traktowanych ATP i Ap_4A wykazano indukcję wybranych genów biorących udział w transdukcji sygnału i reakcję roślin na stres czego nie odnotowano u mutanta *dorn1-3* typu knock-out posiadającym insercje T-DNA w obrębie genu kodującego P2K1/DORN1. Indukcji ekspresji owych genów nie wykazano w liściach *A. thaliana* typu dzikiego pod wpływem CTP i Cp_4C.

Kluczowym osiągnięciem niniejszej pracy jest również wskazanie roślinnego purynoreceptora P2K1/DORN1 jako istotnego białka błonowego w percepcji sygnału wywołanego przez Ap_4A. Jest to pierwsze opisane roślinne białko receptorowe mogące oddziaływać z Ap_4A. Ponadto określono rolę elementów mogących uczestniczyć w transdukcji sygnału prowadzącego do modyfikacji szlaku fenylopropanoidowego u *V. vinifera* i regulacji ruchów aparatów szparkowych u *A. thaliana*. Co więcej, wykazano, że

P2K1/DORN1 nie pełni takiej funkcji w przypadku dinukleotydu pirymidynowego Cp_4C, co świadczyć może o występowaniu u roślin innych białek oddziałujących z tymi nukleotydami.

Streszczenie rozprawy doktorskiej w języku angielskim

As the mobility of plants is impossible, they developed unique defence mechanisms allowing them to respond to adverse environmental conditions. Responses to abiotic and biotic factors are manifested, among others, at molecular level, where the key elements, such as signal perception and its subsequent transduction, occur. Characteristic defence mechanisms of higher plants include closure of the stomata and the activation of phenylpropanoid pathway, resulting in synthesis of related products. Molecules with signalling functions in plant cells include certain nucleotides. Among them, the group of nucleotides named uncommon nucleotides including dinucleoside polyphosphates (Np_nN') and nucleoside 5′-phosphamides (NH_2-pN) can be distinguished.

The overarching aim of this study was to determine the role of Np_nN's and NH_2-pNs nucleotides in signal transduction and phenylpropanoid pathway regulation in grapevine (*Vitis vinifera* L.) and thale cress (*Arabidopsis thaliana* (L.) Heynh.). Results demonstrate that extracellular Np_nN's regulate the phenylpropanoid pathway in *V. vinifera* cv. Monastrell suspension cultured cells depending on the type of nucleoside. Significant increase in the level of accumulation of *trans*-resveratrol and *trans*-piceid in the culture medium were observed after the application of purine (Ap_3A and Gp_3G), pyrimidine (Up_3U and Up_4U) and purine-pyrimidine hybrid (Ap_3U) nucleotides. Moreover, the effect of Np_nN's on the transcript levels of genes encoding enzymes involved in the phenylpropanoid pathway was studied. The increase in stilbene accumulation, as a result of application of Ap_3A, Gp_3G, Up_3U and Up_4U was correlated with a significant rise in *STS1* gene encoding stilbene synthase and *C4H1* gene encoding cinnamate-4-hydroxylase expressions. The same nucleotides induced the expression of the *ABCG44* gene encoding the ABCG44 protein that transports *trans*-resveratrol outside the cell. Pyrimidine Np_nN's, i.e. Cp_3C, Cp_4C and the purine-pyrimidine hybrid Ap_4C, strongly inhibited stilbene biosynthesis, which was associated with a decrease in the expression of *STS1* and *ABCG44* genes while the induction of the expression of the *CCR2* gene encoding a cinnamoyl-CoA reductase controlling lignin biosynthesis was observed. Similarly to studies on the Np_nN' effect, it was shown that NH_2-pNs regulate the phenylpropanoid pathway in *V. vinifera* cv. Monastrell suspension cultured cells which also depends on the type of nucleoside. Significant differences in the level of

accumulation of *trans*-resveratrol and *trans*-piceid in the culture medium were observed after the application of both purine (NH_2-pA, NH_2-pG) and pyrimidine (NH_2-pU, NH_2-pC) NH_2-pNs. It was shown that the studied nucleotides modified the expression of genes controlling stilbene and lignin biosynthesis. The most significant differences in gene expression were observed when NH_2-pC was applied. Furthermore, this nucleotide induced a rapid increase of N-benzoylputrescine (BenPut) level.

Interactions between the signalling molecule and the receptor are the key element in signal perception. A well-known and widely described purine nucleotide plant receptor interacting with eATP, is LecRK-I.9 (L-type lectin receptor-like kinase) (P2K1/DORN1). Despite many indications that extracellular Np_nN's act as signalling molecules, the mechanisms of Np_nN's -induced signal perception and transduction had remained unknown. The research carried out as part of this dissertation demonstrated that P2K1/DORN1 is a critical element of the Ap_4A-induced signal transduction. Furthermore, a significant reduction of the lumen of the stomata was demonstrated when Ap_4A and Cp_4C were applied. However, this process mediated by Cp_4C, occurred independently from P2K1/DORN1. It was shown that reactive oxygen species (ROS), in particular superoxide anion radical ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2), are involved in mediating stomatal closure evoked by Ap_4A and Cp_4C. In addition, the induction of selected genes involved in signal transduction and plant stress response in leaves of *A. thaliana* wild-type, that were treated with ATP and Ap_4A was demonstrated. Such a response was not observed in the *dorn1-3*, *A. thaliana* knock-out mutant carrying a T-DNA insertion within the P2K1/DORN1 encoding gene. Induction of expression of these genes was not demonstrated in *A. thaliana* wild-type leaves under the CTP and Cp_4C treatment.

The most important achievement of the present work is the identification of the plant purinoreceptor P2K1/DORN1 as an important membrane protein involved in Ap_4A-induced signal perception. Furthermore, the role of elements participating in signal transduction leading to modification of the phenylpropanoid pathway in *V. vinifera* and regulation of stomatal apparatus movements in *A. thaliana* was established. In addition, P2K1/DORN1 was shown to have no perception function for the pyrimidine dinucleotide Cp_4C, which indicated that other proteins interacting with this nucleotide are present in plants.

1. Wprowadzenie

Nukleotydy to organiczne związki chemiczne występujące u wszystkich organizmów żywych. Stanowią podstawowy element strukturalny kwasów nukleinowych, odgrywają kluczowe role w wielu procesach metabolicznych oraz uczestniczą w procesie transdukcji sygnału jako cząsteczki sygnalne. Do grupy nukleotydów o ugruntowanej funkcji sygnalnej w komórkach roślinnych zaliczyć można: cykliczny 3′,5′-adenozynomonofosforan (cAMP), cykliczny 3′,5′ - guanozynomonofosforan (cGMP), dinukleotyd nikotynoamidoadeninowy (NAD^+), fosforan NAD^+ ($NADP^+$) i zewnątrzkomórkowy ATP (eATP) (Pietrowska-Borek, Dobrogojski i in., 2020a).

Grupą nukleotydów o mało poznanej funkcji u roślin są nukleotydy nietypowe, a wśród nich dinukleozydopolifosforany (Np_nN') i nukleozydo 5′-fosforamidy (NH_2-pN) będące przedmiotem badań niniejszej rozprawy doktorskiej. Np_nN' zbudowane są z dwóch nukleozydów, połączonych łańcuchem reszt fosforanowych, których liczba waha się od 2 do 7 (Pietrowska-Borek i in., 2020b). Pierwszym Np_nN' zidentyfikowanym w materiale biologicznym w zarodkach krewetek był diguanozynotetrafosforan (Gp_4G) (Finamore i Warner, 1963). Jednakże, najpowszechniej badanymi spośród Np_nN' są diadenozyno 5′,5‴-P^1,P^4-tetrafosforan (Ap_4A) i diadenozyno 5′,5‴-P^1,P^3-trifosforan (Ap_3A). Obecność Ap_4A wykazano po raz pierwszy w 1976 roku w komórkach ssaczych (Rapaport i Zamecnik, 1976). Inne Np_nN' zidentyfikowano w komórkach bakteryjnych, drożdżowych (Lee i in., 1983; Coste i in., 1987), zwierzęcych i ludzkich (Lüthje i Ogilvie, 1983). Ze względu na znaczący wzrost poziomu Np_nN' w komórkach poddawanych stresom, takim jak podwyższona temperatura, etanol czy kadm, molekuły te zakwalifikowano do grupy związków nazywanych „alarmonami” (Lee i in., 1983; Baltzinger i in., 1986; Coste i in., 1987; Pálfi i in., 1991). Istotnym elementem w poznaniu funkcji badanych nukleotydów jest identyfikacja ich efektorów oraz enzymów zarówno syntetyzujących jak i degradujących te molekuły. Elementy te szeroko omówiono w pracy przeglądowej, której jestem współautorem (**Publikacja 1**; Pietrowska-Borek, Dobrogojski i in., 2020a). Nieodłącznym elementem działania molekuły sygnalnej są enzymy kontrolujące jej poziom. Identyfikacja enzymów zaangażowanych w biosyntezę i degradację Np_nN' zarówno w komórkach prokariotycznych jak i eukariotycznych wzmocniła hipotezę o ich funkcji sygnalnej (Ferguson i in., 2020; **Publikacja 1;** Pietrowska-Borek, Dobrogojski i in., 2020a). U roślin dotychczas

zidentyfikowano trzy enzymy katalizujące reakcję syntezy Np_nN'. Są nimi ligaza 4-kumarylo-CoA (EC 6.2.1.12) z *Arabidopsis thaliana* (Pietrowska-Borek i in., 2003) oraz dwie syntetazy fenyloalanylo-tRNA (EC 6.1.1.20) i serylo-tRNA (EC 6.1.1.11) z *Lupinus luteus* (Jakubowski, 1983). Wśród enzymów degradujących Np_nN' wyróżniono substratowo specyficzne i niespecyficzne. Do enzymów specyficznie degradujących Np_nN' należą *asymetryczna* hydrolaza Np_nN' (EC 3.6.1.17), *symetryczna* hydrolaza Np_nN' (EC 3.6.1.41) oraz hydrolaza Np_3N' (EC 3.6.1.29) (Guranowski, 2000). U roślin aktywność *asymetrycznej* hydrolazy Np_nN' wykryto w nasionach *Lupinus luteus* (Jakubowski i Guranowski, 1983), *Helianthus annuus, Cucurbita pepo* (Guranowski, 1990), komórkach pomidora (Feussner i in., 1996), nasionach *Lupinus angustifolius* (Maksel i in., 1998) oraz w *Hordeum vulgare* (Churin i in., 1998). Do roślinnych, niespecyficznych enzymów katalizujących reakcje degradacji Np_nN' należą fosfodiesteraza I (EC 3.1.4.1) z *Lupinus luteus* (Jakubowski, 1983) i pirofosfataza nukleotydowa (EC 3.6.1.9) z bulwy ziemniaka (Bartkiewicz i in., 1984).

Spośród NH_2-pN najczęściej badanym jest adenozyno 5′-fosforamid (NH_2-pA). Do tej pory jego obecność wykazano w mieszaninie nukleotydów wyizolowanych z *Chlorella pyrenoidosa* (Fankhauser i in., 1981). Podobnie jak w przypadku Np_nN', identyfikacja enzymów katalizujących reakcję syntezy oraz degradacji NH_2-pA kontrolujących stężenie tego nukleotydu, zarówno w komórkach organizmów prokariotycznych jak i eukariotycznych, sugeruje jego powszechne występowanie (Fankhauser i in., 1981; Guranowski i in., 2008, 2010; Wojdyła-Mamoń i Guranowski, 2015). Reakcję syntezy NH_2-pA katalizuje adenylylotransferaza-adenylylo siarczano:amoniak (EC 2.7.7.51). Aktywność tego enzymu u roślin wykazano w *Hordeum vulgare, Spinacia oleracea* (Fankhauser i in., 1981) oraz *Lupinus luteus* (Wojdyła-Mamoń i Guranowski, 2015). U roślin rozkład NH_2-pA do AMP i NH_3 katalizowany jest przez hydrolazy (Guranowski i in., 2008, 2010, 2011) natomiast do ADP i NH_3 przez fosforylazę (Guranowski i in., 2011). Białkami posiadającymi zdolność zarówno syntezy jak i degradacji NH_2-pA są enzymy z rodziny białek Fhit (*ang. Fragile histidine triad*; Guranowski i in., 2008).

W odpowiedzi na niekorzystne czynniki środowiskowe w komórkach roślinnych ulega indukcji szereg mechanizmów obronnych mających na celu zwalczanie pojawiających się niepożądanych efektów. U roślin wyższych, jednym z mechanizmów uruchamianych w

stresie wywołanym działaniem zarówno biotycznych jak i abiotycznych czynników jest aktywacja szlaku fenylopropanoidowego dostarczającego metabolitów wtórnych takich jak flawonoidy, ligniny czy stilbeny. Pierwsza reakcja w wyniku której *L*-fenyloalanina zostaje przekształcona do kwasu *trans*-cynamonowego katalizowana jest przez amoniakoliazę fenyloaniny (PAL). Następnie, 4-hydroksylaza kwasu cynamonowego (C4H) katalizuje powstanie kwasu *p*-kumarowego z kwasu *trans*-cynamonowego. Kolejno, w wyniku aktywności ligazy 4-kumarylo-CoA (4CL) kwas *p*-kumarowy ulega przekształceniu do *p*-kumarylo-CoA. Na tym etapie szlak fenylopropanoidowy ulega rozgałęzieniu, a *p*-kumarylo-CoA stanowi substrat wyjściowy dla licznych przemian metabolicznych (Sharma i in., 2019). Wykazano, że nukleotydy nietypowe takie jak Ap_3A, Ap_4A i NH_2-pA mogą modyfikować szlak fenylopropanoidowy w roślinie modelowej *A. thaliana* (Pietrowska-Borek i in., 2011, 2015). Zaobserwowano, że egzogenny Ap_3A i Ap_4A indukowały ekspresję genów *PAL2* i *4CL*. Co więcej, Ap_3A i Ap_4A istotnie zwiększały aktywności enzymów kodowanych przez te geny (Pietrowska-Borek i in., 2011). Ponadto nukleotydy cykliczne (cAMP i cGMP), molekuły o dobrze poznanej funkcji sygnalnej, również wpływały na badany szlak (Pietrowska-Borek i Nuc, 2013). Podobny efekt zaobserwowano pod wpływem przepuszczalnych dla błon komórkowych i nie ulegających rozkładowi przez fosfodiesterazę analogów cyklicznych nukleotydów tj. 8-Br-cAMP i 8-Br-cGMP (Pietrowska-Borek i Nuc, 2013). Wykazano wzrost poziomu transkryptów genów *PAL2*, *4CL1* i genu *CHS*, kodującego syntazę chalkonową oraz aktywności enzymów kodowanych przez te geny (Pietrowska-Borek i Nuc, 2013). Stymulacja ekspresji genów *PAL*, *4CL* oraz genu *CCR2* kodującego reduktazę cynamylo-CoA, enzymu katalizującego reakcje na drodze syntezy lignin, została wykazana również pod wpływem egzogennego NH_2-pA. Jednocześnie zaobserwowano wzrost akumulacji lignin i kwasu salicylowego (Pietrowska-Borek i in., 2015). Grupą fenylopropanoidów wykazujących właściwości antyoksydacyjne są stilbeny a w śród nich *trans*-resweratrol (Sharma i in., 2019). Związek ten zaliczany jest do fitoaleksyn, związków syntetyzowanych w odpowiedzi na stres. Rośliną o dużej zawartości stilbenów, do których należy *trans*-resweratrol jest winorośl właściwa (*Vitis vinifera*). Aktywację szlaku fenylopropanoidowego wykazano również w komórkach kultury zawiesinowej *Vitis vinifera* cv. Monastrell traktowanych Ap_3A (Pietrowska-Borek i in., 2014). Indukcja ekspresji genów *PAL1*, *4CL1*, *C4H1* oraz genu *STS1*, kodującego syntazę stilbenową, w początkowych

punktach czasowych eksperymentu była skorelowana z istotnym wzrostem akumulacji w komórkach stilbenów, *trans*-resweratrolu i *trans*-piceidu. Pomimo identyfikacji enzymów odpowiedzialnych za syntezę i degradację Np_nN' oraz NH_2-pA, jak dotąd brak w literaturze danych potwierdzających występowanie tych nukleotydów w komórkach roślinnych. Jednakże modyfikacja szlaku fenylopropanoidowego u *A. thaliana* i *V. vinifera* pod wpływem Ap_3A i Ap_4A oraz NH_2-pA, sugeruje ich zaangażowanie w odpowiedź na działanie czynników stresowych u roślin.

Coraz więcej badań nad udziałem w odpowiedzi roślin na różne stresy środowiskowe dotyczą innego nukleotydu purynowego jakim jest egzogenny ATP (eATP). Dowiedziono, że nukleotyd ten bierze udział w regulacji wzrostu (Kim i in., 2006; Wu i in., 2007; Riewe i in., 2008; Clark i in., 2010; Tonón i in., 2010; Zhu i in., 2020) i rozwoju rośliny (Reichler i in., 2009; Wu i in., 2018). Ponadto eATP jest zaangażowany w odpowiedź rośliny na stres wywołany czynnikami biotycznymi (Chivasa i in., 2005; Chen i in., 2017; Tripathi i in., 2018; Goodman i in., 2022), i abiotycznymi (Thomas i in., 2000; Sun i in., 2012; Kim i in., 2009; Hou i in., 2018). Wyniki badań nad funkcją eATP u roślin opublikowane w ostatnich latach wskazują na udział tego nukleotydu w mechanizmie zamykania aparatów szparkowych w odpowiedzi na atak chorobotwórczych patogenów takich jak *Pseudomonas syringae* (Chen i in., 2017; Duong i in., 2022) oraz stres wywołany suszą (Wang i in., 2022). Dotychczas zidentyfikowano dwa roślinne błonowe receptory posiadające zdolność wiązania eATP. Białkami tymi są receptory P2K1/DORN1 oraz P2K2/DORN2 należące do błonowych receptorowych kinaz białkowych LecRKs (*ang. L-type lectin receptor kinases*) (Choi i in., 2014; Pham i in., 2020; Cho i in., 2023). Interakcja eATP z receptorem P2K1/DORN1 powoduje wzrost poziomu cząsteczek sygnałowych takich jak tlenek azotu (NO), reaktywnych form tlenu (RFT) oraz cytozolowego wapnia wolnego $[Ca^{2+}]_{cyt}$ (Clark i in., 2010; Tanaka i in., 2010; Chen i in., 2017; Wu i in., 2018). Po rozpoznaniu eATP następuje bezpośrednia fosforylacja błonowej oksydazy NADPH, RBOHD (*ang. Respiratory Burst Oxidase Homolog D*; Chen i in., 2017), której aktywność powoduje wzrost stężenia anionorodnika ponadtlenkowego ($^{\bullet}O_2^-$), który następnie przekształcany jest do nadtlenku wodoru (H_2O_2) (Song i in., 2006). Wykazano, że w zamykaniu aparatów szparkowych pod wpływem eATP, uczestniczą RFT (Chen i in., 2017). Wysokie stężenie RFT, $[Ca^{2+}]_{cyt}$ oraz NO zapoczątkowuje aktywację białkowych kinaz aktywowanych

mitogenami (MAP) (Smékalová i in., 2014; Jiang i in., 2022). Kinazy te tworzą u roślin rozbudowane ścieżki sygnałowe w formie kaskad, które aktywują przez fosforylację inne białka np. czynniki transkrypcyjne. Wykazano, że kinazami MAP zaangażowanymi w transdukcję sygnału w odpowiedzi na eATP są MAPK3 oraz MAPK6 (Choi i in., 2014). Finalnie, w wyniku aktywacji szlaku transdukcji sygnału wywołanego przez eATP u *A. thaliana* dochodzi do zamknięcia aparatów szparkowych oraz, jak wykazano dzięki analizie funkcjonalnej (GO, ang. *Gene Ontology term enrichment*), indukcji ekspresji genów indukowanych stresem oraz genów powiązanych z transdukcją sygnału (Choi i in., 2014). Pomimo wielu przesłanek wskazujących na funkcję sygnalną Np_nN' oraz NH_2-pA, roślinne mechanizmy percepcji i transdukcji sygnału wywołanych przez te molekuły pozostawały nieznane.

2. Hipotezy i cele badań

Przyjęto następujące hipotezy badawcze:

1. Szlak fenylopropanoidowy jest regulowany przez purynowe i pirymidynowe dinukleozydopolifosforany oraz nukleozydo 5′-fosforamidy w komórkach *V. vinifera* cv. Monastrell,
2. Zarówno Np_nN' purynowe (Ap_3A, Ap_4A) jak i pirymidynowe (Cp_3C, Cp_4C) indukują zamykanie aparatów szparkowych u *A. thaliana*,
3. Błonowa receptorowa kinaza białkowa LecRK-I.9 (P2K1/DORN1) jest niezbędna w transdukcji sygnału prowadzącego do zamknięcia aparatów szparkowych wywołanego przez Ap_4A i Cp_4C u *A. thaliana*.

Cele pracy doktorskiej:

Nadrzędnym celem pracy było określenie roli Np_nN' i NH_2-pN w transdukcji sygnału i regulacji szlaku fenylopropanoidowego u winorośli właściwej (*Vitis vinifera* L.) i rzodkiewnika pospolitego (*Arabidopsis thaliana* (L.) Heynh.).

Zasadniczy cel pracy realizowano poprzez następujące cele szczegółowe:

1. Analizę zmian ekspresji genów kodujących wybrane enzymy szlaku fenylopropanoidowego pod wpływem badanych Np_nN' (Ap_3A, Gp_3G, Up_3U, Up_4U, Cp_3C, Cp_4C, Ap_3U, Ap_3C, Ap_4C) i NH_2-pN (NH_2-pA, NH_2-pG, NH_2-pC, NH_2-pU) w zawiesinowej kulturze komórkowej *V. vinifera* cv. Monastrell,
2. Określenie zmian w akumulacji dwóch stilbenów: *trans*-resweratrolu i *trans*-piceidu w komórkach, a także w pożywce pod wpływem wybranych Np_nN' i NH_2-pN w zawiesinowej kulturze komórkowej *V. vinifera* cv. Monastrell,
3. Ocenę wpływu wybranych NH_2-pN na poziom lignin i fenyloamidów w komórkach a także w pożywce w zawiesinowej kulturze komórkowej *V. vinifera* cv. Monastrell,
4. Analizę funkcji Np_nN' purynowych (Ap_3A, Ap_4A) i pirymidynowych (Cp_3C, Cp_4C) w regulacji zamykania aparatów szparkowych u *A. thaliana*,

5. Określenie udziału błonowej receptorowej kinazy białkowej LecRK-I.9 (P2K1/DORN1) w procesie zamykania aparatów szparkowych wywołanym przez Ap_4A i Cp_4C u *A. thaliana,*
6. Określenie udziału RFT ($^{\bullet}O_2^-$ i H_2O_2) jako molekuł pośredniczących w transdukcji sygnału wywołanego przez Ap_4A i Cp_4C prowadzącego do zamykania aparatów szparkowych u *A. thaliana,*
7. Analiza zamian wywołanych ATP, CTP, Ap_4A i Cp_4C w ekspresji genów kodujących kluczowe białka zaangażowane w transdukcję sygnału u *A. thaliana*.

Założono, że wyjaśnienie roli Np_nN' i NH_2-pN w transdukcji sygnału i regulacji szlaku fenylopropanoidowego u roślin pozwoli na identyfikację białek zarówno receptorowych, ścieżek transdukcji sygnału jak i białek enzymatycznych kluczowego szlaku biorącego udział w reakcji roślin na stresy.

3. Omówienie uzyskanych wyników

Kluczowym aspektem w procesie wyjaśniania funkcji potencjalnej molekuły sygnalnej jest identyfikacja efektora oraz elementów szlaków transdukcji sygnału prowadzących do określonej reakcji organizmu. Ostatnie badania na poziomie molekularnym, biochemicznym i fizjologicznym dowodzą o roli sygnalnej nukleotydów takich jak eATP, NH_2-pA i Np_nN' w licznych procesach zachodzących w komórkach roślinnych. Szczegółowy opis i dyskusję nad owym zagadnieniem zawiera praca przeglądowa, której jestem współautorem (**Publikacja 1)**. W pracy tej zaproponowano hipotetyczny szlak transdukcji sygnału wywołanego przez Np_nN' i NH_2-pA u roślin (**Publikacja 1, Fig. 5**), który prowadzi do odpowiedzi roślin na Np_nN' i NH_2-pA w postaci modyfikacji ekspresji genów szlaku fenylopropanoidowego i syntezy kwasu salicylowego u *Arabidopsis thaliana* (Pietrowska-Borek i in., 2011, 2014, 2015). Hipoteza zakładała obecność błonowych receptorów i/lub kanałów transportujących Np_nN' i NH_2-pA do wnętrza komórki roślinnej, zaangażowanie wtórnych przekaźników sygnału i kaskad kinaz powszechnie biorących udział w transdukcji sygnału w komórce.

W celu odpowiedzi na pytanie o uniwersalny charakter uruchamiania mechanizmów obronnych jakim jest indukcja szlaku fenylopropanoidowego przez Np_nN' i NH_2-pN u roślin, przeanalizowano wpływ egzogennych Np_nN' (Ap_3A, Gp_3G, Up_3U, Up_4U, Cp_3C, Cp_4C, Ap_3U, Ap_3C, Ap_4C) i NH_2-pN (NH_2-pA, NH_2-pG, NH_2-pC, NH_2-pU) w zawiesinowej kulturze komórkowej *Vitis vinifera* cv. Monastrell. Ze względu na naturalnie wysoki poziom stilbenów gatunek ten okazał się być dobrym modelem do zaplanowanych eksperymentów. Wzrost akumulacji *trans*-resweratrolu i *trans*-piceidu w pożywkach w porównaniu do kultur kontrolnych zaobserwowano pod wpływem 5 μM nukleotydów purynowych (Ap_3A i Gp_3G), pirymidynowych (Up_3U i Up_4U) oraz puryno-pirymidynowej hybrydy (Ap_3U) do 24 godzin od potraktowania zawiesinowej kultury komórkowej (**Publikacja 2, Fig. 2)**. Analizie poddano również ekspresję genów kodujących wybrane enzymy szlaku fenylopropanoidowego. Po 24 godzinach trwania eksperymentu odnotowano wzrost akumulacji stilbenów w pożywce pod wpływem Ap_3A, Gp_3G, Up_3U i Up_4U co było skorelowane z istotną indukcją ekspresji genu *C4H1* oraz genu *STS1* kodującego enzym katalizujący syntezę *trans*-resweratrolu (**Publikacja 2, Fig. 4)**. Na uwagę zasługuje również

istotny, nawet 5-krotny wzrost ekspresji pod wpływem Ap_3A, Gp_3G, Up_3U i Up_4U genu *ABCG44* kodującego białko ABCG44 transportujące *trans*-resweratrol na zewnątrz komórki (**Publikacja 2, Fig. 5)**. Natomiast inne badane pirymidynowe nukleotydy takie jak Cp_3C, Cp_4C i purynowo-pirymidynowy Ap_4C silnie hamowały ekspresję genu *STS1* (**Publikacja 2, Fig. 4)** i genu *ABCG44* (**Publikacja 2, Fig. 5)** co odzwierciedlało również akumulację stilbenów w pożywce i w komórkach (**Publikacja 2, Fig. 2)**. Co ciekawe, po 72 godzinach od potraktowania zawiesinowej kultury komórkowej *V. vinifera* cv. Monastrell Cp_3C i Cp_4C, zaobserwowano ok. 6-krotny wzrost ekspresji genu *PAL1*, 5-krotny wzrost ekspresji genu *4CL1* oraz 8-krotny wzrost ekspresji genu *CCR2*. Potraktowanie komórek Ap_4C również spowodowało ok. 8-krotny wzrost ekspresji genu *CCR2* po 72 godzinach (**Publikacja 2, Fig. 4)**. Uzyskane wyniki wskazują na odmienny mechanizm modyfikacji szlaku fenylopropanoidowego przez nukleotydy zawierające różne purynowe lub pirymidynowe nukleozydy. Dinukleotydy cytydynowe indukowały szlak syntezy lignin, jednego z odgałęzień szlaku syntezy fenylopropanoidów. Dowiedziono również, że produkty ewentualnego rozpadu tych nukleotydów takie jak AMP, GMP, UMP czy CMP nie wpływały na nagromadzanie stilbenów w pożywkach (**Publikacja 2, Fig. 3)**. W związku z powyższymi obserwacjami kolejną hipotezą była hipoteza mówiąca o udziale w modyfikacji aktywności szlaku fenylopropanoidowego purynowych i pirymidynowych NH_2-pN w zawiesinowej kulturze komórkowej *V. vinifera* cv. Monastrell. Uzyskane wyniki potwierdziły jedną z postawionych hipotez dotyczącą regulacji szlaku fenylopropanoidowego przez purynowe i pirymidynowe Np_nN' i NH_2-pN. Odnotowano istotne różnice akumulacji dwóch stilbenów: *trans*-resweratrolu oraz *trans*-piceidu w komórkach oraz pożywce, zarówno pod wpływem NH_2-pN purynowych (NH_2-pA, NH_2-pG) jak i pirymidynowych (NH_2-pU, NH_2-pC). Różnice w poziomie stilbenów w komórkach oraz pożywce zależały również od czasu traktowania komórek poszczególnymi nukleotydami. Największe zmiany w poziomie *trans*-resweratrolu w pożywce zaobserwowano pod wpływem NH_2-pG, NH_2-pU i NH_2-pC w pierwszym badanym punkcie czasowym, tj. po 6 godzinach od traktowania oraz po 12 godzinach tylko pod wpływem NH_2-pU i NH_2-pC (**Publikacja 3, Fig. 5b)**. Akumulacja *trans*-resweratrolu w pożywce po 24 i 48 godzinach była istotnie indukowana przez NH_2-pA (**Publikacja 3, Fig. 5b)**. Niezależnie od typu NH_2-pN poziom *trans*-resweratrolu w pożywce po 72 godzinach był niski i zbliżony do poziomu w próbie kontrolnej (**Publikacja 3, Fig.**

5b). Istotne statystycznie zmiany w poziomie *trans*-piceidu w pożywce zaobserwowano tylko pod wpływem NH_2-pA po 24 godzinach od traktowania zawiesinowej kultury komórkowej (**Publikacja 3, Fig. 5c)**. Istotną różnicę w akumulacji stilbenów w komórkach zaobserwowano dopiero po 24 godzinach traktowania. Około 1,5-krotny wzrost poziomu *trans*-resweratrolu w komórkach był spowodowany przez NH_2-pA oraz NH_2-pU, podczas gdy pod wpływem NH_2-pC odnotowano ok. 7-krotny spadek w porównaniu do kontroli (**Publikacja 3, Fig. 4b)**. Nie wykazano zmian zawartości *trans*-resweratrolu pod wpływem NH_2-pG po 24 godzinach w porównaniu do próby kontrolnej (**Publikacja 3, Fig. 4b)**. Co ciekawe, po 48 godzinach poziom *trans*-resweratrolu w komórkach pod wpływem NH_2-pA i NH_2-pC uległ ok. 7-krotnej redukcji, podczas gdy NH_2-pG i NH_2-pU nie powodowały istotnych zmian (**Publikacja 3, Fig. 4b)**. W przypadku akumulacji *trans*-piceidu w komórkach wykazano istotny, bo ok. 13-krotny spadek jego akumulacji pod wpływem NH_2-pG po 24 godzinach traktowania, podczas gdy nie zaobserwowano istotnych zmian pod wpływem pozostałych NH_2-pN (**Publikacja 3, Fig. 4c)**. Natomiast, po 48 godzinach zaobserwowano ok. 2-krotny wzrost poziomu *trans*-piceidu w komórkach pod wpływem NH_2-pA i NH_2-pC, podczas gdy nukleotydy NH_2-pG i NH_2-pU nie powodowały istotnych zmian (**Publikacja 3, Fig. 4c)**. Niezależnie od typu NH_2-pN poziom *trans*-resweratrolu i *trans*-piceidu w komórkach po 72 godzinach był niski i zbliżony do poziomu w próbie kontrolnej (**Publikacja 3, Fig. 4)**. Ponadto odnotowano, że ekspresja genów kontrolujących biosyntezę stilbenów oraz lignin była modyfikowana przez badane NH_2-pN. Największą indukcję ekspresji genów kodujących enzymy szlaku fenylopropanoidowego wykazano po 72 godzinach od potraktowania 5 μM NH_2-pN zawiesinowej kultury komórkowej: ok. 8-krotny genu *PAL1* pod wpływem NH_2-pC, a ok. 4-krotny pod wpływem NH_2-pG i NH_2-pU (**Publikacja 3, Fig. 3a)**. Podobne zależności zaobserwowano w przypadku ekspresji genów *4CL1* i *STS1*. Uzyskane dane wykazały, że gen *4CL1* ulegał ok. 10-krotnej indukcji pod wpływem NH_2-pC oraz ok. 3-krotnej pod wpływem NH_2-pG i NH_2-pU (**Publikacja 3, Fig. 3c)**. Około 13-krotną indukcję ekspresji genu *STS1* zaobserwowano pod wpływem NH_2-pC oraz ok. 5-krotną pod wpływem NH_2-pG i NH_2-pU (**Publikacja 3, Fig. 4a)**. Ponadto po 72 godzinach traktowania nukleotydami zawiesinowej kultury komórkowej zaobserwowano ok. 2,5-krotny wzrost ekspresji genu *C4H1* pod wpływem NH_2-pC, podczas gdy ekspresja tego genu uległa obniżeniu pod wpływem pozostałych NH_2-pN (**Publikacja 3, Fig. 3b)**. Na

uwagę zasługuje również podobny, bo ok. 3-krotny wzrost ekspresji genu *4CL1* pod wpływem NH_2-pA (**Publikacja 3, Fig. 3b)**. Ponadto zmiany w poziomie związku N-benzoiloputrescyny (BenPut), jednego z fenyloamidów będących pochodnymi fenylopropanoidów i poliamin, wykazano pod wpływem NH_2-pA, NH_2-pG i NH_2-pC po 24-godzinnach trwania eksperymentu (**Publikacja 3, Fig. 9)**. Z kolei, w pierwszym punkcie czasowym, tj. po 6 godzinach traktowania zawiesinowej kultury komórkowej badanymi nukleotydami, fenyloamid BenPut był wykrywalny na poziomie ok. 0,35 $\mu g\ g^{-1}$ suchej masy (DW) w komórkach *V. vinifera* cv. Monastrell tylko pod wpływem NH_2-pC. Niezależnie od typu NH_2-pN poziom BenPut w komórkach po 48 i 72 godzinach był niski i zbliżony do poziomu w próbie kontrolnej (**Publikacja 3, Fig. 9)**. Co istotne, badane nukleotydy nie wpływały na żywotność komórek (**Publikacja 3, Fig. 6)**, poziom lignin (**Publikacja 3, Fig. 8c)** oraz zmiany w suchej masie komórek (**Publikacja 3, Fig. 7)** w trakcie trwania eksperymentu. Uzyskane wyniki badań nad udziałem Np_nN' i NH_2-pN w kontroli aktywności szlaku fenylopropanoidowego u *V. vinifera* cv. Monastrell (**Publikacja 2 i 3)** oraz na podstawie danych literaturowych wskazujących na ich udział w takim procesie u rośliny modelowej *A. thaliana* (Pietrowska-Borek i in., 2011, 2015) dowodzą, że są to molekuły sygnalne.

Istotnym elementem percepcji sygnału są oddziaływania pomiędzy molekułą sygnalną a receptorem. Poznanym i dobrze opisanym u roślin receptorem nukleotydu purynowego, eATP, jest P2K1/DORN1. Dlatego też kolejną hipotezą jaką poddano weryfikacji w niniejszej pracy doktorskiej jest hipoteza mówiąca o tym, że P2K1/DORN1 może być również receptorem dla purynowych dinukleozydopolifosforanów i brać udział w zamykaniu aparatów szparkowych. Rozpoznawanie eATP przez receptor P2K1/DORN1, a następnie bezpośrednia fosforylacja RBOHD prowadzi do wzrostu RFT, a w konsekwencji zamknięcia aparatów szparkowych (Choi i in., 2014; Chen i in., 2017). Ruchy aparatów szparkowych stanowią modelowy system umożliwiający badania nad transdukcją sygnałów komórkowych oraz śledzenie reakcji rośliny na czynnik stresowy (Jia i Zhang, 2008; Kollist i in., 2014). Podobnie jak w pracach opisujących P2K1/DORN1 jako receptor dla eATP w pierwszych badaniach skupiono się na obserwacji ruchów aparatów szparkowych pod wpływem Ap_4A. Bazując na wynikach badań nad efektem Cp_nC w modyfikacji szlaku fenylopropanoidowego sprawdzono również wpływ nukleotydów pirymidynowych na ruchy

aparatów szparkowych. Spośród nukleotydów purynowych przetestowano Ap_3A, Ap_4A, ADP oraz ATP natomiast pirymidynowych Cp_3C, Cp_4C, CDP oraz CTP. Wykazano, że egzogenne Ap_4A i Cp_4C indukują zamknięcie aparatów szparkowych (**Publikacja 4, Fig. 1)**. Obserwacji tej dokonano po dwugodzinnej inkubacji epidermy liści pobranych z czterotygodniowych roślin *A. thaliana* w roztworze zawierającym 2 mM badane nukleotydy. Podobnego efektu nie zaobserwowano pod wpływem purynowego nukleotydu Ap_3A oraz pirymidynowych CDP, CTP i Cp_3C (**Publikacja 4, Fig. 1)**. Jako kontrolę pozytywną, stymulującą zamykanie aparatów szparkowych zastosowano 2 mM nukleotydy purynowe ADP i ATP oraz 10 μM kwas abscysynowy (ABA) (Choi i in., 2014; Chen i in., 2017). W związku z występowaniem u roślin enzymów degradujących dinukleozydopolifosforany zbadano skład roztworów nukleotydów, w których inkubowano epidermę, pod kątem ich rozpadu do mononukleotydów. Co ciekawe, nie zaobserwowano rozpadu Ap_4A oraz Cp_4C do mononukleotydów, które mogłyby wywoływać zamykanie aparatów szparkowych (**Publikacja 4, Fig. S1)**. Ponadto dowiedziono, iż podobnie do eATP, Ap_4A nie wywołuje zmian w rozwartości aparatów szparkowych w mutancie *dorn1-3* typu knock-out posiadającym insercje T-DNA w obrębie genu kodującego P2K1/DORN1. Tego efektu nie zaobserwowano również pod wpływem Cp_4C (**Publikacja 4, Fig. 2)**. Wyniki te potwierdzają jedną z postawionych hipotez w tej pracy doktorskiej mówiącej o tym, że purynoreceptor P2K1/DORN1 może być również receptorem dla Ap_4A. Ponadto dowiedziono tutaj, że P2K1/DORN1 nie jest zaangażowany w zamykanie aparatów szparkowych wywołane przez pirymidynowy nukleotyd Cp_4C. Jedną z charakterystycznych odpowiedzi roślin na bodźce stresowe jest synteza RFT w przestrzeni apoplastycznej (Macho i Zipfel, 2014; Mittler i in., 2022). Aktywowane są oksydazy NADPH, w tym RBOHD, bezpośrednio przyczyniające się do wzrostu poziomu anionorodnika ponadtlenkowego ($^{\bullet}O_2^-$), który następnie przekształcany jest do H_2O_2 (Song i in., 2006). W celu odpowiedzi na pytanie dotyczące zaangażowania RFT w mechanizmie transdukcji sygnału wywołanego przez badane nukleotydy zastosowano dwie metody. Pierwsza z nich zakładała wykazanie akumulacji $^{\bullet}O_2^-$ i H_2O_2 w liściach poddanych dwugodzinnej inkubacji w 2 mM roztworze nukleotydów purynowych (ATP, Ap_4A) i pirymidynowych (CTP, Cp_4C). Wykorzystując barwienie chlorkiem błękitu tetrazolowego, wykazano zwiększoną akumulację $^{\bullet}O_2^-$ w liściach *A. thaliana* typu dzikiego traktowanych CTP, Ap_4A i Cp_4C w porównaniu do kontroli. Indukcję akumulacji $^{\bullet}O_2^-$ w

liściach mutanta *dorn1-3* zaobserwowano tylko w przypadku traktowania liści nukleotydem Cp_4C **(Publikacja 4, Fig. 3a)**. Następnie, wykorzystując tetrahydrochlorek 3,3′-diaminobenzydyny udokumentowano zwiększoną zawartość H_2O_2 pod wpływem ATP, Ap_4A i Cp_4C w liściach *A. thaliana* typu dzikiego w porównaniu do kontroli. Natomiast w liściach mutanta *dorn1-3* nie wykryto H_2O_2 pod wpływem ATP i Ap_4A. Akumulacja H_2O_2 pod wpływem CTP była wykrywalna na niskim i podobnym poziomie w liściach pochodzących z obu typów roślin **(Publikacja 4, Fig. 3b)**. Druga metoda zakładała pomiar rozwartości aparatów szparkowych po dwugodzinnej inkubacji epidermy liści pobranych z czterotygodniowych roślin *A. thaliana* w 2 mM roztworze nukleotydów purynowych (ATP, Ap_4A) i pirymidynowych (CTP, Cp_4C) w obecności dwóch enzymów antyoksydacyjnych: dysmutazy ponadtlenkowej (SOD) i katalazy (CAT), usuwających RFT w postaci $^{\bullet}O_2^-$ i H_2O_2. Zastosowanie obu enzymów wyeliminowało efekt zamykania aparatów szparkowych przez ATP, Ap_4A i Cp_4C **(Publikacja 4, Fig 4)**. Wyniki te wskazują na zaangażowanie badanych RFT jako molekuł sygnalnych pośredniczących w procesie zamykania aparatów szparkowych przez Ap_4A i Cp_4C.

Jak wykazała analiza funkcjonalna (GO ang. *Gene Ontology term enrichment*), efektem zaobserwowanym pod wpływem działania eATP była również modyfikacja ekspresji genów (Choi i in., 2014). Spośród 574 modulowanych genów pod wpływem eATP, indukcji ekspresji podlegały geny kodujące białka związane z odpowiedzią obronną oraz transdukcją sygnału (Choi i in., 2014). W związku z tym przeprowadzono analizę ekspresji wybranych genów biorących udział w transdukcji sygnału i reakcję roślin na stres u *A. thaliana* zarówno typu dzikiego (Col-0) jak i mutancie *dorn1*-3 pod wpływem badanych nukleotydów purynowych (ATP, Ap_4A) i pirymidynowych (CTP, Cp_4C). Skupiono się na badaniach modyfikacji ekspresji genów kodujących kinazy białkowe SnRK (*SnRK1.1*, *SnRK1.2*, *SnRK2.2*, *SnRK2.3* oraz *SnRK2*.6) (**Publikacja 4, Fig. 5c)**, które powiązane są bezpośrednio z kontrolą poziomu ABA w komórce roślinnej (Soon i in., 2012). U roślin, szczególną rolę w regulacji odpowiedzi na działanie czynników stresowych pełnią kinazy MAP (Boudsocq i in., 2015). Wykazano indukcję ekspresji genu kodującego MAPK6 zarówno pod wpływem ATP jak i Ap_4A (**Publikacja 4, Fig. 5d)**. Wiadomo, że kinazy SnRK grupy 1 (SnRK1), kinazy SnRK grupy 2 (SnRK2) i MAPK oddziałują z czynnikami transkrypcyjnymi (Skalak i in., 2021). Powszechnie znane jest zaangażowanie czynników

transkrypcyjnych ZAT6 i ZAT12 zawierających typowe dla roślin domeny palców cynkowych typu Cys2/His2 oraz domenę EAR (ang. *ERF-associated amphiphilic repression*), w odpowiedzi roślin na kadm i inne czynniki abiotyczne (Opdenakker i in., 2012; Shi i in., 2014; Chen i in., 2016; Dang i in., 2022). Odnotowano, że zarówno ATP i Ap_4A wpływały również na wzrost poziomu transkryptów genów *ZAT6* i *ZAT12* (**Publikacja 4, Fig. 5e)**. Za syntezę RFT w komórkach roślinnych odpowiedzialne są m. in. błonowe oksydazy RBOH (Yoshioka i in., 2016). Zaobserwowano, że pod wpływem Ap_4A silnej indukcji ekspresji ulegał gen *RBOHF*, podczas gdy ten sam nukleotyd wywoływał niewielką zmianę w poziomie ekspresji genu *RBOHD*. Z kolei, pod wpływem ATP silnej indukcji ekspresji ulegał gen *RBOHD*, podczas gdy ten sam nukleotyd wywoływał niewielką zmianę w poziomie ekspresji genu *RBOHF* (**Publikacja 4, Fig. 5a)**. Wykazano, że pod wpływem ATP i Ap_4A poziom transkryptów wyżej wymienionych genów był istotnie zmniejszony w mutancie *dorn1-3* w porównaniu do rośliny typu dzikiego (**Publikacja 4, Fig. 5)**. Białkami niezbędnymi do prawidłowego wzrostu, rozwoju oraz odpowiedzi na czynniki stresowe u roślin są kanały bramkowane cyklicznymi nukleotydami (CNGC). Wykazano wzrost ekspresji genu *CNGC2* pod wpływem Ap_4A w roślinie typu dzikiego, podczas gdy ekspresja tego genu ulegała obniżeniu w mutancie *dorn1-3*. Egzogenny ATP istotnie zmniejszył poziom transkryptów tego genu w obu typach roślin (**Publikacja 4, Fig. 5b)**. Spośród genów analizowanych jako potencjalne geny modyfikowane przez badane nukleotydy purynowe, analizie poddano geny kodujące kinazy SnRK w podpowiedzi na Cp_4C. Nukleotyd pirymidynowy Cp_4C nie modyfikował poziomu ekspresji genów *SnRK1.1*, *SnRK1.2*, *SnRK2.2*, *SnRK2.3*, *SnRK2.6* **(Publikacja 4, Fig. S2)**.

Pomimo wielu przesłanek wskazujących na działanie Np_nN' oraz NH_2-pA jako molekuł sygnalnych, w literaturze brakowało zidentyfikowanych molekularnych elementów zaangażowanych w percepcję oraz transdukcję sygnału wywołanego przez te nukleotydy. W wyniku analizy dostępnych danych literaturowych w 2020 roku zaproponowaliśmy oryginalny model transdukcji sygnału wywołanego przez Np_nN' oraz NH_2-pA **(Publikacja 1, Fig. 5)**. Model zakładał, że nukleotydy mogą być zewnątrzkomórkowymi cząsteczkami sygnałowymi i oddziaływać z komórkami roślinnymi poprzez nieznane wówczas receptory błony plazmatycznej lub mogą być transportowane do wnętrza komórki. Ponadto wiadomo, że ich poziom w komórce roślinnej jest regulowany przez enzymy syntetyzujące i

degradujące (**Publikacja 1**; Pietrowska-Borek, Dobrogojski i in., 2020a). Zakładano, że zarówno zewnątrzkomórkowe, jak i wewnątrzkomórkowe badane nukleotydy nietypowe mogą wpływać na biosyntezę wtórnych przekaźników sygnału oraz hormonów. Hipotetyczny model transdukcji sygnału prowadzący do obserwowanych zmian pod wpływem Np_nN' oraz NH_2-pA w postaci modyfikacji szlaku fenylopropanoidowego zakładał również zaangażowanie kaskady MAP kinaz, które biorą udział w regulacji wzrostu i rozwój roślin, odpowiedzi na fitohormony, regulację cyklu komórkowego oraz odpowiedzi na stresy biotyczne i abiotyczne. Funkcją MAP kinaz jest fosforylacja innych białek np. czynników transkrypcyjnych, wpływając tym samym na poziom transkryptów genów regulowanych przez owe czynniki transkrypcyjne. Rezultatem przeprowadzonych badań w ramach niniejszej rozprawy doktorskiej jest potwierdzenie ówcześnie postawionych hipotez dotyczących funkcji sygnalnej Ap_4A i Cp_4C oraz molekularnych elementów transdukcji sygnału wywołanego przez te nukleotydy **(Publikacja 4)**. Wykazano, że molekułami sygnalnymi pośredniczącymi w zamykaniu aparatów szparkowych przez Ap_4A i Cp_4C są RFT ($^{\bullet}O_2^-$ i H_2O_2). Ponadto zidentyfikowano pierwszy roślinny receptor, P2K1/DORN1, zaangażowany w proces zamykania aparatów szparkowych wywołany przez Ap_4A u *A. thaliana*. Udowodniono również, że P2K1/DORN1 nie uczestniczy w percepcji sygnału indukowanego przez Cp_4C. Wykazano również, że zamykaniu aparatów szparkowych pod wpływem Ap_4A towarzyszy istotna indukcja ekspresji kluczowych genów zaangażowanych w procesy obronne przed niekorzystnymi czynnikami środowiskowymi. Podobnych obserwacji dokonano dla ATP. Interesującym jest fakt, że nukleotyd pirymidynowy Cp_4C nie wpływał na indukcję ekspresji badanych genów. Wyniki przeprowadzonych badań wchodzące w skład publikacji stanowiących cykl prac w niniejszej rozprawie doktorskiej dostarczają nowych danych wyjaśniających funkcje sygnalne dinukleozydopolifosforanów i nukleozydo 5′-fosforamidów oraz wskazują elementy transdukcji sygnału przez nie wywołanego. Po raz pierwszy w literaturze zidentyfikowano białko błonowe, purynoreceptor, który jest kluczowym elementem percepcji sygnału dla Ap_4A. Uzyskane wyniki pozwoliły na uzupełnienie wielu hipotetycznych punktów w modelu transdukcji sygnału zaproponowanego w **Publikacji 1** (Pietrowska-Borek, Dobrogojski i in., 2020a). Uzupełniony model przedstawiono w **Publikacji 4**, **Fig. 6** (Dobrogojski i in., 2023). Dalsze badania mające na celu identyfikację efektorów oraz elementów szlaków transdukcji sygnału

prowadzących do wykazanych w niniejsze pracy reakcji roślin, będą stanowiły kluczowy aspekt w procesie wyjaśniania funkcji sygnalnej pozostałych Np_nN' i NH_2-pN.

4. Podsumowanie i wnioski

Badania nad rolą Np_nN' i NH_2-pN w transdukcji sygnału i regulacji szlaku fenylopropanoidowego u winorośli właściwej (*Vitis vinifera* L.) i rzodkiewnika pospolitego (*Arabidopsis thaliana* (L.) Heynh.) doprowadziły do sformułowania następujących konkluzji:

1. Potwierdzono, że egzogenne Np_nN' regulują szlak fenylopropanoidowy w zawiesinowej kulturze komórkowej *V. vinifera* cv. Monastrell zależnie od typu nukleozydu (**Publikacja 2**):
    - wzrost akumulacji stilbenów w pożywce pod wpływem Ap_3A, Gp_3G, Up_3U i Up_4U był skorelowany z indukcją ekspresji genu *C4H1*, *STS1* oraz genu *ABCG44*,
    - pirymidynowe Np_nN' tj. Cp_3C, Cp_4C oraz puryno-pirymidynowy nukleotyd Ap_4C, silnie hamowały biosyntezę stilbenów co związane było z obniżeniem ekspresji genów *PAL1*, *4CL1* i *STS1* przy jednoczesnej indukcji ekspresji genu *CCR2* kodującego reduktazę cynamylo-CoA kontrolującą biosyntezę lignin.
2. Potwierdzono, że NH_2-pN regulują szlak fenylopropanoidowy zawiesinowej kultury komórkowej *V. vinifera* cv. Monastrell zależnie od typu nukleozydu (**Publikacja 3**):
    - zarówno purynowe (NH_2-pA, NH_2-pG) jak i pirymidynowe (NH_2-pU, NH_2-pC) NH_2-pN modyfikowały ekspresję genów kontrolujących biosyntezę stilbenów i lignin,
    - spośród testowanych NH_2-pN, NH_2-pC wykazał największą indukcję ekspresji genów kodujących enzymy szlaku fenylopropanoidowego, takich jak *PAL1*, *C4H1*, *4CL1* i *STS1*. Ponadto nukleotyd ten indukował szybki wzrost poziomu N-benzoilputrescyny (BenPut),
    - nie wykazano zamian zawartości lignin w komórkach *V. vinifera* w porównaniu do kontroli pod wpływem badanych NH_2-pN,
    - nie zaobserwowano negatywnego wpływu badanych NH_2-pN na żywotność komórek *V. vinifera.*

3. Wykazano, że błonowa receptorowa kinaza białkowa LecRK-I.9 (P2K1/DORN1) jest kluczowym elementem w percepcji i transdukcji sygnału wywołanego przez Ap_4A (**Publikacja 4**):
    - egzogenne Ap_4A i Cp_4C istotnie zmniejszały stopień rozwarcia aparatów szparkowych u *A. thaliana*, podczas gdy Ap_3A i Cp_3C nie wywołały podobnego efektu,
    - receptor P2K1/DORN1 nie uczestniczy w percepcji i transdukcji sygnału prowadzącego do zamknięcia aparatów szparkowych wywołanego przez Cp_4C,
    - molekułami sygnalnymi pośredniczącymi w zamknięciu aparatów szparkowych przez Ap_4A i Cp_4C są RFT ($^{\bullet}O_2^-$ i H_2O_2),
    - zamykaniu aparatów szparkowych pod wpływem Ap_4A towarzyszy istotna indukcja ekspresji genów *SnRK1.1*, *SnRK1.2*, *SnRK2.2*, *SnRK2.3*, *SnRK2.6*, *RBOHD*, *RBOHF*, *MAPK6*, *ZAT6*, *ZAT12*. Ekspresja wyżej wymienionych genów uległa obniżeniu w mutancie *dorn1-3* w porównaniu do roślin typu dzikiego. Podobne obserwacje odnotowano pod wpływem ATP,
    - zmian w ekspresji genów *SnRK1.1*, *SnRK1.2*, *SnRK2.2*, *SnRK2.3*, *SnRK2.6*, nie odnotowano pod wpływem CTP oraz Cp_4C.

Kluczowym osiągnięciem niniejszej pracy jest wskazanie roślinnego purynoreceptora P2K1/DORN1 jako istotnego białka błonowego w percepcji sygnału wywołanego przez Ap_4A. Jest to pierwsze opisane roślinne białko receptorowe mogące oddziaływać z Ap_4A. Ponadto określono rolę elementów mogących uczestniczyć w transdukcji sygnału prowadzącego do modyfikacji szlaku fenylopropanoidowego u *V. vinifera* i regulacji ruchów aparatów szparkowych u *A. thaliana*. Co więcej, wykazano, że P2K1/DORN1 nie pełni takiej funkcji w przypadku dinukleotydu pirymidynowego Cp_4C, co świadczyć może o występowaniu u roślin innych białek oddziałujących z tymi nukleotydami.

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Oświadczenia współautorów

Uniwersytet Przyrodniczy w Poznaniu
ul. Dojazd 11
60-632 Poznań
tel. +48 61 848 72 02
e-mail: kbib@up.poznan.pl

WYDZIAŁ ROLNICTWA,
OGRODNICTWA
I BIOINŻYNIERII

Katedra Biochemii i Biotechnologii

prof. UPP dr hab. Małgorzata Pietrowska-Borek
Katedra Biochemii i Biotechnologii
Uniwersytet Przyrodniczy w Poznaniu
ul. Dojazd 11
60-632 Poznań
malgorzata.pietrowska-borek@up.poznan.pl

Poznań, 26.01.2024 r.

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr. Jędrzejem Dobrogojskim

stanowiących podstawy rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr. Jędrzeja Dobrogojskiego poniższych publikacji do Jego rozprawy doktorskiej oświadczam, że mój udział w przygotowaniu poszczególnych polegał na:

1. **Pietrowska-Borek M.**, Dobrogojski J., Sobieszczuk-Nowicka E., Borek S. 2020. New insight into plant signaling: extracellular ATP and uncommon nucleotides. *Cells*, 9, 345

   - współtworzeniu koncepcji pracy, wiodącym udziale w przygotowaniu pierwszej wersji manuskryptu, współprojektowaniu wszystkich figur, udziale w przygotowaniu wersji manuskryptu po uwagach i komentarzach recenzentów, w tym udziale w przygotowaniu odpowiedzi na recenzje.

2. **Pietrowska-Borek M.**, Wojdyła-Mamoń A., Dobrogojski J., Młynarska-Cieślak A., Baranowski M.R., Dąbrowski J.M., Kowalska J., Jemielity J., Borek S., Pedreño M.A., Guranowski A. 2020. Purine and pyrimidine dinucleoside polyphosphates differentially affect the phenylpropanoid pathway in *Vitis vinifera* L. cv. Monastrell suspension cultured cells. *Plant Physiology and Biochemistry*, 147, 125-132

   - opracowaniu koncepcji i planowaniu eksperymentów, realizacji części badań, opiece merytorycznej podczas wykonywania badań, analizie i interpretacji wyników, wiodącym udziale w przygotowaniu pierwszej wersji manuskryptu, udziale w przygotowaniu wersji manuskryptu po uwagach i komentarzach recenzentów, w tym udziale w przygotowaniu odpowiedzi na recenzje.

3. **Pietrowska-Borek M.**, Dobrogojski J., Wojdyła-Mamoń A.M., Romanowska J., Gołębiewska J., Borek S., Murata K., Ishihara A., Pedreño M.Á., Guranowski A. 2021. Nucleoside 5'-Phosphoramidates Control the Phenylpropanoid Pathway in *Vitis vinifera* Suspension-Cultured Cells. *International Journal of Molecular Sciences*, 22, 13567

   - opracowaniu koncepcji pracy, zaprojektowaniu badań, realizacji części badań, opiece merytorycznej podczas wykonywania badań, analizie i interpretacji wyników, udziale w przygotowaniu pierwszej wersji manuskryptu, udziale w przygotowaniu wersji manuskryptu po uwagach i komentarzach recenzentów, w tym udziale w przygotowaniu odpowiedzi na recenzje.

4. Dobrogojski J., Nguyen V.H., Kowalska J., Borek S., **Pietrowska-Borek M.** 2023. The plasma membrane purinoreceptor P2K1/DORN1 is essential in stomatal closure evoked by extracellular diadenosine tetraphosphate (Ap_4A) in *Arabidopsis thaliana*. *International Journal of Molecular Sciences*, 24, 16688

   - opracowaniu koncepcji badań, zaplanowaniu i opiece merytorycznej podczas wykonywania badań, analizie i interpretacji otrzymanych wyników, przeprowadzeniu analizy statystycznej, udziale w przygotowaniu pierwszej wersji manuskryptu, współprojektowaniu i współtworzeniu wszystkich rycin oraz udziale w przygotowaniu wersji manuskryptu po uwagach i komentarzach recenzentów, w tym udziale w przygotowaniu odpowiedzi na recenzje.

Małgorzata Pietrowska-Borek

UNIWERSYTET IM. ADAMA MICKIEWICZA W POZNANIU
Wydział Biologii
Zakład Fizjologii Roślin

Sławomir Borek, dr hab., prof. UAM

Poznań, 28 stycznia 2024

Uniwersytet im. Adama Mickiewicza w Poznaniu
Wydział Biologii
Zakład Fizjologii Roślin
ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr. Jędrzejem Dobrogojskim stanowiących podstawę rozprawy doktorskiej

1. Pietrowska-Borek M, Dobrogojski J, Sobieszczuk-Nowicka E, **Borek S**. 2020. New insight into plant signaling: extracellular ATP and uncommon nucleotides. *Cells*, 9, 345, doi:10.3390/cells9020345

   Mój udział obejmował wykonanie wszystkich figur, merytoryczny i edytorski współudział w redagowaniu tekstu oraz wkład w przygotowanie odpowiedzi na recenzje i wprowadzenie zmian w końcowej wersji manuskryptu.

2. Pietrowska-Borek M, Wojdyła-Mamoń A, Dobrogojski J, Młynarska-Cieślak A, Baranowski MR, Dąbrowski JM, Kowalska J, Jemielity J, **Borek S**, Pedreño MA, Guranowski A. 2020. Purine and pyrimidine dinucleoside polyphosphates differentially affect the phenylpropanoid pathway in *Vitis vinifera* L. cv. Monastrell suspension cultured cells. *Plant Physiology and Biochemistry*, 147, 125-132, doi:10.1016/j.plaphy.2019.12.015

   Mój udział polegał na wykonaniu analizy statystycznej, merytorycznym i edytorskim współudziale w redagowaniu tekstu, przygotowaniu odpowiedzi na recenzje oraz wprowadzeniu zmian w ostatecznej wersji manuskryptu.

ul. Uniwersytetu Poznańskiego 6
61-614 Poznań
tel. +48 61 829 5893, borek@amu.edu.pl
www.ibe.amu.edu.pl/zfr

3. Pietrowska-Borek M, Dobrogojski J, Wojdyła-Mamoń AM, Romanowska J, Gołębiewska J, **Borek S**, Murata K, Ishihara A, Pedreño MÁ, Guranowski A. 2021. Nucleoside 5'-phosphoramidates control the phenylpropanoid pathway in *Vitis vinifera* suspension-cultured cells. *International Journal of Molecular Sciences*, 22, 13567, doi:10.3390/ijms222413567

   Moje zaangażowanie w powstanie tej publikacji obejmowało wykonanie analizy statystycznej oraz współudział w przygotowaniu odpowiedzi na recenzje i wprowadzeniu zmian w finalnej wersji manuskryptu.

4. Dobrogojski J, Nguyen VH, Kowalska J, **Borek S**, Pietrowska-Borek M. 2023. The plasma membrane purinoreceptor P2K1/DORN1 is essential in stomatal closure evoked by extracellular diadenosine tetraphosphate (Ap_4A) in *Arabidopsis thaliana*. *International Journal of Molecular Sciences*, 24, 16688, doi:10.3390/ijms24231668

   Mój udział polegał na współprojektowaniu i wykonaniu figury 6, merytorycznym i edytorskim współudziale w redagowaniu tekstu oraz zaangażowaniu w przygotowanie odpowiedzi na recenzje i wprowadzenie zmian w ostatecznej wersji manuskryptu.

Sławomir Borek

RESEARCH UNIVERSITY
EPUCUR
ul. Uniwersytetu Poznańskiego 6
61-614 Poznań
tel. +48 61 829 5893, borek@amu.edu.pl
www.ibe.amu.edu.pl/zfr/

Poznań, 19.01.2024

prof. UAM dr hab. Ewa Sobieszczuk-Nowicka

Zakład Fizjologii Roślin,
Wydział Biologii,
Uniwersytet im. Adama Mickiewicza w Poznaniu, Uniwersytetu Poznańskiego 6, 61-614 Poznań,
email: ewa.sobieszczuk-nowicka@amu.edu.pl

Oświadczam, że mój udział w przygotowaniu pracy:

Pietrowska-Borek M., Dobrogojski J., **Sobieszczuk-Nowicka E.**, Borek S. 2020. New insight into plant signaling: extracellular ATP and uncommon nucleotides. *Cells*, 9, 345 polegał na współtworzeniu koncepcji pracy oraz zaangażowaniu w dyskusję i powstanie manuskryptu.

Poznań, 19.01.2024
Ewa Sobieszczuk-Nowicka

Poznań, 19.01.2024

Prof. dr hab. Andrzej Guranowski

Katedra Biochemii i Biotechnologii,
Wydział Rolnictwa, Ogrodnictwa i Bioinżynierii,
Uniwersytet Przyrodniczy w Poznaniu, Dojazd 11, 60-632 Poznań,
email: andrzej.guranowski@up.poznan.pl

Oświadczam, że mój udział w przygotowaniu pracy:

Pietrowska-Borek M., Wojdyła-Mamoń A., Dobrogojski J., Młynarska-Cieślak A., Baranowski M.R., Dąbrowski J.M., Kowalska J., Jemielity J., Borek S., Pedreño M.A., **Guranowski A.** 2020. Purine and pyrimidine dinucleoside polyphosphates differentially affect the phenylpropanoid pathway in *Vitis vinifera* L. cv. Monastrell suspension cultured cells. *Plant Physiology and Biochemistry*, 147, 125-132 polegał na zaangażowaniu w dyskusję nad uzyskanymi wynikami oraz przygotowanie manuskryptu.

Pietrowska-Borek M., Dobrogojski J., Wojdyła-Mamoń A.M., Romanowska J., Gołębiewska J., Borek S., Murata K., Ishihara A., Pedreño M.Á., **Guranowski A.** 2021. Nucleoside 5'-Phosphoramidates Control the Phenylpropanoid Pathway in *Vitis vinifera* Suspension-Cultured Cells. *International Journal of Molecular Sciences*, 22, 13567 polegał na współtworzeniu koncepcji badań, zaangażowaniu w dyskusję wyników i w powstanie manuskryptu.

A. Guranowski

Murcia, 22.01.2024

Maria Angeles Pedreño, Prof.

Department of Plant Biology,
Faculty of Biology,
University of Murcia, 30100 Murcia, Spain,
email: mpedreno@um.es

I declare that I have contributed to the article:

Pietrowska-Borek M., Wojdyła-Mamoń A., Dobrogojski J., Młynarska-Cieślak A., Baranowski M.R., Dąbrowski J.M., Kowalska J., Jemielity J., Borek S., **Pedreño M.A**., Guranowski A. 2020. Purine and pyrimidine dinucleoside polyphosphates differentially affect the phenylpropanoid pathway in *Vitis vinifera* L. cv. Monastrell suspension cultured cells. *Plant Physiology and Biochemistry*, 147, 125-132 by providing the plant material and being involved in writing the manuscript.

Pietrowska-Borek M., Dobrogojski J., Wojdyła-Mamoń A.M., Romanowska J., Gołębiewska J., Borek S., Murata K., Ishihara A., **Pedreño M.Á**., Guranowski A. 2021. Nucleoside 5'-Phosphoramidates Control the Phenylpropanoid Pathway in *Vitis vinifera* Suspension-Cultured Cells. *International Journal of Molecular Sciences*, 22, 13567 by providing the plant material and being involved in writing the manuscript.

PEDREÑO GARCIA MARIA ANGELES - 22947266M

Firmado digitalmente por PEDREÑO GARCIA MARIA ANGELES - 22947266M
Fecha: 2024.01.22 10:50:10 +01'00'

Warszawa, 19.01.2024

Prof. dr hab. Jacek Jemielity
email: j.jemielity@cent.uw.edu.pl

Centrum Nowych Technologii,
Uniwersytet Warszawski, Stefana Banacha 2c, 02-097 Warszawa,

mgr inż. Agnieszka Młynarska-Cieślak
email: agnieszka.czajkowska-nowak@fuw.edu.pl

mgr Marek Rafał Baranowski
email: marek.baranowski@fuw.edu.pl

mgr Jakub Michał Dąbrowski
email: marek.baranowski@fuw.edu.pl

Zakład Biofizyki,
Wydział Fizyki,
Uniwersytet Warszawski, Pasteura 5, 02-093 Warszawa,

Oświadczamy, że nasz udział w przygotowaniu pracy:

Pietrowska-Borek M., Wojdyła-Mamoń A., Dobrogojski J., **Młynarska-Cieślak A.**, **Baranowski M.R.**, **Dąbrowski J.M.**, Kowalska J., **Jemielity J.**, Borek S., Pedreño M.A., Guranowski A. 2020. Purine and pyrimidine dinucleoside polyphosphates differentially affect the phenylpropanoid pathway in *Vitis vinifera* L. cv. Monastrell suspension cultured cells. *Plant Physiology and Biochemistry*, 147, 125-132 był związany z syntezą dinukleozydopolifosforanów tj. Up_3U, Up_4U, Cp_3C, Cp_4C, Ap_3U, Ap_3C, Ap_4C, Ap_3A oraz Gp_3G.

UNIWERSYTET WARSZAWSKI
WYDZIAŁ FIZYKI
Instytut Fizyki Doświadczalnej
ZAKŁAD BIOFIZYKI
ul. Pasteura 5, 02-093 Warszawa
tel. 22 55 32 304

Warszawa, 19.01.2024

dr hab. Joanna Kowalska

Zakład Biofizyki,
Wydział Fizyki,
Uniwersytet Warszawski, Pasteura 5, 02-093 Warszawa,
email: joanna.kowalska@fuw.edu.pl

Oświadczam, że mój udział w przygotowaniu pracy:

Pietrowska-Borek M., Wojdyła-Mamoń A., Dobrogojski J., Młynarska-Cieślak A., Baranowski M.R., Dąbrowski J.M., **Kowalska J.**, Jemielity J., Borek S., Pedreño M.A., Guranowski A. 2020. Purine and pyrimidine dinucleoside polyphosphates differentially affect the phenylpropanoid pathway in *Vitis vinifera* L. cv. Monastrell suspension cultured cells. *Plant Physiology and Biochemistry*, 147, 125-132 polegał na syntezie dinukleozydopolifosforanów tj. Up_3U, Up_4U, Cp_3C, Cp_4C, Ap_3U, Ap_3C, Ap_4C, Ap_3A oraz Gp_3G.

Dobrogojski J., Nguyen V.H., **Kowalska J.**, Borek S., Pietrowska-Borek M. 2023. The plasma membrane purinoreceptor P2K1/DORN1 is essential in stomatal closure evoked by extracellular diadenosine tetraphosphate (Ap_4A) in *Arabidopsis thaliana*. *International Journal of Molecular Sciences*, 24, 16688 polegał na syntezie dinukleozydopolifosforanów (Np_3N') użytych w badaniach oraz zaangażowaniu w korektę manuskryptu.

Joanna Kowalska

Poznań, 19.01.2024

mgr inż. Anna Wojdyła-Mamoń

Katedra Biochemii i Biotechnologii,
Wydział Rolnictwa, Ogrodnictwa i Bioinżynierii,
Uniwersytet Przyrodniczy w Poznaniu, Dojazd 11, 60-632 Poznań,
email: anna.wojdyla@up.poznan.pl

Oświadczam, że mój udział w przygotowaniu pracy:

Pietrowska-Borek M., **Wojdyła-Mamoń A.**, Dobrogojski J., Młynarska-Cieślak A., Baranowski M.R., Dąbrowski J.M., Kowalska J., Jemielity J., Borek S., Pedreño M.A., Guranowski A. 2020. Purine and pyrimidine dinucleoside polyphosphates differentially affect the phenylpropanoid pathway in *Vitis vinifera* L. cv. Monastrell suspension cultured cells. *Plant Physiology and Biochemistry*, 147, 125-132 polegał na realizacji części badań dotyczących analizy akumulacji dwóch stilbenów: *trans*-resweratrolu i *trans*-piceidu w komórkach, a także w pożywce (Fig. 2.) pod wpływem testowanych Np_nN' w zawiesinowej kulturze komórkowej *V. vinifera*.

Pietrowska-Borek M., Dobrogojski J., **Wojdyła-Mamoń A.M.**, Romanowska J., Gołębiewska J., Borek S., Murata K., Ishihara A., Pedreño M.Á., Guranowski A. 2021. Nucleoside 5'-Phosphoramidates Control the Phenylpropanoid Pathway in *Vitis vinifera* Suspension-Cultured Cells. *International Journal of Molecular Sciences*, 22, 13567 polegał na realizacji części badań dotyczących analizy akumulacji dwóch stilbenów: *trans*-resweratrolu i *trans*-piceidu w komórkach (Fig. 4b i Fig. 4c), a także w pożywce (Fig. 5b i Fig. 5c) pod wpływem NH_2-pN w zawiesinowej kulturze komórkowej *V. vinifera*. Ponadto byłam zaangażowana w badania nad zawartością lignin w komórkach *V. vinifera* traktowanych NH_2-pN (Fig. 8c).

Tottori, 19.01.2024

Atsushi Ishihara, Ph.D.

Faculty of Agriculture,
Department of Life and Environmental Agricultural Sciences,
Tottori University, Tottori 680-8553, Japan,
email: aishihara@tottori-u.ac.jp

&

Koichi Murata

Graduate School of Agriculture,
Tottori University, Tottori 680-8553, Japan,
email: yoba.sean24@gmail.com

We declare that we have contributed to the article:

Pietrowska-Borek M., Dobrogojski J., Wojdyła-Mamoń A.M., Romanowska J., Gołębiewska J., Borek S., **Murata K.**, **Ishihara A.**, Pedreño M.Á., Guranowski A. 2021. Nucleoside 5′-Phosphoramidates Control the Phenylpropanoid Pathway in *Vitis vinifera* Suspension-Cultured Cells. *International Journal of Molecular Sciences*, 22, 13567 by performing experiments on the determination of phenylamide concentrations.

Tottori, 19.01.2024

Atsushi Ishihara, Ph.D.

Faculty of Agriculture,
Department of Life and Environmental Agricultural Sciences,
Tottori University, Tottori 680-8553, Japan,
email: aishihara@tottori-u.ac.jp

&

Koichi Murata

Graduate School of Agriculture,
Tottori University, Tottori 680-8553, Japan,
email: yoba.scan24@gmail.com

K. murata

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Umeå, 19.01.2024

dr Justyna Gołębiewska

Department of Chemistry,
Umeå University, 901 87, Umeå, Szwecja,
email: justyna.golebiewska@umu.se

Oświadczam, że mój udział w przygotowaniu pracy:

Pietrowska-Borek M., Dobrogojski J., Wojdyła-Mamoń A.M., Romanowska J., **Gołębiewska J.**, Borek S., Murata K., Ishihara A., Pedreño M.Á., Guranowski A. 2021. Nucleoside 5′-Phosphoramidates Control the Phenylpropanoid Pathway in *Vitis vinifera* Suspension-Cultured Cells. *International Journal of Molecular Sciences*, 22, 13567 polegał na przeprowadzeniu syntezy i oczyszczania nukleozydo 5′-fosforamidów.

Justyna Gołębiewska

Poznań, 19.01.2024

dr Joanna Romanowska

Zakład Chemii Komponentów Kwasów Nukleinowych,
Instytut Chemii Bioorganicznej,
Polska Akademia Nauk, 61-704 Poznań,
email: joarom@ibch.poznan.pl

Oświadczam, że mój udział w przygotowaniu pracy:

Pietrowska-Borek M., Dobrogojski J., Wojdyła-Mamoń A.M., **Romanowska J.**, Gołębiewska J., Borek S., Murata K., Ishihara A., Pedreño M.Á., Guranowski A. 2021. Nucleoside 5'-Phosphoramidates Control the Phenylpropanoid Pathway in *Vitis vinifera* Suspension-Cultured Cells. *International Journal of Molecular Sciences*, 22, 13567 polegał na opracowaniu metody chemicznej syntezy nukleozydo 5'-fosforamidów oraz przeprowadzaniu ich syntezy i oczyszczania.

Joanna Romanowska

Warsaw, 19.01.2024

Van Hai Nguyen, M.Sc.

Division of Biophysics,
Faculty of Physics,
University of Warsaw, Pasteura 5, 02-093 Warsaw,
email: v.nguyen5@uw.edu.pl

I declare that I have contributed to the article:

Dobrogojski J., **Nguyen V.H.**, Kowalska J., Borek S., Pietrowska-Borek M. 2023. The plasma membrane purinoreceptor P2K1/DORN1 is essential in stomatal closure evoked by extracellular diadenosine tetraphosphate (Ap_4A) in *Arabidopsis thaliana*. *International Journal of Molecular Sciences*, 24, 16688 by synthesizing the dinucleoside polyphosphates (Np_nN') used in the study and participated in reviewing and editing the manuscript.

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mgr Jędrzej Dobrogojski

Katedra Biochemii i Biotechnologii,
Wydział Rolnictwa, Ogrodnictwa i Bioinżynierii,
Uniwersytet Przyrodniczy w Poznaniu, Dojazd 11, 60-632 Poznań,
email: jedrzej.dobrogojski@up.poznan.pl

Oświadczam, że mój udział w przygotowaniu pracy:

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Pietrowska-Borek M., Wojdyła-Mamoń A., **Dobrogojski J.**, Młynarska-Cieślak A., Baranowski M.R., Dąbrowski J.M., Kowalska J., Jemielity J., Borek S., Pedreño M.A., Guranowski A. 2020. Purine and pyrimidine dinucleoside polyphosphates differentially affect the phenylpropanoid pathway in *Vitis vinifera* L. cv. Monastrell suspension cultured cells. *Plant Physiology and Biochemistry*, 147, 125-132 polegał na realizacji części badań laboratoryjnych dotyczących analizy zmian ekspresji genów kodujących kluczowe enzymy szlaku fenylopropanoidowym (Fig. 4.) oraz akumulacji dwóch stilbenów: *trans*-resweratrolu i *trans*-piceidu w komórkach, a także w pożywce (Fig. 2.) pod wpływem testowanych Np,N′ w zawiesinowej kulturze komórkowej *Vitis vinifera*.

Pietrowska-Borek M., **Dobrogojski J.**, Wojdyła-Mamoń A.M., Romanowska J., Gołębiewska J., Borek S., Murata K., Ishihara A., Pedreño M.Á., Guranowski A. 2021. Nucleoside 5′-Phosphoramidates Control the Phenylpropanoid Pathway in *Vitis vinifera* Suspension-Cultured Cells. *International Journal of Molecular Sciences*, 22, 13567 polegał na realizacji części badań laboratoryjnych dotyczących analizy zmian ekspresji genów kodujących kluczowe enzymy szlaku fenylopropanoidowym (Fig. 3., Fig. 4a, i Fig. 8) oraz akumulacji dwóch stilbenów: *trans*-resweratrolu i *trans*-piceidu w komórkach (Fig. 4b i Fig. 4c), a także w pożywce (Fig. 5b i Fig. 5c) pod wpływem NH_2-pN w zawiesinowej kulturze komórkowej *Vitis vinifera*. Dokonałem również oceny żywotności komórek *V. vinifera*, wyniki której przedstawiono na Fig. 6.

Dobrogojski J., Nguyen V.H., Kowalska J., Borek S., Pietrowska-Borek M. 2023. The plasma membrane purinoreceptor P2K1/DORN1 is essential in stomatal closure evoked by extracellular diadenosine tetraphosphate (Ap_4A) in *Arabidopsis thaliana*. *International Journal of Molecular Sciences*, 24, 16688 polegał na współprojektowaniu badań, realizacji i analizie badań laboratoryjnych oraz zaangażowaniu w analizę statystyczną, wizualizację i powstanie pierwotnej wersji manuskryptu.

Dobrogojski

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cells

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Review

New Insight into Plant Signaling: Extracellular ATP and Uncommon Nucleotides

Małgorzata Pietrowska-Borek [1,*], Jędrzej Dobrogojski [1], Ewa Sobieszczuk-Nowicka [2] and Sławomir Borek [2]

[1] Department of Biochemistry and Biotechnology, Faculty of Agronomy and Bioengineering, Poznań University of Life Sciences, Dojazd 11, 60-632 Poznań, Poland; dobrogojski@gmail.com

[2] Department of Plant Physiology, Faculty of Biology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland; evaanna@amu.edu.pl (E.S.-N.); borek@amu.edu.pl (S.B.)

* Correspondence: małgorzata.pietrowska-borek@up.poznan.pl

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Abstract: New players in plant signaling are described in detail in this review: extracellular ATP (eATP) and uncommon nucleotides such as dinucleoside polyphosphates (Np_nN's), adenosine 5′-phosphoramidate (NH_2-pA), and extracellular NAD^+ and $NADP^+$ (eNAD(P)$^+$). Recent molecular, physiological, and biochemical evidence implicating concurrently the signaling role of eATP, Np_nN's, and NH_2-pA in plant biology and the mechanistic events in which they are involved are discussed. Numerous studies have shown that they are often universal signaling messengers, which trigger a signaling cascade in similar reactions and processes among different kingdoms. We also present here, not described elsewhere, a working model of the Np_nN' and NH_2-pA signaling network in a plant cell where these nucleotides trigger induction of the phenylpropanoid and the isochorismic acid pathways yielding metabolites protecting the plant against various types of stresses. Through these signals, the plant responds to environmental stimuli by intensifying the production of various compounds, such as anthocyanins, lignin, stilbenes, and salicylic acid. Still, more research needs to be performed to identify signaling networks that involve uncommon nucleotides, followed by omic experiments to define network elements and processes that are controlled by these signals.

Keywords: adenosine 5′-phosphoramidate; adenosine 5′-tetraphosphate; diadenosine 5′,5‴-tetraphosphate; dinucleoside polyphosphates; eATP; eNAD(P)$^+$

1. Introduction

Plant signaling is a set of phenomena that enables the transduction of external and internal signals into physiological responses such as modification of enzyme activity, cytoskeleton structure, and gene expression. It is known that in plants there exist mechanisms involved in the signal transduction pathways. Plants have evolved signaling networks providing reactions to environmental stimuli through signaling proteins such as plasma membrane receptors and ion transporters and by cascades of kinases and other enzymes up to effectors. For many years plant hormones were considered to be dominant molecules in plant signaling. Nowadays this term embraces many other compounds including second messengers, such as cytosolic Ca^{2+} [1], reactive oxygen (ROS) and nitrogen species (RNS) [2] or cyclic nucleotides such as adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) [3]. Nowadays there is more and more information about synthesis, degradation, and function of cAMP and cGMP in plant [3–5], and they are currently accepted as key signaling molecules in many processes in plants including growth and differentiation, photosynthesis, and biotic and abiotic defense [6]. However, recently it was shown that nucleotides, such as ATP and (di)nucleoside polyphosphates, also can play signaling roles in plant cells. In this review, we focus on

the extracellular ATP (eATP) and uncommon nucleotides, such as mono- (p_nNs) and dinucleoside polyphosphates (Np_nN's), and their new function as signaling molecules.

2. eATP as a Signaling Molecule

Adenosine 5'-triphosphate (ATP), as well as the other nucleoside triphosphates, are established as agents providing energy in various reactions inside cells, both in animal and plant organisms [7]. As ATP is omnipresent in all living cells, it is often called the essential energy currency molecule. In the 1970s it was first hypothesized that ATP might be released into the extracellular environment and act as a signaling compound for animal cells [8]. However, it is worth mentioning that the first effect of eATP on a cell was noted much earlier, i.e., in 1929, during research on heart muscle contraction [9]. In animals, three possible ways of ATP release from the cells into the extracellular matrix have been proposed; they involve multiple channels, transporters, and exocytosis. The interest in the eATP signaling function accelerated after the first purinoreceptor was cloned and characterized in rat brain tissue [10]. Currently, eATP, as well as some of the other nucleotides, are considered as signaling molecules mediating numerous animal cellular processes. For decades the role of nucleotides as signaling molecule functioning similarly in plants as it was demonstrated in animals was viewed with skepticism. A real breakthrough in this topic came with the discovery of the existence of a plant transmembrane receptor protein with serine/threonine kinase activity having a high affinity for extracellular nucleotides [11]. However, the evidence for the mechanism of ATP release from the cytosol into the extracellular matrix in plants appeared earlier [12–15]. In plants, there are several possible ways of ATP outlet. The ATP release triggered by environmental stimuli appears via the wounded cell membrane [16], exocytosis [12], the p-glycoprotein (PGP1) belonging to the ATP-binding cassette ABC transporters [17], and plasma membrane-localized nucleotide transporters (PM-ANT1) [18]. The receptor-eATP interaction begins a cascade reaction that leads to further downstream physiological changes protecting the plant against both biotic and abiotic stresses but also guarantees proper plant growth and development [19].

In order to maintain proper cell growth and function, eATP concentration must be controlled. During regeneration after the stress factor has disappeared, eATP degradation is conducted by hydrolytic enzymes called apyrases [20]. The human apyrases are the best characterized and described apyrases among different kingdoms. Their cellular localization includes plasma membrane, Golgi apparatus, and endoplasmic reticulum. The human apyrases present in plasma membrane show ecto-apyrases activity mediating regulation of the eATP in the extracellular environment. The *Arabidopsis thaliana* apyrase family consists of seven enzymes among which two closely related ones, APY1 and APY2, are the most extensively characterized. These enzymes mediate the luminal glycosylation and can be a component of regulation of the eATP level. It was demonstrated that these two enzymes are an integral component of the Golgi apparatus membrane where they indirectly control the eATP level by modulating the luminal concentration of ATP in secretory vesicles [21–23] (Figure 1). Both APY1 and APY2 are also essential enzymes for proper plant growth and development. These processes are auxin-dependent. Amoeg various factors, auxin transport also depends on the expression of the genes encoding APY1 and APY2. Suppression of the APY1 and APY2 expression causes dwarfism, impaired polar auxin transport and eATP over-accumulation in *Arabidopsis thaliana* [24,25]. However, it was suggested that some fractions of the APY1 and APY2 population with ecto-apyrases activity might by localized also in the plasma membrane [19]. Although there are many data regarding subcellular localization of *Arabidopsis* apyrases (APY1 and APY2), the apyrases from soybean (GS52), pea (PsAPY1), and potato (StAPY3) occur outside the cell (ecto-apyrases) [26–28]. A study conducted by Wu and co-workers indicated that the externally applied APY1 and APY2 inhibitors cause the increase of eATP and physiological changes typical for the plant reaction to stress [24]. Furthermore, there are some reports showing particular plant species secreting individual apyrase members out of the endomembrane system. Taking all these circumstances into account, the existence of a plasma membrane-localized *Arabidopsis thaliana* apyrase (Figure 1) cannot be excluded [29].

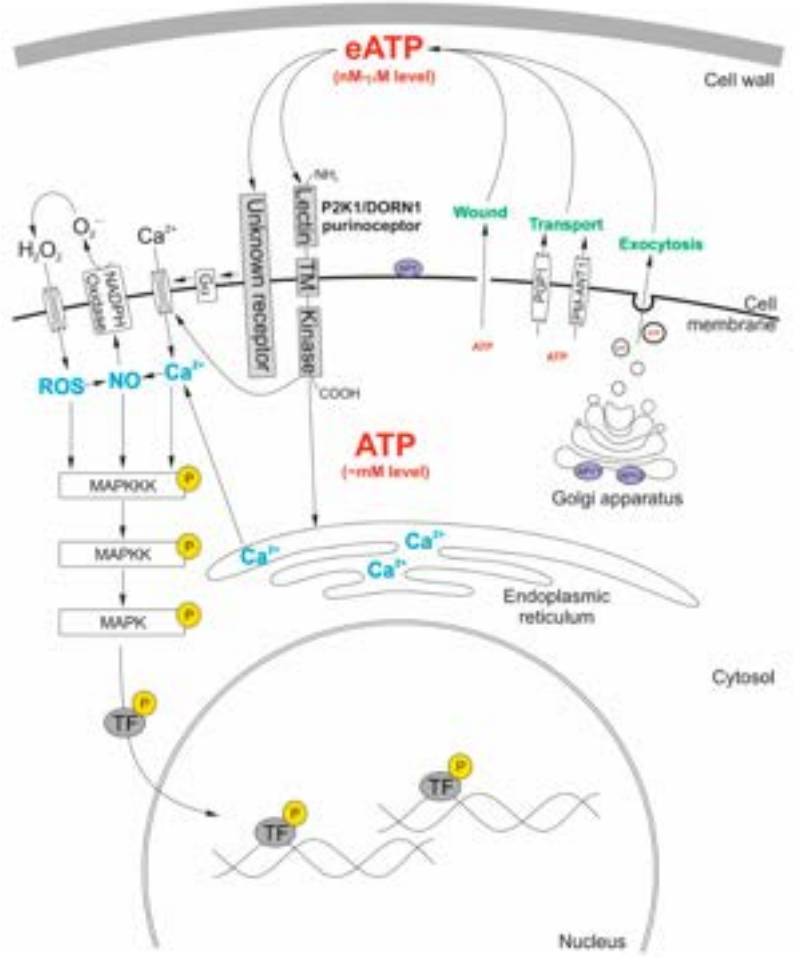

Figure 1. Model of changes occurring in the plant cell triggered by the extracellular ATP (eATP). In this model, three possible ways of ATP release into the extracellular matrix are demonstrated. It considers the wounded cell membrane, exocytosis, and two transporters: the p-glycoprotein (PGP1) belonging to the ATP-binding cassette ABC transporters, and the plasma membrane-localized nucleotide transporters (PM-ANT1). Two apyrases, APY1, and APY2, localized in the Golgi apparatus membrane of *Arabidopsis thaliana* regulate the concentration of eATP. Additionally, the hypothesized apyrase (APY) located at the extracellular surface of the plasma membrane can decrease eATP concentration directly in the extracellular matrix. The released eATP acts as a signaling molecule triggering elevation of the cytosolic Ca^{2+} level by activation of the P2K1 receptor, which in turn activates the Ca^{2+} channel. The hypothetical non-P2K1 receptor, whose binding with eATP leads to activation of the Gα subunit of the heterotrimeric G-protein, activates the cell membrane Ca^{2+} channel. High cytosolic Ca^{2+} concentration causes an increase in production of nitric oxide (NO), reactive oxygen species (ROS), and mitogen-activated protein kinases (MAPKs), which finally leads to various physiological responses. The ROS boosted production is due to the activation of the RBOHD subunit of the plasma membrane-localized NADPH oxidase. The contribution of the transcription factors in the regulation of gene expression is of high importance.

2.1. Plant eATP Receptors

The eATP animal receptors were discovered in 1976 [30]. Initially, they were called 'purinergic receptors'. The name was changed to 'nucleotide receptors', as both purine and pyrimidine nucleotides trigger their activation [31]. These receptors belong to two groups, P1 and P2, but only P2 receptors are activated by ATP [32]. P2 receptors are divided into two classes: ligand-gated ion channels (P2X) and G protein-coupled (P2Y) receptors [33]. It is important to emphasize that P2X and P2Y receptors do not exist in plant organisms [34]. For many years it was hard to prove the existence of a similar receptor in plants although there were papers indicating changes in plants' development, growth and response to stresses under exogenously applied ATP [11]. Research on *dorn1* (DOes not Respond to Nucleotides) mutants of *Arabidopsis thaliana* revealed the first plant receptor with a high affinity to bind eATP. Mutants showed a lower cytosolic Ca^{2+} level, lack of mitogen-activated protein kinases (MAPKs) activation and as a consequence decline of the defense-related gene expression level. Initially, the newly discovered receptor was named DORN1, but later the name was changed to P2K1 because the research showed that the *DORN1* gene encodes the L-type lectin receptor-like kinase I.9 (LecRK-I.9). This receptor has three domains: an extracellular ATP-binding lectin domain, a single transmembrane domain, and an intracellular kinase domain (Figure 1). In the case of *dorn1* mutants defective in the kinase domain, eATP is not able to connect with the receptor and as a result is not able to trigger downstream responses. Decreased expression of the *LecRK-I.9* gene caused weaker eATP responses, while intensified *LecRK-I.9* gene expression enhanced eATP responses [35]. DORN1 is a plant purine receptor that belongs to the lectin-receptor kinase family and is denoted as P2K1 to distinguish it from the animal P2 receptors: P2X and P2Y [11,36,37]. Interestingly, *P2K1* gene expression level is relatively high during the major stages of plant development [37], suggesting the essential role of the eATP in processes such as seedling growth, stomata movement, pollen tube development, root hair growth, gravitropism, and biotic and abiotic stress responses [38].

Recently, the existence of another, unidentified, non-P2K1 receptor with affinity to eATP was suggested [39]. It was reported that *Arabidopsis thaliana dorn1* null mutants demonstrated an increased level of cytosolic Ca^{2+} under the exogenously applied ATP. It was a result of the heterotrimeric G-protein G_α subunit activation followed by the opening of a plasma-membrane Ca^{2+} channel. This finding suggests new, P2K1-independent responses to eATP, involved among others in the root-bending mechanism [39]. Moreover DORN1 could underpin several calcium-related responses but it may not be the only receptor for eATP in *Arabidopsis thaliana* [40].

2.2. Plant eATP Signal Transduction Pathway

In plants, eATP takes part in cell signaling as a messenger, which triggers a signaling cascade, when binding to the P2K1 receptor (Figure 1). Cytosolic nitric oxide (NO), Ca^{2+}, and ROS form a secondary messenger trio, which occurs in various signaling pathways leading to the transient phosphorylation of MAPK, especially MPK3 and MPK6, and expression of defense-related genes [41]. The multibranched reaction starts with the elevation of the cytosolic Ca^{2+} level triggered by eATP, leading to the accumulation of MAPK and NO, as well as to the phosphorylation and activation of the RBOHD (respiratory burst oxidase homolog protein D) subunit of the plasma membrane-localized NADPH oxidase [42]. This enzyme catalyzes the synthesis of extracellular ROS like superoxide (O^{2-}), which is then converted into hydrogen peroxide (H_2O_2) in the extracellular milieu [16,33,43]. Extracellular H_2O_2 crosses the double-layered plasma membrane via various channels among which aquaporins are distinguishable [44]. Subsequently, ROS trigger changes in the expression of nuclear genes, responsible for defense responses [16], root hair growth [12] or regulation of Na^+, H^+, and K^+ levels. Consequently, a disturbance in the ion homeostasis may lead to the mitochondria-independent type of programmed cell death by activation of the caspase-like proteases [45].

2.3. eATP Involvement in Plant Responses to Biotic and Abiotic Stresses

Aside from different eATP-induced factors and signaling molecules contributing to the plant resistance against pathogens and various abiotic stresses, classical defense hormones such as jasmonate, ethylene, and salicylic acid are of high importance [38]. Based on the pathogens' lifestyles two groups of these organisms are distinguishable: biotrophs and necrotrophs. Interestingly, the efficiency of the used phytohormone against these two groups of pathogeneses differs. Salicylic acid induces plant defense against biotrophic pathogens, whereas jasmonate and ethylene are indispensable when necrotrophic pathogens and herbivorous insects attack the plant. Furthermore, salicylic acid regulates pathogen-induced systemic acquired resistance (SAR), whereas jasmonate and ethylene play a crucial role in rhizobacteria-mediated induced systemic resistance (ISR) [46]. Transcriptomic research of *Arabidopsis thaliana* mutants defective in the jasmonate, ethylene, and salicylic acid signaling pathway, which were treated with ATP, revealed crosstalk in the signaling of the typical plant hormones and eATP. These results showed that from among all of the defense-related genes, up to 50% were induced by eATP in cooperation with the typical plant defense hormones. This finding suggests a complex network of the plant defense mechanism that needs to be explored more deeply at different levels [38]. The contribution of transcription factors to ATP-responsive transcription is also considered. It was demonstrated that a calmodulin-binding transcription activator (CAMTA3) and MYC transcription factors are required for proper defense-related gene transcription, whose expression is induced by eATP [38].

In order to examine the importance of eATP in the plant reaction to pathogens two approaches might be considered: either alteration of the P2K1 receptor or manipulation at the eATP level. Overexpression of the P2K1 gene resulted in increased plant resistance to the bacterial pathogen *Pseudomonas syringae* and the oomycete pathogen *Phytophthora brassicae* [47,48]. Plants treated with ATP were found to be protected against various organisms such as the fungal pathogen *Botrytis cinerea* [7] and the bacterial pathogen *Pseudomonas syringae* [48]. Although the complete mechanisms of eATP signal transduction through the P2K1 receptor remain unclear, a plenitude of evidence shows the involvement of eATP in plant resistance to biotic stresses.

Studies show that eATP level, as well as P2K1 activity, are also of high importance in the plant-fungus symbiosis. Interactions of the filamentous root endophyte *Serendipita indica* with various experimental plant hosts, including *Arabidopsis thaliana* and *Hordeum vulgare*, have been examined. *Serendipita indica* colonization was found to be beneficial for plants, reflected in plant growth enhancement, assimilation of nitrate and phosphate improvement and better tolerance to both abiotic and biotic stresses [49]. Although *Serendipita indica* penetrates the host's root cells, massive plant cell death does not occur. It is because of an enzymatically active ecto-5′-nucleotidase (E5′NT) enzyme secreting by *Serendipita indica* which is capable of hydrolyzing nucleotides in the apoplast. By the hydrolyzation of ATP, ADP, and AMP, *Serendipita indica* E5′NT modifies the eATP level, leading to switching off the plant defense reactions which promote proper fungal accommodation [50]. Moreover, it was observed that the rhizobial nodule factor stimulates the release of ATP outside the cell by the root hair tips of *Medicago truncatula* [12]. Treatment of plants with eATP caused also the change their susceptibility to pathogen infection [16]. It is hypothesized that ecto-apyrases may decrease the eleveted level of eATP upon symbiont infection and thereby prevents the activation of plant defense pathways that could limit symbiont invasion [51].

Several studies indicate the role of eATP in the response to different types of abiotic stresses. In addition to mechanical stimuli caused by wounding or touch, ATP is also released in response to treatment with molecules such as abscisic acid and L-glutamate [15,52]. A similar reaction was observed during both osmotic and salt stress [12,15,53] as well as under cadmium treatment [54]. Consequently, eATP accumulation triggers plant physiological changes leading to enhancement of resistance. It involves rapid closure of leaf stomata [55], probable seedling viability enhancement [56] and modification of root growth direction when encountering an obstacle [34]. In addition, hypertonic salt stress interferes with the photosynthesis machinery by decreasing the levels of maximal efficiency

of photosystem II and depleting the photochemical quenching [54]. Moreover, abiotic stress caused by cadmium triggers a rapid increase in lipid peroxidation but also higher antioxidant and lipoxygenase activities in *Arabidopsis thaliana* cells [54]. These reactions are associated with boosted synthesis of jasmonic acid, which is one of the most important molecules involved in the different stress responses [57]. Nowadays, one of the major anthropogenic pollutants with the highest level of threat to human health is cadmium [58]. It is also considered as one of the most phytotoxic elements among heavy metals, causing a reduction in crop biomass by disrupting photosynthesis and respiration [59]. In addition, cadmium can induce other physiological changes including induction of oxidative stress by boosting the production of ROS [58], modifications in gene expression [60,61], as well as changes in enzyme activity [62], hence activating plant defense.

3. Extracellular Pyridine Nucleotides

The pyridine nucleotides nicotinamide adenine dinucleotide (NAD^+) and NAD^+ phosphate ($NADP^+$) are commonly occurring electron carriers that are involved in metabolic reactions as well as intracellular signaling [63,64]. It is known that in plants $NAD(P)^+$ can be released outside the cell [65]. Extracellular $NAD(P)^+$ ($eNAD(P)^+$) induces the expression of pathogen-related genes and the resistance to *Pseudomonas syringae* in *Arabidopsis thaliana* through pathways involving calcium- and salicylic acid-mediated defense signaling. It was also indicated that $eNAD(P)^+$ induces transcriptional and metabolic changes in *Arabidopsis thaliana* similar to those caused by pathogen infection [66]. Moreover, the expression of the human $NAD(P)^+$-hydrolyzing ecto-enzyme CD38 in *Arabidopsis thaliana* partially compromises systemic acquired resistance (SAR) and this suggests that $eNAD(P)^+$ can be a SAR signal molecule [67]. Recent studies on the function of $eNAD(P)^+$ focus on understanding their role in plants and on identifying their receptor(s). Analysis of transcriptome changes in *Arabidopsis thaliana* evoked by $eNAD^+$ identified a lectin receptor kinase (LecRK) LecRK-I.8 as a potential $eNAD^+$ receptor. LecRK-I.8 is located in the plasma membrane, has kinase activity, and specifically binds only NAD^+, but not $NADP^+$, ATP, ADP or AMP. Moreover, the expression of LecRK-I.8 is induced by the $eNAD^+$ [68]. Another LecRK that can be a potential receptor for $eNAD(P)^+$ is LecRK-VI.2, which was identified in *Arabidopsis thaliana*. LecRK-VI.2 is constitutively associated with Brassinosteroid Insensitive1-Associated Kinase1 (BAK1) and it was shown that complex LecRK-VI.2/BAK1 is involved in SAR [69].

4. Uncommon Nucleotides as Signaling Molecules

4.1. Mononucleoside Polyphosphates

4.1.1. Structure and Occurrence of Mononucleoside Polyphosphates

Despite mono- and dinucleoside polyphosphates having been discovered in the middle of the twentieth century, our knowledge about their biological function is still poor, especially in plants. Mononucleoside polyphosphates (p_nNs) contain a nucleoside and oligophosphate chain. Examples of these nucleotides are adenosine 5′-tetraphosphate (p_4A, pppp$A$, Figure 2) and adenosine 5′-pentaphosphate (p_5A, ppppp$A$). Both of them were discovered in commercial preparations of ATP obtained from bovine cells [70,71], horse muscle [72] and yeast [73]. Additionally, other purine and pyrimidine p_4Ns were found as contamination of various nucleoside triphosphates (NTP) preparations: p_4G [74,75], p_4U [76], and p_4C [77]. The existing of p_4A and p_5A was confirmed in biological materials such as rat liver [78,79], rabbit and horse muscle [80], bovine adrenal medulla [80–83], rabbit thrombocytes [84], and *Saccharomyces cerevisiae* [85]. However, the concentration of p_4A in the above-mentioned animal samples was about 2 µM, but in chromaffin granules from the adrenal medulla it was about 800 µM, and it was even up to 4 orders of magnitude and 300-fold lower than ATP concentration, respectively [83]. Until now there is no information about the content of p_nNs in the plant tissues.

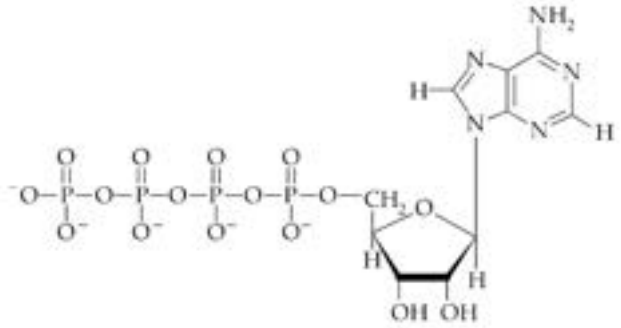

Figure 2. Structure of adenosine 5′-tetraphosphate (p_4A).

4.1.2. Synthesis and Degradation of Mononucleoside Polyphosphates

The level of p_nNs in a cell depends on its biosynthesis and degradation, but also p_nNs may be a product of the degradation of some dinucleoside polyphosphates ($Np_nN's$). Enzymes that can synthesize p_nNs in vitro can be grouped into two categories: aminoacyl-tRNA synthetases (AARS) and non-aminoacyl-tRNA synthetases (non-AARS) [86]. Among enzymes synthesizing p_nNs only one belongs to the AARS and it is lysyl-tRNA synthetase (LysRS) from *Escherichia coli*, which can synthesize p_4A [87,88]. The majority of the enzymes belong to the non-AARS. Table 1 presents non-plant enzymes synthesizing p_nNs.

Table 1. Non-plant enzymes synthesizing mononucleoside polyphosphates (p_nNs).

Enzyme	Organism	Reaction (E, Enzyme)	References
Lysyl-tRNA synthetase (EC 6.1.1.6)	*Escherichia coli*	1st step: E + lysine + pppA ↔ ↔ E-lysyl-pA + PP$_i$ 2nd step: E-lysyl–pA + ppp → → pppA + lysine + E	[87–89]
Luciferase EC 1.13.12.7	*Photinus pyralis*	1st step: E + luciferin + pppA → → E-luciferin–pA + PP$_i$ 2nd step: E-luciferin–pA + (p)ppp → → (p)pppA+ luciferin + E	[90]
UTP:glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	*Saccharomyces cerevisiae*	1st step: glucose-1-P + pppU → → Upp-glucose + PP$_i$ 2nd step: Upp-glucose + (p)ppp → → (p)pppU + glucose-1-P	[100]
Phosphoglycerate kinase (EC 2.7.2.3)	*Saccharomyces cerevisiae*	1,3-ppGly + pppA ↔ ↔ 3-pGly + ppppA	[80,101]
Adenylate kinase (EC 2.7.4.3)	Rabbit and pig muscles	ppA + pppA ↔ pA + ppppA	[102]
Succinyl-CoA synthetase (EC 6.2.1.5)	*Escherichia coli*	E-P + pppA ↔ E + ppppA	[103]
Acetyl-CoA synthetase (EC 6.2.1.1)	*Saccharomyces cerevisiae*	acetyl–pA + ppp ↔ acetate + ppppA	[104,105]
Acyl-CoA synthetase (EC 6.2.1.3)	*Pseudomonas fragi*	acyl–pA + ppp ↔ fatty acid + ppppA	[106,107]
DNA ligase (EC 6.5.1.1)	T4 phage, *Pyrococcus furiosus*	E-pA + ppp ↔ E + ppppA	[105,108–110]
RNA ligase (EC 6.5.1.3)	T4 phage	E-pA + (p)ppp ↔ E + (p)pppA	[111]
UDP-MurNAc-L-alanine:D-glutamate ligase (EC 6.3.2.9)	*Escherichia coli*	E-acyl-P + pppA ↔ ppppA + acyl + E	[112]

Among enzymes that can degrade p_nNs some of them exhibit low substrate specificity, for example, alkaline (EC 3.1.3.1) and acid phosphatases (EC 3.1.3.2), that release phosphate residues up to adenosine [113]. Apyrase (EC 3.6.1.5) also can cut off phosphate residues, but only to

AMP [113]. Phosphodiesterase I (EC 3.1.4.1) degrades p_4A to triphosphate and AMP [100]. Additionally, adenosine-phosphate deaminase from *Aspergillus oryzae* and *Helix pomatia* is involved in p_4A metabolism, converting it into inosine 5′-tetraphosphate (p_4I) [114].

As mentioned above, p_nNs can accumulate in the cell as a result of the degradation of Np_nN's. The way in which p_4A can accumulate in a cell is the degradation of diadenosine 5′,5‴-pentaphosphate (Ap_5A) and diadenosine 5′,5‴-hexaphosphate (Ap_6A). The degrading enzymes include phosphodiesterase I (EC 3.1.4.1) occurring in prokaryotes and eukaryotes [115,116], symmetrical dinucleoside tetraphosphatase (EC 3.6.1.41) from bacteria [117], dinucleoside tetraphosphate phosphorylase (EC 2.7.7.53) from *Saccharomyces cerevisiae* and *Euglena gracilis* [118,119], and dinucleoside triphosphatase (EC 3.6.1.29) among others from *Lupinus luteus* [120].

Despite the lack of knowledge about the occurrence and the concentration of mononucleoside polyphosphates in higher plants, there are described a few enzymes which can synthesize p_nNs. All of them are listed in Table 2. The first described plant enzyme that synthesizes p_nNs is 4-coumarate:CoA ligase (4CL2) from *Arabidopsis thaliana* that catalyzes the reaction of the synthesis of both p_4A and p_5A [121]. This enzyme is a branch point in the phenylpropanoid pathway that leads to the biosynthesis of flavonoids, lignin, and stilbenes. It is known that the phenylpropanoid pathway is involved in plant responses to numerous environmental stimuli, especially under biotic and abiotic stresses [122]. Another enzyme synthesizing p_4A is jasmonate:amino acid synthetase from *Arabidopsis thaliana* (JAR1) [123]. JAR1 is involved in the function of jasmonic acid (JA) as a plant hormone and it catalyzes the synthesis of several JA-amido conjugates, the most important of which appears to be jasmonic acid-isoleucine. Both of the above-mentioned plant enzymes belong to the acyl–adenylate-forming firefly luciferase superfamily [124] which catalyzes a two-step reaction. During the first step, acyl and ATP form an acyl-adenylate intermediate with the simultaneous release of pyrophosphate (PP_i). In the second step, 4CL in the absence of CoA catalyzes the formation of uncommon mononucleoside polyphosphates such as p_4A and p_5A [121].

Table 2. Plant enzymes synthesizing mononucleoside polyphosphates (p_nNs).

Enzyme	Organism	Reaction (E, Enzyme)	References
4-Coumarate:CoA ligase (4CL2) (EC 6.2.1.12)	*Arabidopsis thaliana*	1st step: E + coumarate + ATP → → E-coumaroyl–pA + PP_i 2nd step: E-coumaroyl–pA + (p)ppp → → (p)pppA + coumarate + E	[121]
Jasmonate:amino acid synthetase (JAR1) (EC 6.3.2.52)	*Arabidopsis thaliana*	1st step: E + jasmonate + ATP → → E-jasmonyl–pA + PP_i 2nd step: E-jasmonyl–pA + ppp → → pppA + jasmonate + E	[123]

In plants there also exists an enzyme degrading p_4A. It is nucleoside tetraphosphate hydrolase (EC 3.6.1.14) occurring in *Lupinus luteus* seeds. This enzyme hydrolyzes both p_4A and p_4G with the same rate while p_5A is degraded up to 200-fold more slowly than p_4A and p_4G [120].

4.2. Adenosine 5′-Phosphoramidate

Structure, Occurrence, and Metabolism of Adenosine 5′-Phosphoramidate

Among naturally occurring uncommon mononucleotides is adenosine 5′-phosphoramidate (NH_2-pA, Figure 3). This compound is believed to occur in all organisms; however, so far NH_2-pA has only been detected among cellular nucleotides purified from the green alga *Chlorella pyrenoidosa* [125]. Similarly to p_nNs, the level of NH_2-pA in a cell is enzymatically controlled. It is known that NH_2-pA can be synthesized by adenylyl sulfate:ammonia adenylyltransferase (EC 2.7.7.51) in the algae *Chlorella pyrenoidosa*, *Euglena gracilis*, amoeba *Dictyostelium discoideum*, bacteria *Escherichia coli*, and in higher plants *Hordeum vulgare*, *Spinacia oleracea* [126], and *Lupinus luteus* [127]. This transferase catalyzes the following reaction: SO_4-pA + NH_4^+ → NH_2-pA + SO_4^{2-} + $2H^+$.

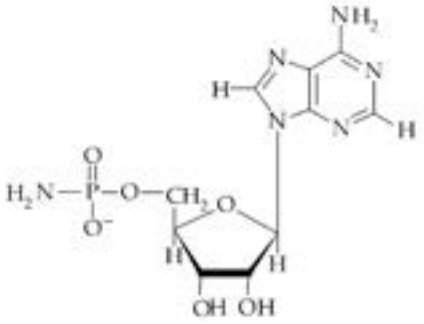

Figure 3. Structure of adenosine 5′-phosphoramidate (NH_2-pA).

The supposition that NH_2-pA is a ubiquitous compound and that its concentration is enzymatically controlled may be supported by the existence of various enzymes that catalyze the cleavage of NH_2-pA to ammonia and AMP (pA) by hydrolysis [128–133], or to ammonia and ADP (ppA) by phosphorolysis [130]. Both the synthesis [127] and the degradation [131] of NH_2-pA can be controlled by HIT (histidine triad) proteins. One of the proteins belonging to the HIT family proteins is Fhit (fragile histidine triad). Based on the mechanism of the action of Fhit protein, which is able to hydrolyze the P-N bond in adenosine phosphoimidazolide [134], it is hypothesized that other uncommon nucleotides can be substrates for human and *Arabidopsis thaliana* Fhit. Other proteins belonging to HIT family proteins are Hint protein (having the activity of NH_2-pA hydrolase) and GalT proteins (having the specific activity of nucleoside monophosphate transferase) [135]. The protein Fhit from human and *Arabidopsis thaliana* exhibited the activity attributed to adenylyl sulfate sulfohydrolase and nucleoside phosphoamidases releasing AMP from adenosine 5′-phosphosulfate (SO_4-pA) and NH_2-pA, respectively. Fhit protein also catalyzes the hydrolysis of the P-F bond in the synthetic nucleotide adenosine 5′-monophosphofluoride (F-pA) releasing AMP [136]. Recently it has been shown that NH_2-pA can also be synthesized by Fhit proteins from *Lupinus luteus* seeds and *Arabidopsis thaliana*. It catalyzes the ammonolysis of SO_4-pA, leading to the formation of NH_2-pA [127].

4.3. Dinucleoside Polyphosphates

4.3.1. Structure and Occurrence of Dinucleoside Polyphosphates

Dinucleoside polyphosphates (Np_nN's) consist of an oligophosphate chain that links the two 5′-esterified nucleosides. Among Np_nN's that are most frequently tested are adenine nucleotides: diadenosine triphosphate (Ap_3A) and diadenosine tetraphosphate (Ap_4A, Figure 4). The first reports about the existence of Np_nN's come from the 1950s. It was discovered that during the chemical synthesis of adenosine-uridine monophosphate (ApU) there were synthesized byproducts such as diadenosine diphosphate (Ap_2A) and diuridine diphosphate (Up_2U) [137]. Nevertheless, the first Np_nN discovered in the biological material was diguanosine tetraphosphate (Gp_4G). The presence of Gp_4G and Gp_3G was observed in biological preparations in encysted gastrulae of the brine shrimp and *Artemia salina* in millimolar concentrations [138,139]. The most common Np_nN is Ap_4A, which for the first time was detected in rat liver, and its concentration was estimated at 30 nM [97]. Subsequently Ap_4A and other Np_nN's have been observed in submicromolar concentrations in many investigated cells and tissues [140,141]. Np_nNs were identified in bacteria [142,143], yeast [144,145], and animal and human cells [140,146–150]. So far, Np_nN's have not been identified in plants.

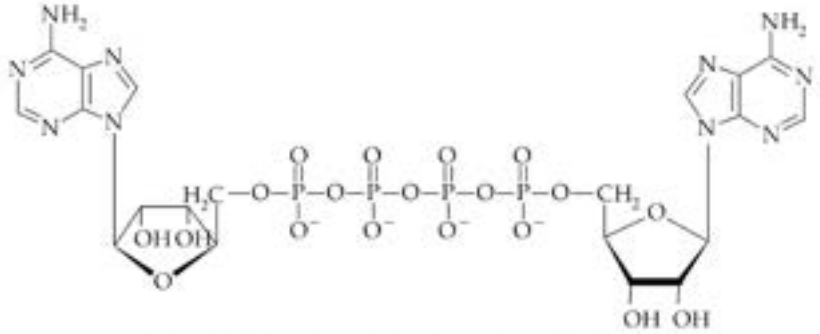

Figure 4. Structure of diadenosine 5', 5'''-tetraphosphate (Ap_4A).

4.3.2. Synthesis and Degradation of Dinucleoside Polyphosphates

The level of Np_nN's in cells may be a result of the synthesis and degradation of these compounds. Table 3 lists non-plant enzymes synthesizing various Np_nN's. Enzymes synthesizing Np_3N's are some ligases [89,92,106,121,151,152], luciferase from *Photinus pyralis* [153], and some transferases [100,119,154]. Among aminoacyl-tRNA synthetases, the most effective in Ap_nN synthesis are lysyl- (EC 6.1.1.6), phenylalanyl- (EC 6.1.1.20), alanyl- (EC 6.1.1.7), and prolyl- (EC 6.1.1.15) t-RNA synthetases [86]. Moreover, the RNA-dependent RNA polymerase elongation complex from hepatitis C virus (HCV) can use in vitro nucleoside triphosphates (NTPs) to excise the terminal nucleotide in nascent RNA and mismatched ATP, UTP, or CTP could mediate excision of 3'-terminal CMP to generate the dinucleoside tetraphosphate products Ap_4C, Up_4C, and Cp_4C, respectively [155].

Table 3. Non-plant enzymes synthesizing dinucleoside polyphosphates (Np_nN's).

Enzyme	Organism	Reaction (E, Enzyme)	References
Luciferase (EC 1.13.12.7)	*Photinus pyralis*	1st step: E + luciferin + pppA ↔ ↔ E-luciferin–pA + PP_i 2nd step: E-luciferin–pA + pppN → → AppppN+ luciferin + E	[89]
GTP:GTP guanylyltransferase (EC 2.7.7.45)	*Artemia salina*, *Saccharomyces cerevisiae*	1st step: E + pppN ↔ E–pN + PP_i 2nd step: E–pN + pppN → N_4pppN' + E	[154]
UTP:glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	*Saccharomyces cerevisiae*	1st step: glucose-1-P + pppU → → Upp-glucose + PP_i 2nd step: Upp-glucose + pppN → → UpppN + glucose-1-P	[100]
Acyl-CoA synthetase (EC 6.2.1.3)	*Pseudomonas fragi*	1st step: Fatty acid + pppA ↔ ↔ acyl–pA + PP_i 2nd step: acyl–pA + pppA → → AppppA + fatty acid	[108,107]
RNA ligase (EC 6.5.1.3)	T4 phage	E-pA + pppN ↔ E + Ap_3N	[111]
DNA ligase (EC 6.5.1.1)	T4 phage, *Pyrococcus furiosus*	E-pA + pppN ↔ E + Ap_4N	[105,108–110]
Lysyl-tRNA synthetase (EC 6.1.1.6)	*Escherichia coli*	1st step: E + lysine + pppA ↔ ↔ lysyl–pA + PP_i 2nd step: E:lysyl–pA + pppN → → AppppN + lysine + E	[86]

So far, only three enzymes synthesizing Np_nN's have been described in plants (Table 4). Among aminoacyl-tRNA synthetases only phenylalanyl- (EC 6.1.1.20) and seryl-tRNA (EC 6.1.1.11) synthetases from *Lupinus luteus* [152] synthesize Ap_nN. The mechanism of the synthesis of Ap_nNs catalyzed by plant aminoacyl-tRNA synthetases is based on the formation of aminoacyl–pA and the transfer of

adenylate to pppN [152]. The third enzyme which is involved in the synthesis of Ap_nN in plant cells is 4coumarate-CoA ligase (EC 6.2.1.12) [121].

Table 4. Plant enzymes synthesizing dinucleoside polyphosphates (Np_nN's).

Enzyme	Organism	Reaction (E, Enzyme)	References
Phenylalanyl-tRNA synthetase (EC 6.1.1.20)	*Lupinus luteus*	1st step: E + phenylalanine + pppA ↔ ↔ phenylalanyl–pA + PPi 2nd step: E:phenylalanyl–pA + pppN → → ApppppN + phenylalanine + E	[152]
Seryl-tRNA synthetase (EC 6.1.1.11)	*Lupinus luteus*	1st step: E + serine + pppA ↔ ↔ seryl–pA + PPi 2nd step: E:seryl–pA + pppA → → AppppA + serine + E	[152]
4-Coumarate:CoA ligase (4CL2) (EC 6.2.1.12)	*Arabidopsis thaliana*	1st step: E + coumarate + pppA → → E:coumaroyl–pA + PPi 2nd step: E:coumaroyl–pA + pppN → → NppppN+ coumarate + E	[121]

Enzymes degrading Np_nN's may be divided into substrate-specific and substrate non-specific. Substrate-specific enzymes degrading Np_nN's include, among others, *asymmetrical* Np_4N hydrolase (EC 3.6.1.17), *symmetrical* Np_4N hydrolase (EC 3.6.1.41), and dinucleoside triphosphate (Np_3N) hydrolase (EC 3.6.1.29) [156]. In plants, the activity of *asymmetrical* Np_4N hydrolase was detected in *Lupinus luteus* seeds [157], *Helianthus annuus* and marrow (*Cucurbita pepo*) seeds [158], tomato cells [159], *Lupinus angustifolius* seeds [160] and in *Hordeum vulgare* [161]. Among Np_4N's, only Ap_4A can be degraded by the *asymmetrical* Np_4N hydrolase giving ATP. The substrate non-specific enzymes in plants are phosphodiesterase I (EC 3.1.4.1) from *Lupinus luteus* [157] and nucleotide pyrophosphatase (EC 3.6.1.9) from potato tuber [162].

4.4. Function of Uncommon Nucleotides

The cellular level of Np_nN's is modified under various physiological and pathological conditions and the compounds have been suggested as intracellular messengers in diverse cellular processes [163,164]. The increased concentration of Ap_4A was correlated with high cellular proliferation rates [165] or some phases of the cell cycle [165,166]. A dramatic increase of levels of Np_4N and various Np_nN's has been observed in cells subjected to stresses, such as elevated temperature, ethanol or cadmium [143–145,167]. A very interesting function of uncommon nucleotides is their potential function as alarmones [168]. However, so far no clear metabolic or molecular targets or receptors of the postulated alarm signaled by the Np_nN's have been experimentally demonstrated. Alarmones are intracellular signaling molecules that are produced under adverse environmental factors. They regulate gene expression at the transcriptional level. Nucleotides (p)ppGpp and ppGpp have such a role in bacteria [169]. The results of recent studies on the role of uncommon nucleotides focus on microorganisms and animals, including man. It has been demonstrated that Np_nN's may accumulate in cells under stress conditions. In cells of *Saccharomyces cerevisiae* and *Escherichia coli* under elevated temperature and cadmium ions an increased level of Np_nN's was observed. The intracellular concentrations of these molecules vary from 10^{-9} M in a basal metabolic state to 10^{-4} M when the organisms are subjected to stresses, such as heat shock or exposure to cadmium [143,167]. In cyanobacteria the same effects are a result of thermal shock and heavy metals [145]. In *Salmonella*, the increased synthesis of Np_nN's was caused by ethanol [142]. Studies performed on orange fruit showed that under high temperature enzymes involved in Np_nN' metabolism were activated and an extremely large increase in bis(5′-adenosyl) triphosphatase protein content was observed. These results suggest intensive synthesis of Ap_3A under high temperature [170]. Another group of enzymes involved in Np_nN' metabolism is Nudix (nucleoside diphosphate linked to x) hydrolases (NUDTs). An increase of transcript and protein NUDT7 levels was found in *Arabidopsis thaliana* plants under biotic stress evoked by *Pseudomonas syringae* avrRpt2 [171]. In *Arabidopsis thaliana*, there was also observed a quick

increase in NUDT7 content under the effect of ozone and pathogen infection [172]. Recent studies on AtNUDT19 indicate the involvement of this hydrolase in the photo-oxidative stress response by regulating photosynthesis, the antioxidant system and the synthesis of salicylic acid—a signaling molecule involved in the response to biotic and abiotic stresses [173]. Additionally, it is suggested that barley NUDTs respond to abiotic stress [174]. It is also known that NUDTs bind RNA and participate in the regulation of gene expression in animals and plants [175–177].

Studies on the function of Ap_4A in *Escherichia coli* have shown that it may be a damage metabolite and a few proteins binding Ap_4A in these bacteria were identified [178]. Recent studies on the function of Ap_4A have shown that aminoglycoside antibiotics induced synthesis of Ap_4A in *Escherichia coli* and it caused bacterial cell killing by these antibiotics [179]. Lysyl-tRNA synthetase plays a key role in MITF (microphthalmia-associated transcription factor) transcriptional activity via Ap_4A as an important signaling molecule in mast cells [180,181]. Studies on chronic myelogenous leukemia cells showed that NUDT2 disruption elevates Ap_4A and down-regulates immune response and cancer promotion genes [182]. Ap_3A and Ap_4A induce activation of a signaling pathway that results in increase of proliferation of vascular smooth muscle cells by stimulation of ERK1/2(MAP kinase) [183]. It is known that Np_nN's can act as neurotransmitters and influence the vascular system through purinergic receptors. There is also evidence that Ap_4A inhibits the initiation of DNA replication. It is also proposed that Ap_4A acts as an important inducible ligand in the DNA damage response to prevent the replication of damaged DNA [184]. In contrast, the increase in the ratio of Ap_3A/Ap_4A in human HL60 cells can induce apoptosis [164].

So far there are only a few papers describing the role of uncommon nucleotides in plant cells. It was shown that the phenylpropanoid pathway may be affected by exogenous uncommon nucleotides [185–188]. The phenylpropanoid pathway is a source of secondary metabolites which are very important in plant responses to biotic and abiotic stresses [122]. The first data showed that Ap_3A and Ap_4A regulate the expression of genes and the activity of enzymes involved in the phenylpropanoid pathway in seven-day old *Arabidopsis thaliana* seedlings [185]. Ap_3A and Ap_4A caused an increase in the activity of phenylalanine ammonia-lyase (PAL) and 4:coumarate-CoA ligase (4CL) just 10 min after application. Analysis of *PAL* gene expression showed that there was strong induction (about 70-fold) of *PAL2* gene expression by Ap_3A and Ap_4A. Additionally, PAL activity was significantly enhanced by Ap_3A (about 9-fold). Moreover, it has been shown that the activity of 4CL and the *4CL* gene expression level were higher in seedlings treated with Ap_3A and Ap_4A [185]. These results may indicate a dual role of 4CL in the plant response to stress factors. Firstly, this enzyme synthesizes Np_nN's, i.e., compounds that can play a function as alarmones [121], and secondly, it synthesizes secondary metabolites minimizing the adverse effects of stresses in plant cells [189]. Also, Ap_3A caused changes in the gene expression level and the activity of chalcone synthase (CHS) in *Arabidopsis thaliana* seedlings. This enzyme catalyzes the synthesis of chalcone, which is the precursor of secondary metabolites—flavonoids [185]. These data strongly indicate the signaling role of Np_nN's in plants. The inductive effect of Ap_3A on gene expression of the phenylpropanoid pathway proteins has also been confirmed in a cell suspension of *Vitis vinifera* cv. Monastrell. A synergistic effect evoked by Ap_3A and cyclodextrins in *trans*-resveratrol biosynthesis has been demonstrated [186]. It may suggest the involvement of Np_nN's in the plant response to stress. Interestingly, cyclodextrins act as elicitors in their chemical similarity to the alkyl-derivatized pectic oligosaccharides, which are released from the cell wall during fungal infection [190]. It is known that one of the defense strategies of higher plants against biotic and abiotic stresses is activation of the phenylpropanoid pathway leading to enhanced production of various phenylpropanoid compounds, such as flavonoids [191,192], lignin [193,194], anthocyanins [194–196], and salicylic acid [197] (Figure 5). Recently the differences in the regulation of gene expression of the phenylpropanoid pathway enzymes and phenylpropanoid accumulation by purine or pyrimidine Np_nN's in *Vitis vinifera* suspension cell culture were described. The pyrimidine dinucleotides, such as Cp_3C, Cp_4C, and Ap_4C, markedly (6- to 8-fold) induced the expression of the gene coding the cinnamoyl-CoA:NADP oxidoreductase (CCR) that controls lignin biosynthesis.

The most effective in stilbene accumulation was Up_4U, but other pyrimidine dinucleotides (Cp_3C, Cp_4C, and Ap_4C) strongly inhibited the biosynthesis of these phenylpropanoids [188].

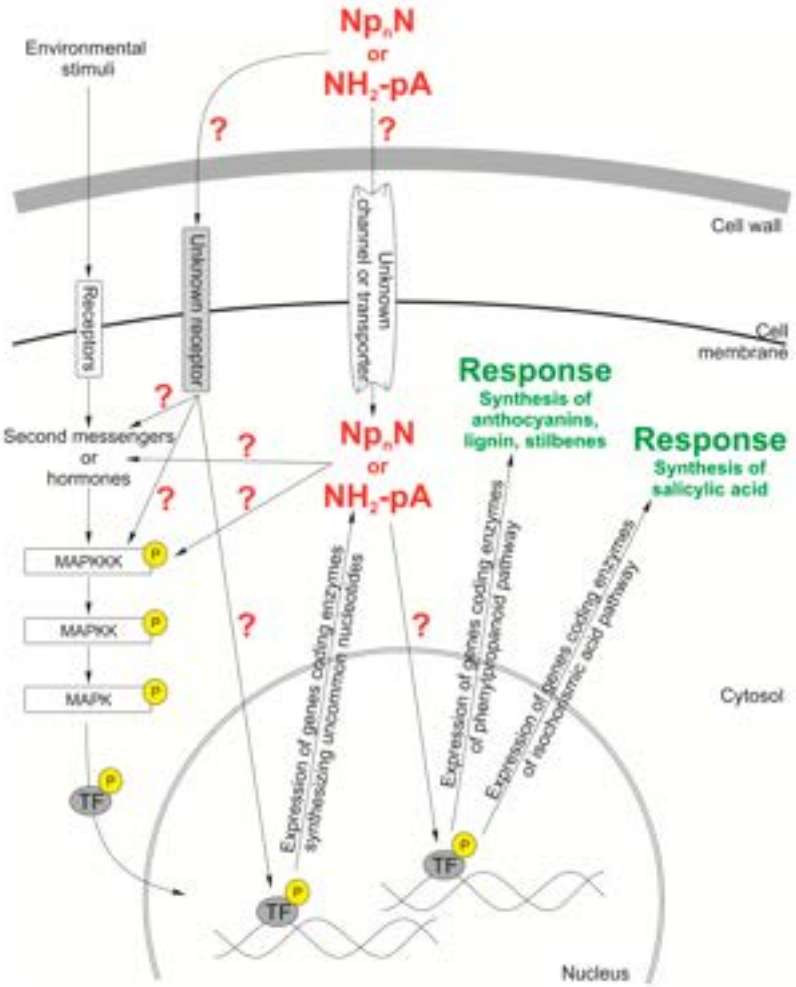

Figure 5. Hypothetical working model of Np_nN' and NH_2-pA signaling network in a plant cell. Dinucleoside polyphosphate (Np_nN) and adenosine 5′-phosphoramidate (NH_2-pA) trigger induction of the phenylpropanoid and the isochorismic acid pathways yielding metabolites protecting plant against various types of stresses. Plant cells respond to environmental stimuli by intensification of the production of various compounds, such as anthocyanins, lignin, stilbenes, and salicylic acid. Question marks indicate the hypothetical components of the signaling network.

As described above, in plants, the presence of enzymes degrading and synthesizing another uncommon nucleotide, NH_2-pA, has been described [127,132]. It was evidenced that NH_2-pA regulates the expression of genes coding enzymes of the phenylpropanoid pathway, such as PAL,

cinnamate-4-hydroxylase (C4H), 4CL, CHS, CCR, and isochorismate synthase (ICS) in *Arabidopsis thaliana* seedlings. CCR is an enzyme involved in the biosynthesis of lignin, whereas ICS is involved in the biosynthesis of a signaling molecule—salicylic acid. Among the analyzed genes the strongest induction in gene expression caused by NH_2-pA was observed for *CCR2* (approx. 4-fold). This was also accompanied by an increased level of lignin in the seedlings. Another important effect caused by NH_2-pA was an increase in the *ICS* gene expression and a significant increase (2-fold) in the level of free salicylic acid. In view of the fact that salicylic acid is one of the signaling molecules involved in the response of plants to biotic stress, the induction of the synthesis of salicylic acid by NH_2-pA may suggest the interaction of both these compounds in plant responses to stresses [187].

Another uncommon nucleotide occurring in plants is nicotinic acid adenine dinucleotide phosphate (NAADP). The synthesis of NAADP is not fully understood. However, it is supposed that it can be synthesized by adenosine-5′- diphosphateribosyl-cyclase (EC 3.2.2.5) [198]. This nucleotide is involved in calcium signaling in many organisms including plants [199–201]. NAADP-mediated calcium release has been shown in the microsomal vesicles of red beets (*Beta vulgaris*) and cauliflower (*Brassica oleracea*). Analysis of sucrose gradient-separated cauliflower microsomes revealed that the NAADP-sensitive Ca^{2+} pool was derived from the endoplasmic reticulum [199].

4.5. Uncommon Nucleotide Signal Transduction Pathways in Plants

As yet there has been no information about signal transduction pathways or receptors activated by uncommon nucleotides. Based on the data existing in the literature about the involvement of these molecules in different biochemical and physiological processes in bacteria, fungi, animal, and plant organisms, we propose putative signaling pathways induced by uncommon nucleotides in plant cells (Figure 5). It is hypothesized that uncommon nucleotides may be signal molecules synthesized by pathogens outside the cell and interact with plant cells through unknown plasma membrane receptor(s). These nucleotides can also be transported inside the plant cell by unknown plasma membrane transporter(s). In addition, their level in the plant cell can be regulated by synthesizing and degrading enzymes. Both the extracellular and intracellular uncommon nucleotides may affect the biosynthesis of other plant cell signal molecules, e.g., secondary messengers and hormones that can engage MAPK cascades, which are involved in plant growth and development, cellular responses to hormones, regulation of the cell cycle, and responses to biotic and abiotic stresses, such as pathogen infection, wounding, low temperature, drought, high salinity, metals, and ROS. Moreover, it is known that MAPK cascades can regulate gene transcription by activation or repression of transcription factors. In addition, MAPK transcript levels in *Arabidopsis thaliana* seedlings were shown to increase in a time-dependent manner following exposure to Cu and Cd [202]. Many MAPK cascades respond to hormones such as abscisic acid, jasmonic acid, salicylic acid, ethylene, auxins, and brassinosteroids. Usually, signaling molecules participate in distinct signaling pathways, resulting in the formation of a cross-talking network that co-ordinates responses to different stresses [202–204]. We postulate that MAPK cascades can be involved in the signal transduction pathway triggered by uncommon nucleotides. So far, it is only known that extracellular uncommon nucleotides can induce gene expression of the phenylpropanoid pathway, the accumulation of phenylpropanoids, and the synthesis of salicylic acid. However, components of the nucleotide transduction pathway are still not known (Figure 5).

5. Conclusions

Plants are not able to escape from their abiotic (cold, heat, etc.) or their biotic (herbivores, insects) environment [205]. Furthermore, their food uptake and gas exchange take place through external surfaces (leaves: light and CO_2, roots: ions and water). Plants must, therefore, possess systems to exchange information throughout the entire plant to ensure the coordination of plant development and defense. Evidence suggests that in plants information exchange relies on at least two different systems: one involving molecules that are transported within the plant and another that uses electrical

and/or hydraulic signals to carry the information throughout the entire organism [205]. The systems for transmitting this information are complex and involve multiple components, which are far from being understood. For many years plant hormones were considered to be dominant molecules in plant signaling. Nowadays, this term is applied to many more compounds. It is suggested that dynamic changes in the level of second messengers, such as Ca^{2+}, ROS, and NO, serve as signatures for both intracellular signaling and cell-to-cell communications. These second messenger signatures work in concert with physical signal signatures (such as electrical and hydraulic signals) to create a "lock and key" mechanism that triggers an appropriate response to various stresses [206]. Part of the system, new players in plant signaling, are chemical signals such as extracellular ATP and uncommon nucleotides described and discussed in this work. These molecules are essential for proper coordination of processes in particular organs as well as responses to internal and external signals playing analogical or similar functions in plants and in animals. The data obtained during the last few years suggest that in plants both purine and pyrimidine dinucleotides should be considered as members of a family of naturally occurring stress signaling molecules. So far, our knowledge of these signals in plants is still insufficient to clearly understand their signaling role. The mechanisms of signal perception and transduction are unknown. Further studies are required, which should aim at describing the signaling networks involving uncommon nucleotides, and also perform omic experiments to identify the network components and processes that are regulated by these signals.

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Abbreviations

1,3-ppGly	1,3-Diphosphoglycerate
3-pGly	3-Phosphoglycerate
4CL	4-coumarate:CoA ligase
AARS	Aminoacyl t-RNA synthetase
ABC transporter	ATP-binding cassette ABC transporter
AMP, pA	Adenosine 5′-monophosphate
Ap_3A, ApppA	Diadenosine 5′,5‴-P^1,P^3-triphosphate
Ap_4A, AppppA	Diadenosine 5′,5⁗-P^1,P^4-tetraphosphate
Ap_4C	P^1-(5′-Adenosyl)-P^4-(5′-cytidyl)-tetraphosphate
ApU	Adenosine-uridine monophosphate
APY1 and APY2	Apyrase 1 and 2
C4H	Cinnamate-4-hydroxylase
cAMP	Adenosine 3′,5′-cyclic monophosphate
CAMTA3	Calmodulin-binding transcription activator
CCR	Cinnamoyl-CoA:NADP oxidoreductase
cGMP	Guanosine 3′,5′-cyclic monophosphate
CHS	Chalcone synthase
Cp_3C	Dicytosine 5′,5‴-P^1,P^3-triphosphate
Cp_4C	Dicytosine 5′,5⁗-P^1,P^4-tetraphosphate
DORN1	Does not Respond to Nucleotide

E	Enzyme
eATP	Extracellular ATP
eNAD$^+$	Extracellular NAD$^+$
eNADP$^+$	Extracellular NADP$^+$
Fhit	Fragile histidine triad
F-pA	Adenosine 5′-O-phosphorofluoridate
Gp_3G	Diguanosine 5′,5‴-P^1,P^3-triphosphate
Gp_4G	Diguanosine 5′,5‴-P^1,P^4-tetraphosphate
HIT	Histidine triad
ICS	Isochorismate synthase
ISR	Induced systemic resistance
JAR1	Jasmonate:amino acid synthetase
LecRK-I.9	L-type lectin receptor-like kinase I.9
LysRS	Lysyl-tRNA synthetase
MAPK	Mitogen-activated protein kinases
MITF	Microphthalmia-associated transcription factor
MYC	Transcription factors
NAADP	Nicotinic acid adenine dinucleotide phosphate
NH_2-pA	Adenosine 5′-phosphoramidate
NO	Nitric oxide
Non-AARS	Non-aminoacyl t-RNA synthetase
Np_nN'	Dinucleoside polyphosphate
NTP, pppN	Nucleoside 5′-triphosphate
NUDT	Nudix hydrolase
Nudix	Nucleoside diphosphate linked to x (x: any moiety)
P2K1	P2 receptor kinase 1
P2X	Ligand-gated ion channel
P2Y	G protein-coupled receptor
P_3, ppp	Triphosphate
P_4, pppp	Tetraphosphate
p_4A, ppppA	Adenosine 5′-tetraphosphate
p_4C	Cytosine 5′-tetraphosphate
p_4G	Guanosine 5′-tetraphosphate
p_4U	Uridine 5′-tetraphosphate
p_5A, pppppA	Adenosine 5′-pentaphosphate
PAL	Phenylalanine ammonia-lyase
PGP1	p-Glycoprotein 1
PM-ANT1	Plasma membrane-localized nucleotide transporters
p_nN	Nucleoside 5′-polyphosphate
ppGpp	Guanosine 3′-diphosphate 5′-diphosphate
PP_i	Pyrophosphate
(p)ppGpp	Guanosine 3′-diphosphate 5′-triphosphate
RBOHD	Respiratory burst oxidase homolog protein D
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
STS	Stilbene synthase
TF	Transcription factor
Up_2U	Diuridine 5′,5‴-P^1,P^2-diphosphate
UTP, pppU	Uridine 5′-triphosphate

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Publikacja 2

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Research article

Purine and pyrimidine dinucleoside polyphosphates differentially affect the phenylpropanoid pathway in *Vitis vinifera* L. cv. Monastrell suspension cultured cells

Małgorzata Pietrowska-Borek[a,*], Anna Wojdyła-Mamoń[a], Jędrzej Dobrogojski[a], Agnieszka Młynarska-Cieślak[b], Marek R. Baranowski[b], Jakub M. Dąbrowski[b], Joanna Kowalska[b], Jacek Jemielity[c], Sławomir Borek[d], Maria Angeles Pedreño[e], Andrzej Guranowski[a]

[a] Department of Biochemistry and Biotechnology, Poznań University of Life Sciences, Dojazd 11, 60-632, Poznań, Poland
[b] Division of Biophysics Institute of Experimental Physics, Faculty of Physics University of Warsaw, Żwirki i Wigury 93, 02-089, Warsaw, Poland
[c] Centre of New Technologies, University of Warsaw, Banacha 2c, 02-097, Warsaw, Poland
[d] Department of Plant Physiology, Adam Mickiewicz University Poznań, Uniwersytetu Poznańskiego 6, 61-614, Poznań, Poland
[e] Department of Plant Biology, Faculty of Biology, University of Murcia, Campus de Espinardo, 30100, Murcia, Spain

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ABSTRACT

It is known that the concentration of dinucleoside polyphosphates (Np_nN's) in cells increases under stress and that adverse environmental factors induce biosynthesis of phenylpropanoids, which protect the plant against stress. Previously, we showed that purine Np_nN's such as Ap_3A and Ap_4A induce both the activity of enzymes of the phenylpropanoid pathway and the expression of relevant genes in *Arabidopsis* seedlings. Moreover, we showed that Ap_3A induced stilbene biosynthesis in *Vitis vinifera* cv. Monastrell suspension cultured cells. Data presented in this paper show that pyrimidine-containing Np_nN's also modify the biosynthesis of stilbenes, affecting the transcript level of genes encoding key enzymes of the phenylpropanoid pathway and of these, Up_4U caused the most effective accumulation of *trans*-resveratrol in the culture media. Similar effect was caused by Ap_3A and Gp_3G. Other pyrimidine Np_nN's, such as Cp_3C, Cp_4C, and Ap_4C, strongly inhibited the biosynthesis of stilbenes, but markedly (6- to 8-fold) induced the expression of the cinnamoyl-CoA reductase gene that controls lignin biosynthesis. Purine counterparts also clearly induced biosynthesis of *trans*-resveratrol and *trans*-piceid, but only slightly induced the expression of genes involved in lignin biosynthesis. In cells, Up_3U caused a greater accumulation of *trans*-resveratrol and *trans*-piceid than did Up_4U. Each of the Np_nN's studied induced expression of the gene encoding the resveratrol transporter VvABCG44, which operates within the *Vitis vinifera* cell membrane. AMP, GMP, UMP, and CMP, potential products of Np_nN degradation, did not affect the accumulation of stilbenes. The results obtained strongly support that Np_nN's play a role as signaling molecules in plants.

1. Introduction

Dinucleoside polyphosphates (Np_nN's, where N and N' are nucleosides and n represents the number of phosphate residues in the oligophosphate chain that esterifies these nucleosides at their 5' positions) occur in all organisms. The first Np_nN discovered in biological material was diguanosine tetraphosphate (Gp_4G), which was observed in encysted gastrulae of the brine shrimp *Artemia franciscana* (Finamore and Warner, 1963). Later, diadenosine tetraphosphate (Ap_4A) was detected in mammalian cells (Rapaport and Zamecnik, 1976). Other Np_nN's were identified in bacteria, yeast (Lee et al., 1983; Coste et al., 1987), and animal and human cells (Lüthje and Ogilvie, 1983). The intracellular concentrations of these molecules vary from 10^{-9} M in a basal metabolic state up to 10^{-4} M when the organisms are under stress, such as heat shock or exposure to cadmium (Lee et al., 1983; Coste et al., 1987). The concentration of Np_nN's in the cell can be regulated by different

Abbreviations: PAL, phenylalanine:ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate:coenzyme A ligase; CHS, chalcone synthase; STS, stilbene synthase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; Ap_3A, P^1,P^3-Di-(adenosyl-5')-triphosphate; Gp_3G, P^1,P^3-Di-(guanosyl-5')-triphosphate; Up_4U, P^1,P^4-Di-(uridyl-5')-polyphosphate; Cp_4C, P^1,P^4-Di-(cytidyl-5')-polyphosphate; Ap_4U, P^1-(5'-Adenosyl)-P^4-(5'-uridyl)-polyphosphate; Ap_4C, P^1-(5'-Adenosyl)-P^4-(5'-cytidyl)-polyphosphate

* Corresponding author.
E-mail address: malgorzata.pietrowska-borek@up.poznan.pl (M. Pietrowska-Borek).

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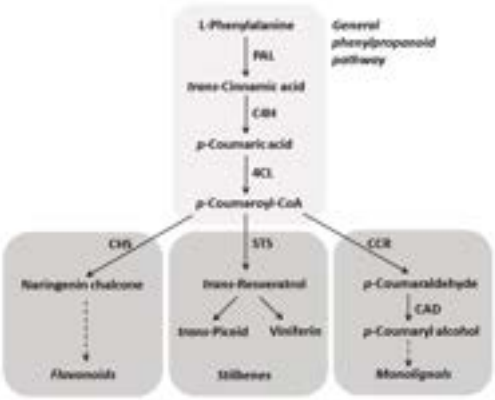

Fig. 1. Simplified diagram of the phenylpropanoid pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase, STS, stilbene synthase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase.

enzymes. The synthesis of Np_nN's can be carried out by certain ligases (Zamecnik et al., 1966; Goerlich et al., 1982; Pietrowska-Borek et al., 2003; for reviews see: McLennan, 1992; Fraga and Fontes, 2011), transferases (Guranowski et al., 2004, 1988), firefly luciferase (Guranowski et al., 1990) and hepatitis C virus RNA-dependent RNA polymerase (Jin et al., 2013). So far, plant ligases known to exhibit such a capacity include phenylalanyl- and seryl-tRNA synthetases from yellow lupin (Jakubowski, 1983) and 4-coumarate-CoA ligase from *Arabidopsis thaliana* (Pietrowska-Borek et al., 2003). Despite the ability of these plant enzymes to synthesize Np_nN's, it has not yet been proved that these dinucleotides accumulate in plants. Enzymes able to degrade Np_nN's occur in all kinds of organisms and may be divided into specific and nonspecific hydrolases and phosphorylases (Guranowski, 2000).

Among secondary metabolites are those that are synthesized from L-phenylalanine *via* the phenylpropanoid pathway (Fig. 1). The first step is mediated by phenylalanine ammonia-lyase (PAL) giving *trans*-cinnamic acid. In the next step, *trans*-cinnamic acid is converted to *p*-coumaric acid by cinnamic acid 4-hydrolase (C4H). The latter compound, by the action of 4-coumarate:CoA ligase (4CL), yields *p*-coumaroyl-CoA. This compound is a branching point of the phenylpropanoid pathway. The *p*-coumaroyl-CoA may condense with three molecules of malonyl-CoA initiating either flavonoid or stilbene biosynthesis, catalyzed by chalcone synthase (CHS) and stilbene synthase (STS) respectively. The *p*-coumaroyl-CoA may also be converted into coumaraldehyde by cinnamoyl-CoA reductase (CCR), initiating the pathway leading to lignan and lignin (Fig. 1). It is known that in plants the synthesis of secondary metabolites in the phenylpropanoid pathway is induced by many environmental stresses (Dixon and Paiva, 1995) and involves induction of the relevant genes. In fact, enhanced expression of *PAL1*, *PAL2*, and *PAL4* was observed in *A. thaliana* during lignification caused by biotic and abiotic stresses (Mouradov et al., 2010; Oh et al., 2003; Raes et al., 2003). Elevated expression of *PAL1* and *PAL2* was also observed in *Arabidopsis* leaves exposed to low temperature and nitrogen deficiency, where an increased level of some flavonoids was detected (Olsen et al., 2008). Expression of phenylpropanoid biosynthesis-related genes was also induced in *Vitis vinifera* cells by cyclodextrins applied alone or in combination with methyl jasmonate, which caused a synergistic effect (Lijavetzky et al., 2008). Also, in *Vitis* cells, cyclodextrins caused early induction of expression of *PAL*, *C4H*, and 4CL (Zamboni et al., 2009). The ability of cyclodextrins to act as elicitors resides in their chemical similarity to the alkyl-derivatized pectic oligosaccharides which are released from the cell walls during fungal infection (Ilbi et al., 2006). Also, *Vitis pseudoreticulata* leaves infected with *Erysiphe necator* showed induction of *PAL*, *C4H*, and *4Cl* (Weng et al., 2014). On the other hand, induction of *At4CL1*, *At4CL2*, and *At4CL3* was observed in *A. thaliana* leaves exposed to UV irradiation and after inoculation with *Peronospora parasitica* (Ehlting et al., 1999). Another gene of the phenylpropanoid pathway that is induced by the pathogen is *AtCCR2* (Lauvergeat et al., 2001). Also the expression of *CCR2* was induced in *V. vinifera* cell cultures by cyclodextrins (Lijavetzky et al., 2008). Induction of *STS* gene expression in *V. vinifera* cells was observed under pathogen attack (Weng et al., 2014; Xu et al., 2010) as well as by cyclodextrins alone (Lijavetzky et al., 2008; Zamboni et al., 2009) or in combination with methyl jasmonate (Lijavetzky et al., 2008) or with Ap_3A (Pietrowska-Borek et al., 2014). Moreover, stilbene synthesis in berries (Gatto et al., 2008) and leaves can be elicited by fungal infection (Adrian et al., 1997; Langcake and Pryce, 1976), UV irradiation (Langcake and Pryce, 1977; Suzuki et al., 2015), ozone (Schubert et al., 1997), and heavy metals (Adrian et al., 1996) in *Vitis*.

Secondary metabolites are often accumulated in the cell, but some of them are exported to the extracellular space by the ABC cell membrane transporter family (Yazaki, 2006); therefore, ABC transporters are considered to be associated with biotic and abiotic stress resistance in plants and with the transport of phytoalexins (Zamboni et al., 2009). It is known that ABC transporters play an important role in pathogen resistance, and in plants they seem to take part in the plant defense response (Campbell et al., 2003). One such ABC transporter is VvABCG44, which was found in cells of *Vitis vinifera* and is responsible for the export of *trans*-resveratrol. Expression of *VvABCG44* was induced by UV irradiation, leading to resveratrol accumulation in the cells (Suzuki et al., 2014). Translocation of resveratrol outside the cells has two main objectives: to mediate the defense response against pathogens and to avoid the intracellular accumulation of this compound to cytotoxic levels (Zamboni et al., 2009).

We previously showed that certain diadenosine polyphosphates (Ap_3A and Ap_4A) induced expression of genes and enzymes of the phenylpropanoid pathway in *A. thaliana* seedlings (Pietrowska-Borek et al., 2011). We also demonstrated that exogenous Ap_3A, but not Ap_4A, induced the biosynthesis of *trans*-resveratrol in *V. vinifera* suspension cultured cells. Moreover, Ap_3A in combination with cyclodextrins evoked a synergistic effect in *trans*-resveratrol biosynthesis (Pietrowska-Borek et al., 2014). This compound is a stilbene derivative reported to accumulate in plants in response to environmental stresses (Chong et al., 2009). Since it is also known that the content of Np_nN's is clearly elevated in bacteria under stresses such as heat shock or cadmium (Lee et al., 1983; Conte et al., 1987) and that these compounds may be considered as signaling molecules (Kisselev et al., 1998), we have postulated that Np_nN's may be a part of the signaling cascade involved in plant responses to biotic and abiotic stresses. We also wondered whether purely pyrimidine Np_nN's or purine-pyrimidine hybrids might also modulate the phenylpropanoid pathway. This paper presents the results of our studies on the effects exerted by different exogenously applied Np_nN's on the accumulation of *trans*-resveratrol and *trans*-piceid in *V. vinifera* cv. Monastrell suspension cultured cells and in the spent culture medium. We have also measured the expression of phenylpropanoid biosynthesis-related genes and the expression of the *VvABCG44* gene encoding the *trans*-resveratrol cell membrane transporter.

2. Materials and methods

2.1. Plant material, culture media, and experimental conditions

Vitis vinifera L. cv. Monastrell calli were established as previously described (Calderon et al., 1993). The cell suspension was obtained by adapting the callus material to liquid culture maintained in the dark at

23 °C. Callus tissue (40 g) was dispersed in 200 mL of liquid medium containing Gamborg B5 basal salt supplemented with casein hydrolysate (0.25 g L^{-1}), 1-naphthaleneacetic acid (0.1 mg L^{-1}), kinetin (0.2 mg L^{-1}), sucrose (20 g L^{-1}) and Morel vitamins (Befché-Navarro et al., 2012) pH adjusted at 6.0. The culture was grown in 500 mL flasks on a rotary shaker at 110 rpm in the dark at 23 °C. The following Np_nN's were applied: purine dinucleotides (Ap_3A, Gp_3G), pyrimidine dinucleotides (Up_3U, Up_4U, Cp_3C, Cp_4C), and mixed (Ap_3U, Ap_3C, Ap_4C) at a final concentration of 5 μM. Also the effect of 5 μM nucleoside monophosphates, such as AMP, GMP, UMP, and CMP (products of Np_nN degradation), was investigated. The control cells, as well as the Np_nN'- and NMP- treated cells, were collected up to 72 h after treatment. The parameters of concentration of Np_nN's and time points of experiment we selected based on our previous works (Pietrowska-Borek et al., 2011, 2014). Cells were separated from the culture medium by filtration under a gentle vacuum, rapidly washed with cold distilled water, frozen in liquid nitrogen, and kept at −80 °C until use. The spent culture media were collected and the content of *trans*-resveratrol and *trans*-piceid was determined.

2.2. Np_nN's chemical synthesis

The Up_3U, Up_4U, Cp_3C, Cp_4C, Ap_3U, Ap_3C, Ap_4C, Ap_3A, and Gp_3G dinucleotides were synthesized taking advantage of metal chloride-mediated pyrophosphate bond formation between an appropriate nucleotide and imidazole-activated nucleotide subunit (Kadokura et al., 1997). Details of the protocols of Np_nN's chemical synthesis and their characterization by HRMS, ^1H NMR and ^{31}P NMR are given in the Supplementary data (Methods S1).

2.3. Quantification of *trans*-resveratrol and *trans*-piceid in cells and spent culture medium

Aliquots of the spent medium were diluted with methanol to a final concentration of 80% (v/v). One gram of fresh cells was extracted overnight with 4 mL of methanol at 4 °C with continuous shaking and the extract clarified by centrifugation at 14000*g* for 10 min. Reversed-phase analytical HPLC was used to separate stilbenes. The samples were injected on a LiChrospher 100 RP-18 column (250 × 4 mm, 5 μm; Merck) and eluates analyzed with a photodiode array detector (PDA) set at an interval of 220–600 nm. The absorbance of the eluate was monitored at 306 nm. Gradient elution was performed as described previously (Pietrowska-Borek et al., 2014). Identification of the chromatographic peaks was performed by comparison to commercial standards of *trans*-resveratrol and *trans*-piceid (Sigma-Aldrich).

2.4. Gene expression analyses

RNA from *Vitis vinifera* cv. Monastrell cells was extracted using the RNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase (Qiagen) according to the manufacturer's protocol. Evaluation of RNA purity and cDNA synthesis were performed as described earlier by Pietrowska-Borek et al. (2014). The quantitative real-time PCR reaction was carried out in 20 μL of reaction mixture composed of cDNA, primers and master mix (Taq Universal SYBR Green Supermix (Bio-Rad) using a CFX96 Real-Time PCR Detection System (Bio-Rad). The specific primers for *Vitis vinifera* genes (*PAL1*, *C4H1*, *4CL1*, *CHS1*, *STS1*, *CCR2*, *CAD1*, and *VvABCG44*) and GenBank accession numbers are presented in Table 1. The comparative C_T method for relative quantification was used with *Efα1* as the endogenous control. The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta CT}$ (Schmittgen and Livak, 2008).

2.5. Statistical analysis

Quoted values for *trans*-resveratrol and *trans*-piceid content and mRNA levels are the means of 3 replicates ± standard deviation. Statistical significance of the differences was tested with ANOVA software using Tukey's HSD multiple range test ($p < 0.05$).

3. Results

3.1. Accumulation of stilbenes

In our previous work, we showed that exogenous Ap_3A, but not Ap_4A, induced accumulation of *trans*-resveratrol both in *Vitis vinifera* cells and in the spent culture medium (Pietrowska-Borek et al., 2014). Therefore, we wondered whether other Np_nN's (purine, pyrimidine, and hybrid compounds) might affect the content of *trans*-resveratrol and *trans*-piceid in cells and culture media. The accumulation of both stilbenes in cells was induced the most by Up_3U, and only slightly less by Ap_3U or Up_4U during the first 24 h of exposure. The highest content of *trans*-resveratrol in cells treated with these dinucleotides was observed after 6 h, reaching 300–700 μg g^{-1} dry weight (DW), a level 1.5- to 2.5-fold higher than in control cells (Fig. 2). The content of *trans*-piceid was highest at 6 and 24 h, reaching about 200 μg g^{-1} DW (Fig. 2). Interestingly, all the cytosine-containing dinucleotides exerted a strong inhibitory effect on the cellular accumulation of stilbenes throughout the duration of the treatment; *trans*-resveratrol and *trans*-piceid were about 400- and 300-fold lower, respectively, than in control cells (Fig. 2).

The highest concentration of *trans*-resveratrol in the spent culture medium of 9–17 mg L^{-1} (7- to 13-fold higher than in the control) was attained after 6 h treatment with Ap_3A, Gp_3G, Ap_3U, Up_3U or Up_4U. It was similar after 12 h, but subsequently decreased to undetectable levels (Fig. 2). The *trans*-resveratrol content of the medium increased after exposure to Cp_3C, Cp_4C, Ap_3C and Ap_4C, from about 0.5 mg L^{-1} at 6 h (2- to 12-fold lower than control) up to 10 mg L^{-1} at 12 h (4-fold higher than control) and 24 h (2- to 8-fold higher than control). However, at 48 and 72 h we observed a drastic decrease in the content of this stilbene down to an undetectable level (Fig. 2). A similar effect was observed for *trans*-piceid accumulation in the medium. It slowly increased in samples treated with Ap_3A, Gp_3G, Ap_3U, Up_3U or Up_4U, reaching 2 mg L^{-1} by 6–24 h. In each case it was 2- to 6-fold higher than in the control. The content of *trans*-piceid in the spent medium increased up to 24 h in samples treated with Cp_3C and Cp_4C but it was always considerably lower than in the controls and over the subsequent few hours it decreased to an undetectable level (Fig. 2).

None of the potential products of Np_nN degradation, such as AMP, GMP, UMP, and CMP, had any appreciable effect on the accumulation of *trans*-resveratrol and *trans*-piceid in cells or in the spent medium. (Fig. 3).

3.2. Expression of phenylpropanoid biosynthesis-related genes

We analyzed the expression of phenylpropanoid biosynthesis-related genes after 24 and 72 h of dinucleotide exposure. These two time points were selected based on our data on the accumulation of stilbenes in both cells and culture medium; at 24 h, the content of *trans*-resveratrol and *trans*-piceid was generally high, while at 72 h the content of both stilbenes was at its lowest or even undetectable, especially in the spent medium (Fig. 2). Based on previous studies (Lijavetzky et al., 2008; Figueiredo et al., 2008; Pietrowska-Borek et al., 2014) we choose one gene (*PAL1*, *C4H1*, *4CL1*, *STS1*, *CAD1*, and *CCR2*). After 24 h of Np_nN' treatment, the relative expression of *PAL1* evoked by the various purine and pyrimidine dinucleotides differed. In Cp_3C- or Cp_4C-treated cells, *PAL1* expression was about 5-fold lower than in controls while Gp_3G, Up_3U, Ap_3U, and Up_4U all induced *PAL1* expression about 2-fold more (Fig. 4). However at 72 h, *PAL1* expression was significantly (6-fold) elevated in Cp_3C- and Cp_4C-treated cells compared to controls.

Table 1
Primer sequences used for quantitative real-time polymerase chain reaction (Ljavetzky et al., 2008; Figueiredo et al. 2008; Pietrowska-Borek et al., 2014).

Gene symbol	GeneBank ID	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon length (bp)
PAL1	XM_002281763.2	CCGAACCGAATCAAGGACTG	GTTCCAGCCACTGAGACAATC	183
C4H1	XM_002266202.1	TCCAAGTCACCGAGCCTGAT	GCAGGAATGTCATAGCCACC	109
4CL1	XM_002272746.2	CTGATGCCGCTGTTGTTTCG	GCAGGATTTTACCCGATGGA	198
STS1	XM_002264419.2	CGCCAGGAGATAATCACTGCT	GCACCAGGCATTTCTACACC	134
CHS1	EC996578.1	GTCCCAGGGTTGATTTCCAA	TCTCTTCCTTCAGACCCAGTT	157
CCR2	XM_002273418.3	ACAGCATGACGACTCTCTTCG	AGTGACAAGGGCTGGATTGA	182
CAD1	100247381	GGAGGGATGAAGGAGACACA	CTTTCAGCGTGTTGCCAATG	166
VvABCG44	AB910387.1	TAGGAGTGGTTGCAGCTGTG	TTTTGCTCGGTGTGACTTCTT	114
Rlx1	XP_002284964.1	GAACTGGGTGCTTGATAGGC	AACCAAAATATCCGGAGTAAAAGA	164

The other pyrimidine nucleotides, Up_3U and Up_4U, also elevated the expression of *PAL1* at 72 h, but this increase was less spectacular, exceeding the level observed in controls by only about 2- and 1.6-fold, respectively. A very similar effect of Np_nN's on *4CL1* expression was observed. At 24 h Gp_3G, Up_3U, or Up_4U caused a slight increase in *4CL1* expression, whereas Ap_3U, Cp_3C, Cp_4C, and Cp_4A diminished it about 5-fold compared to controls. With Cp_3C and Cp_4C, we observed around 5-fold higher expression of *4CL1* after 72 h (Fig. 4). However, a different picture emerged with *C4H1* expression. In fact, at 24 h the induction of *C4H1* expression caused by the purine dinucleotides Ap_3A and Gp_3G and by some pyrimidines (Up_3U and Up_4U) was about 3- to 4-fold higher than in control cells; however, after 72 h, the expression was reduced by each of these Np_nN's (Fig. 4).

We also examined the transcription of genes encoding key enzymes leading to the biosynthesis of flavonoids, stilbenes, and monolignols (Fig. 1). Expression of the chalcone synthase gene (*CHS1*), the branching point of flavonoid biosynthesis, was evaluated at 24 and 72 h, but it was not detected. The same result was previously observed in *V. vinifera* suspension cultured cells by Ljavetzky et al. (2008) and by Pietrowska-Borek et al. (2014), strongly suggesting that flavonoids are not synthesized in the dark in cells of this plant species. We also studied the effect of Np_nN's on the expression of *STS1*, which encodes stilbene synthase, a key enzyme leading to stilbenes. Ap_3A, Gp_3G, and Up_3U induced *STS1* expression about 6-fold at 24 h, but Up_4U was even more effective, causing a 9-fold induction. In contrast, Ap_3U, Cp_3C, Cp_4C, and Ap_4C strongly decreased the expression of *STS1* (Fig. 4). At 72 h each of the Np_nN's evoked an increase in the expression of *STS1*, but no more than 2-fold compared to the controls (Fig. 4). The final branch of the phenylpropanoid pathway we considered was monolignol biosynthesis (Fig. 1). At 24 h the expression of *CCR2* was slightly elevated by almost

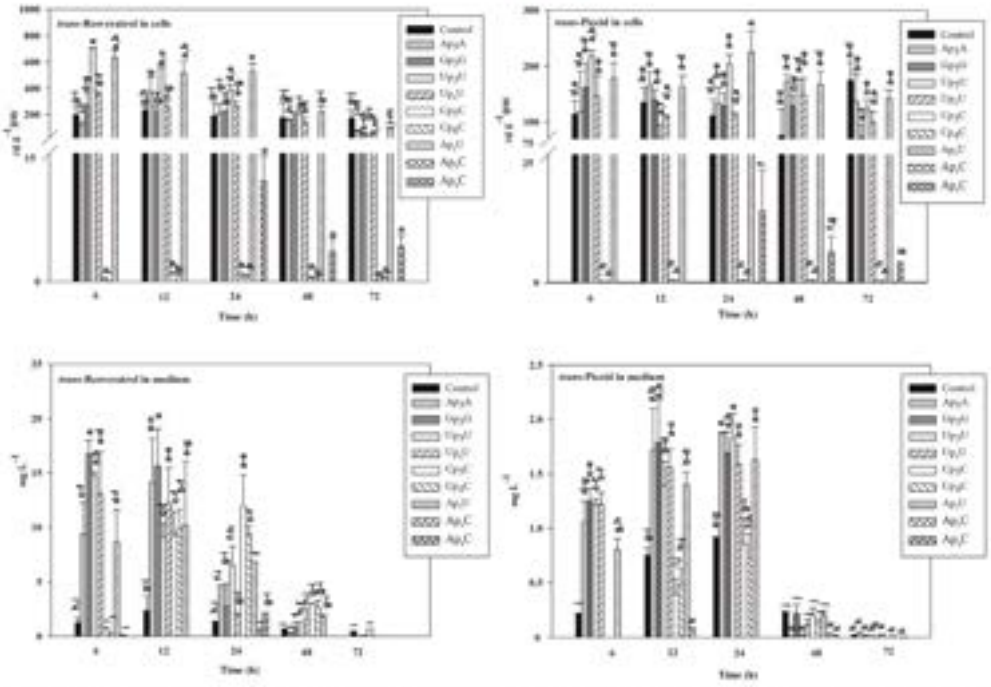

Fig. 2. Accumulation of *trans*-resveratrol and *trans*-piceid in cells of *Vitis vinifera* cv. Monastrell and in the spent medium of the suspension culture treated with 5 μM dinucleoside polyphosphates. Values are the mean ± standard deviation of three replicates. Values without a common superscript are significantly different according to the ANOVA statistical analysis and Tukey's HSD multiple range test ($p < 0.05$).

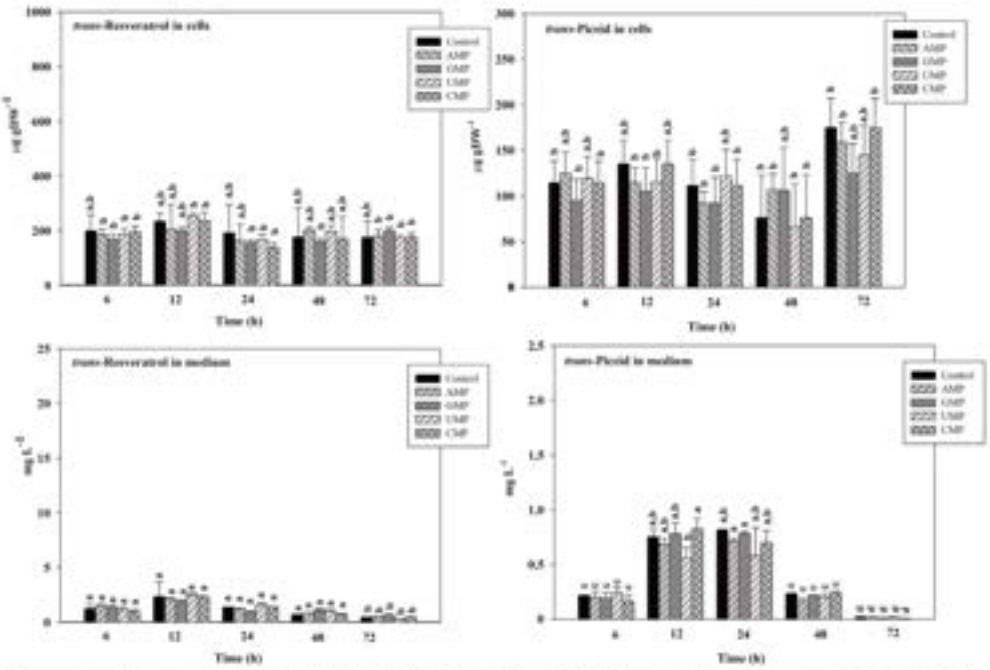

Fig. 3. Accumulation of *trans*-resveratrol and *trans*-piceid in cells of *Vitis vinifera* cv. Monastrell and in the spent medium of the suspension culture treated with 5 μM nucleoside monophosphates. Values are the mean ± standard deviation of three replicates. Values without a common superscript are significantly different according to the ANOVA statistical analysis and Tukey's HSD multiple range test ($p < 0.05$).

all Np_nN's with the exception of Ap_2U, which caused a clear decrease in expression (Fig. 4). At 72 h Cp_2C, Cp_4C, and Ap_4C elevated *CCR2* expression about 8-fold compared to controls (Fig. 4). The effect on *CAD1* expression was not so pronounced as observed with *CCR2*, with only a slight induction caused by Ap_2A, Gp_2G, Up_2U, or Up_4U at 24 h. Other dinucleotides caused a small decrease in *CAD1* expression. At 72 h each of the Np_nN's caused only a slight increase in the expression of *CAD1* not exceeding 1.5-fold compared to controls (Fig. 4).

3.3. Expression of ATP-binding cassette (ABC) transporter (*VvABCG44*) gene

The occurrence of *trans*-resveratrol and *trans*-piceid in the spent culture medium (Fig. 2) encouraged us to check the expression of the gene encoding the resveratrol-transporting protein previously identified in *V. vinifera* cell membranes (Suzuki et al., 2015). We found that 24 h after the addition of Ap_2A, Gp_2G, Up_2U or Up_4U to the medium there was a 3- to 5-fold increase in *VvABCG44* expression (Fig. 5). Cp_2C and Cp_4C only slightly elevated *VvABCG44* expression, but neither Ap_2U nor Ap_4C had any effect. At 72 h each of the Np_nN's caused induction of *VvABCG44* expression, but no more than 2- to 2.5-fold compared to controls (Fig. 5).

4. Discussion

Metabolism of Np_nN's *in vivo* is still poorly understood. Currently, the biochemistry of these compounds seems to be neglected; therefore for the most comprehensive discussion on this issue see the reviews published two decades ago (Guranowski, 2000; McLennan, 2000). Our earlier findings that Ap_2A and/or Ap_4A can function as *alarmones* in plants encouraged us to carry out studies of the potential effects evoked by other, non-adenylylated Np_nN's in a convenient model – grape cells grown in suspension. Such an investigation was originally proposed when we summarized the results of our very first investigations of the effects exerted on plants by dinucleotides (Pietrowska-Borek et al., 2011, 2014). The findings described in this paper show that purine and pyrimidine Np_nN's control phenylpropanoid metabolism in different ways.

trans-Resveratrol is an inducible phytoalexin in *Vitis* species with known functions in plants, especially in plant-pathogen and plant-herbivore relationships, and in plants subjected to abiotic stresses. A number of studies, especially those concerning grapevine inoculated with various pathogens, have established a positive correlation between stilbene levels and pathogen resistance. Analysis of the potential production of stilbenes by grapevine genotypes revealed that resistance to the necrotrophic pathogen *Botrytis cinerea* was strongly correlated with the production of resveratrol and ε-viniferin (Chong et al., 2009).

Among the pyrimidine Np_nN's, those containing cytosine behaved differently from their uridine counterparts. Surprisingly, both Cp_2C and Cp_4C strongly inhibited the accumulation of *trans*-resveratrol in *Vitis* cells, the level being almost 400-fold lower than in controls. The same was found for *trans*-piceid accumulation. We also observed inhibition of the biosynthesis of stilbenes by the hybrid purine/pyrimidine dinucleotides Ap_2C and Ap_4C. *trans*-Piceid accumulation was undetectable or 20-fold lower than in controls, respectively. However, the secretion of *trans*-resveratrol into the culture medium after Cp_4C and Cp_4C treatment was significantly higher compared to controls up to 48 h (Fig. 2). These results may suggest the early involvement of cytidine

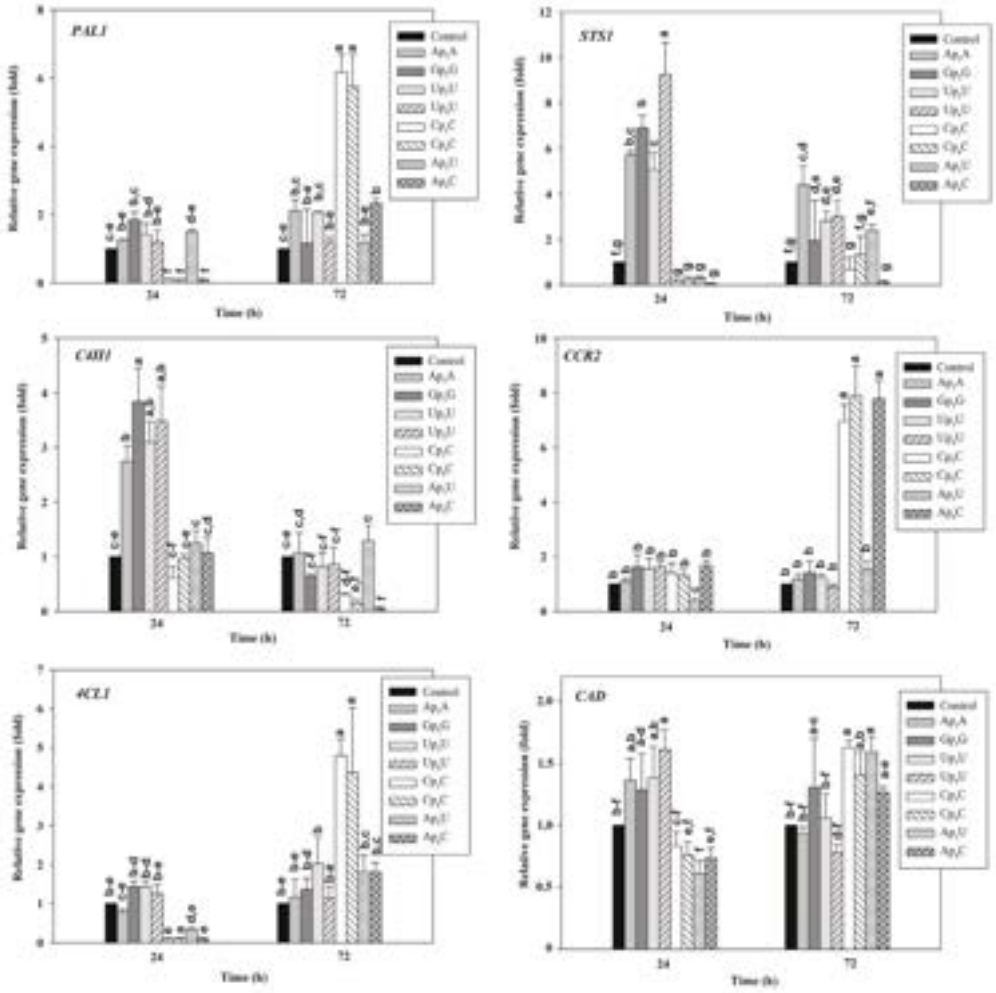

Fig. 4. Expression of phenylpropanoid biosynthesis-related genes in cells of *Vitis vinifera* cv. Monastrell treated with 5 μM dinucleoside polyphosphates. Values are the mean ± standard deviation of three replicates. Values without a common superscript are significantly different according to the ANOVA statistical analysis and Tukey's HSD multiple range test ($p < 0.05$). Total RNA was reverse-transcribed into cDNA and used as a template for real-time quantitative PCR reaction as described in the Experimental procedures. Specific primers were designed for *PAL1*, *C4H1*, *4CL1*, *STS1*, *CCR2*, *CAD1*, and for *EFα1* (elongation factor 1-alpha) that was used as an endogenous control. Relative transcript levels were normalized to *EFα1* mRNA. The expression levels of *PAL1*, *C4H1*, *4CL1*, *STS1*, *CCR2*, and *CAD1* in the control cells were set to 1. *PAL1*, phenylalanine ammonia-lyase; *C4H1*, cinnamate-4-hydroxylase; *4CL1*, 4-coumarate:CoA ligase; *STS1*, stilbene synthase; *CCR2*, cinnamoyl-CoA reductase; *CAD1*, cinnamyl alcohol dehydrogenase.

dinucleotides in the response to environmental stresses and rapid export of stilbenes from plant cells to the extracellular space. We observed that during the experiment the concentrations of *trans*-resveratrol and *trans*-piceid in the spent medium decreased despite their biosynthesis in the cell at a constant level. This could be the result of the conversion of resveratrol to one or more possible metabolites, e.g. viniferins or/and pterostilbene, a dimethylated form of resveratrol. This derivative exhibits higher activity in inhibiting fungal growth than resveratrol itself (Pezet et al., 2004). It is worth mentioning here that none of the nucleoside monophosphates (NMPs) affected *trans*-resveratrol or *trans*-piceid accumulation either in cells or in the culture medium (Fig. 3).

As mentioned previously, the export of *trans*-resveratrol and *trans*-piceid into the growth medium encouraged us to determine whether Np_nN's affect the expression of the membrane transporter *VvABCG44*. Two major classes of transporter proteins have been identified: the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters. Members of both classes can have broad and overlapping substrate specificities for natural, toxic compounds and can be regarded as a first-line defense barrier in survival mechanisms. In plant pathogens, these transporters can play an essential role in protection against

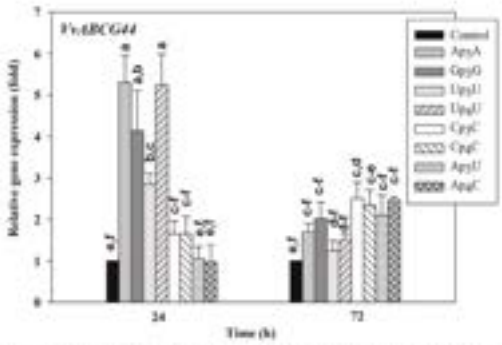

Fig. 5. Expression of ATP binding cassette (ABC) transporter (*VvABCG44*) gene in cells of *Vitis vinifera* cv. Monastrell treated with 5 μM dinucleoside polyphosphates. Relative transcript levels were normalized to *EFα1* (elongation factor 1-alpha) mRNA. The expression level of *VvABCG44* in the control cells was set to 1. Values are the mean ± standard deviation of three replicates. Values without a common superscript are significantly different according to the ANOVA statistical analysis and Tukey's HSD multiple range test ($p < 0.05$).

plant defense compounds during pathogenesis (Del Sorbo et al., 2000). Recent studies on *trans*-resveratrol transport out of grapevine cells showed that also tau class glutathione-*S*-transferase GSTU-2 is a part of machinery for transporting *trans*-resveratrol to the extracellular medium (Martínez-Márquez et al., 2017). In this study, we tested expression of a transporter gene belonging to the ABC transporters - *VvABCG44*. We found that 24 h of treatment by adenine-, guanine- and uridine-containing dinucleotides caused the most effective induction of this gene – up to 6-fold higher than in the control group (Fig. 5). We also investigated the effects exerted by Np_4N's on genes involved in the biosynthesis of other important phenylpropanoid metabolites. The same nucleotides also showed the greatest induction of *C4H1* and *STS1* at 24 h, whereas the cytidine-containing dinucleotides strongly induced expression of *PAL1*, *4CL1*, and the lignin biosynthetic gene *CCR2* at 72 h (Fig. 4).

During defense responses, the accumulation of lignin or lignin-like phenolic compounds was shown to occur in a variety of plant-microbe interactions. Defense lignin accumulated by an elicitor treatment was shown to be significantly different from lignin in vascular tissues, suggesting that lignin biosynthesis is differentially regulated (Bhuiyan et al., 2009). Recent studies on grapevine green shoots infected with *Lasiodiplodia theobromae* showed that the phenylpropanoid biosynthetic pathway is involved in the response to *L. theobromae* infection where a large number of enzymes associated with lignin biosynthesis were induced, including transcripts encoding PAL, 4CL, and CCR enzymes (Zhang et al., 2019). Signaling pathways regulating plant responses to environmental factors are not fully understood. Several studies have been conducted on grapevine suspension cultured cells to investigate elicitor signal transduction leading to defense reactions, including stilbene and monolignol biosynthesis (Chong et al., 2009; Zhang et al., 2019). In this system, several plant secondary messengers, especially calcium and active oxygen species, as well as the plant hormone jasmonic acid are involved in the regulation of stilbene production (Chong et al., 2009). One can anticipate that Np_4N's will now also attract the attention of researchers carrying out such investigations. It is known that both purine and pyrimidine dinucleotides are synthesized by enzymes from viruses (Jin et al., 2013), bacteria, fungi, plants, and animals (Fraga and Fontes, 2011).

The data obtained in this study allow us to postulate that both purine and pyrimidine dinucleotides should be considered as members of a family of naturally occurring stress signaling molecules, at least in plants, and that they function by a variety of mechanisms. We trust that our findings will be followed by different "omic" experiments to shed more light on the physiological functions of these nucleotides. At the very least, they appear to participate in the response to environmental stimuli not only by secretion of stilbenes but also by biosynthesis of monolignols. This process may be significant in the response of plants to pathogen attack and suggests the formation of a lignin barrier. Future lines of investigation will include the response of plants to dinucleoside polyphosphates synthesized by microorganisms and determination of the molecular targets of these dinucleotides.

Authors' contributions

MPB conceived and designed the study, performed the majority of experiments, analyzed results wrote the bulk of manuscript; AWM and JD carried out some of the experiments; ACM, MRB, JMD, JK, JJ synthesized the dinucleoside polyphosphates; SB performed the statistical analysis and was involved in writing of the manuscript; MAP provided the plant material and was involved in writing the manuscript, AG was involved in the discussion and writing of the manuscript. All authors have read and approved the final version of the manuscript.

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Declaration of competing interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.12.015.

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Np_nN's chemical synthesis

List of content

1. Experimental Section

1.1. General Information

Cytidine and adenosine 5'-monophosphate (free acid form) were purchased from Sigma Aldrich. Cytidine 5'-monophosphate was synthesized as described by Yoshikawa *et al.* (1969). The reaction then quenched by addition of 10 volumes of water and adjusted to pH 7 with solid $NaHCO_3$. Imidazolide derivatives of 5'-CMP, 5'-AMP and Cytidine 5'-diphosphate were synthesized as described previously (Kore and Parmar, 2006). Pyrophosphate diimidazolide was synthesized as described by Ziemniak *et al.* (2015).

Purification of CMP, CDP, ADP, and final compounds was performed by ion-exchange chromatography on a DEAE Sephadex A-25 (HCO_3^- form) column. First, the column was loaded with the reaction mixture and washed thoroughly with water until the eluate did not precipitate with $AgNO_3$ solution (to remove residual solvents and non-binding reagents). Nucleotides were eluted using a 0–1.2 M linear gradient of triethylammonium bicarbonate (TEAB) in deionized water. Collected fractions were analyzed spectrophotometrically at 260 nm. Fractions containing nucleotides, as confirmed by RP HPLC and MS analysis, were combined, evaporated with repeated additions of ethanol (96%) (to decompose TEAB) and acetonitrile (to remove residual water). Products isolated using this procedure were isolated as triethylammonium salts. The final products were additionally purified by semi preparative RP HPLC using a Discovery RP Amide C-16 HPLC column (25 cm×21.2 mm, 5 µm, flow rate 5.0 mL min^{-1}) with UV detection. After repeated freeze-drying products were isolated as ammonium salts.

Analytical RP HPLC was performed using SUPELCO Supelcosil LC-18-T HPLC column (4.6×250 mm, 5 µm, flow rate 1.3 mL min^{-1}) with a linear gradient of 50% methanol in buffer A (ammonium acetate buffer, pH 5.9, 0.05 M) in 15 min and UV-detection at 254 nm. Structures of final compounds were confirmed by high-resolution mass spectrometry (HRMS-ESI) and ^1H and ^{31}P NMR spectroscopy. Mass spectra were recorded with LTQ Orbitrap Velos (Thermo Scientific) spectrometer. NMR spectra were recorded at 25 °C with a BRUKER AVANCE III HD spectrometer at 500 MHz (^1H NMR) and 202 MHz (^{31}P NMR). ^1H NMR chemical shifts were calibrated to sodium 3-trimethylsilyl-[2,2,3,3-D4]propionate (TSP) in D_2O and for ^{31}P NMR to H_3PO_4 (20%) in D_2O as an external standard. ^1H NMR signal assignments and identification were supported by COSY spectra analysis. The raw NMR spectroscopic data were processed using MestReNova v 12.0.2-20910 Software.

1.2. General procedure

An appropriate triethylammonium salt of nucleoside mono-, di- or triphosphate (2 equiv.) was dissolved in DMSO or DMF with addition of $ZnCl_2$ (4 equiv.). The mixture was stirred at room temperature for 5 min. Then the imidazolide derivative of adenosine or cytidine 5'-phosphate (1 equiv.) was added, followed by a second portion of $ZnCl_2$ (4 equiv.). The reaction was analyzed by RP HPLC and quenched by addition of 10 volumes of an aqueous solution of EDTA and $NaHCO_3$ (10 g/l and 5 g/l, respectively).

1.1. Synthesis of uridine 5'-diphosphate (UDP)

Uridine 5'-diphosphate was obtained according to the general procedure starting from orthophosphate (336 mg, 1.69 mmol, 4 equiv.), uridine 5'-monophosphate imidazolide (167 mg, 0.42 mmol, 1 equiv.) and zinc chloride (8 equiv.) in DMF (2.5 mL). After purification by DEAE Sephadex compound was isolated as an triethylamonium salt (142 mg, 0.20 mmol, 48%).

1.3. Synthesis of cytidine 5'-triphosphate (CTP)

Cytidine 5'-triphosphate was obtained according to the general procedure starting from cytidine 5'-monophosphate imidazolide sodium salt (200 mg, 0.51 mmol, 1 equiv.), triethylamine pyrophosphate (610 mg, 1.27 mmol, 2.5 equiv.), zinc chloride (555 mg, 4.08 mmol, 8 equiv.), and DMSO (4 mL). After purification by DEAE Sephadex compound was isolated as an triethylamonium salt (231 mg, 2030 mOD, 0.29 mmol, 56%).

^{1}H NMR (500 MHz, D_2O, 25°C): δ 8.06 (d, $J_{H,H}$ 7.7 Hz, 1 H, H6), 6.21 (d, $J_{H,H}$ 7.7 Hz, 1 H, H5), 6.00 (d, $J_{H,H}$ 4.5 Hz, 1 H, H1'), 4.42 – 4.39 (m, 1 H, H2'), 4.36 – 4.34 (m, 1 H), 4.32 – 4.22 (m, 3 H) ppm; **^{31}P NMR (202 MHz, D_2O, 25°C):** δ -9.86 (d, $J_{P,P}$ 19.7 Hz, 1 P), -10.50 (d, $J_{P,P}$ 19.3 Hz, 1 P), -22.13 – -22.43 (m, 1 P, P_β) ppm.

2. Synthesis of dinucleotides

2.1. Compound 1 (Cp_3C)

Compound **1** was obtained according to the general procedure starting from cytidine 5'-diphosphate triethylammonium salt (100 mg, 0.20 mmol, 2 equiv.), cytidine 5'-monophosphate imidazolide sodium salt (42 mg, 0.11 mmol, 1 equiv.), zinc chloride (110 mg, 0.86 mmol, 8 equiv.), and DMSO (2 mL). After purification by ion-exchange chromatography the isolated product was contaminated with cytidine 5'-diphosphate (CDP), which has the same negative net charge. After additional purification by semi-preparative HPLC compound **1** was isolated as an ammonium salt (14 mg, 151 mOD, 0.01 mmol, 19%).

^{1}H NMR (400 MHz, D_2O, 25°C): δ 8.12 (d, $J_{H,H}$ 7.8 Hz, 2 H, H6), 6.24 (d, $J_{H,H}$ 7.8 Hz, 2 H, H5), 5.94 (d, $J_{H,H}$ 4.2 Hz, 2 H, H1'), 4.38 – 4.35 (m, 2 H, H2'), 4.33 – 4.21 (m, 8 H) ppm; **^{31}P NMR (202 MHz, D_2O, 25°C):** δ -10.58 – -10.78 (m, 2 P, P_α), -22.31 (t, $J_{P,P}$ 18.7, 1 P, P_β) ppm; **HR-MS (ESI), *m/z*:** Calculated for $C_{18}H_{26}N_6O_{18}P_3^-$ ([M-H]$^-$): 707.05219; Found: 707.05452.

2.2. Compound 2 (Cp_4C)

Compound **2** was synthesized following the general procedure starting from cytidine 5'-triphosphate triethylammonium salt (97 mg, 0.20 mmol, 2 equiv.), cytidine 5'-monophosphate imidazolide sodium salt (32 mg, 0.08 mmol, 1 equiv.), and zinc chloride (88 mg, 0.65 mmol, 8 equiv.) in DMSO (2 mL). After purification by ion-exchange chromatography the isolated product was contaminated with cytidine 5'-triphosphate (CTP), which has the same negative net charge. After additional purification by semi-preparative HPLC compound **2** was isolated as ammonium salt (27 mg, 371 mOD, 0.03 mmol, 36%).

^{1}H NMR (500 MHz, D_2O, 25°C): δ 8.07 (d, $J_{H,H}$ 7.6 Hz, 2 H, H6), 6.24 (d, $J_{H,H}$ 7.6 Hz, 2 H, H5), 5.96 (d, $J_{H,H}$ 4.1 Hz, 2 H, H1'), 4.38 (m, 2 H, H2'), 4.34–4.22 (m, 6 H) ppm; **^{31}P NMR (202 MHz, D_2O, 25°C):** δ -8.01– -9.43 (m, 2 P, P_α), -20.96– -22.58 (m, 2 P, P_β) ppm; **HR-MS (ESI), *m/z*:** Calculated for $C_{18}H_{27}N_6O_{21}P_4^-$ ([M-H]$^-$): 787.01852; Found: 787.02145.

2.3. Compound 3 (Ap_3C)

Compound **3** was obtained according to the general procedure starting from cytidine 5'-diphosphate (40 mg, 0.08 mmol, 2 equiv.), adenosine 5'-monophosphate imidazolide (20 mg, 0.04 mmol, 1 equiv.), and zinc chloride (39 mg, 0.32 mmol, 8 equiv.) in DMSO (1 mL). After purification by ion-exchange chromatography product was isolated as triethylammonium salt (27 mg, 295 mOD, 0.02 mmol, 38%). After additional purification by semi-preparative HPLC compound **3** was isolated as ammonium salt (10 mg, 153 mOD, 19%).

^{1}H NMR (500 MHz, D_2O, 25°C): δ 8.50 (s, 1 H), 8.23 (s, 1 H), 7.79 (d, $J_{H,H}$ 7.7 Hz, 1 H, H6), 6.11 (d, $J_{H,H}$ 6.1 Hz, 1 H, H1'$_A$), 5.87–5.84 (m, 2 H, H5 and H1'$_C$), 4.75 (dd, $J_{H,H}$ 6.1, 5.1 Hz, 2 H, H2'$_A$), 4.54 (dd, $J_{H,H}$ 5.0, 3.2 Hz, 1 H, H3'$_A$), 4.41 – 4.37 (m, 1 H, H4'$_A$), 4.31 – 4.18 (m, 8 H) ppm; **^{31}P NMR (202 MHz, D_2O, 25°C):** δ -9.44 - -9.91 (m, 2 P, P_α), -21.22 (t, $J_{P,P}$ 19.0, 1 P, P_β) ppm; **HR-MS (ESI), *m/z*:** Calculated for $C_{19}H_{28}N_8O_{17}P_3^-$ ([M-H]$^-$): 731.06342; Found: 731.06465 .

2.4. Compound 4 (Ap_4C)

Compound **4** was obtained according to the general procedure starting from cytidine 5'-triphosphate (47 mg, 0.08 mmol, 2 equiv.), adenosine 5'-monophosphate imidazolide (20 mg, 0.04 mmol, 1 equiv.), and zinc chloride (40 mg, 0.32 mmol, 8 equiv.) in DMSO (1 mL). After purification by ion-exchange chromatography the product was isolated as triethylammonium salt (30 mg, 638 mOD, 0.03 mmol, 38%). After additional purification by semi-preparative HPLC compound **4** was isolated as an ammonium salt (18 mg, 260 mOD, 0.01 mmol, 16%).

^{1}H NMR (500 MHz, D_2O, 25°C): δ 8.54 (s, 1 H), 8.25 (s, 1 H), 7.90 (d, $J_{H,H}$ 7.6 Hz, 1 H, H6), 6.11 (d, $J_{H,H}$ 6.3 Hz, 1 H, H1'$_A$), 6.03 (d, $J_{H,H}$ 7.7 Hz, 1 H, H5), 5.91 (d, $J_{H,H}$ 4.3, 1 H, H1'$_C$), 4.80 (1H, H2'$_A$ overlapped with HDO), 4.59 (dd, $J_{H,H}$ 5.2, 3.0 Hz, 1 H, H3'$_A$), 4.41 – 4.38 (m, 1 H, H4'$_A$), 4.37 – 4.33 (m, 1 H), 4.32 – 4.20 (m, 8 H) ppm; **^{31}P NMR (202 MHz, D_2O, 25°C):** δ -22.24 - -11.59 (m, 2 P), -22.99 – -23.37 (m, 2 P) ppm; **HR-MS (ESI, *m/z*):** Calculated for $C_{19}H_{27}N_8O_{22}P_4^-$ ([M-H]$^-$): 811.02975; Found: 811.03076.

2.5. Compound 5 (Up_3U)

Compound **5** was obtained according to the general procedure starting from orthophosphate (20 mg, 0.10 mmol, 1 equiv.), uridine 5'-monophosphate imidazolide (121 mg, 0.31 mmol, 3 equiv.) and zinc chloride (8 equiv.) in DMF (1.8 mL). After purification by DEAE Sephadex compound precipitated with ice cold acetone solution of $NaClO_4$ and isolated as an sodium salt (64.4 mg, 0.08 mmol, 83%).

^{1}H NMR (400 MHz, D_2O): δ 7.97 (d, $J_{H,H}$ 8.2 Hz, 2 H, H5$_{U1}$, H5$_{U2}$); 5.98 (br s, 2 H, H6$_{U1}$, H6$_{U2}$), 5.97 (d, $J_{H,H}$ 2.1 Hz, 2 H, H1'$_{U1}$, H1'$_{U2}$),), 4.40 – 4.34 (m, 4 H, H3'$_{U1}$, H2'$_{U1}$, H3'$_{U2}$, H2'$_{U2}$), 4.29 – 4.23 (m, 6 H, H5'$_{U1}$, H5"$_{U1}$, H4'$_{U2}$, H4'$_{U2}$, H5'$_{U2}$, H5"$_{U2}$) ppm; **^{31}P NMR (162 MHz, D_2O):** δ -8.49 (2 P, Pα_{γ}), -20.04 (1 P, P$_{\beta}$) ppm.

2.6. Compound 6 (Ap_3U)

Compound **6** was obtained according to the general procedure starting from uridine 5'-diphosphate (10 mg, 0.01 mmol, 1 equiv.), adenosine 5'-monophosphate imidazolide (37 mg, 0.09 mmol, 6 equiv.) and zinc chloride (8 equiv.) in DMF (0.7 mL). After purification by DEAE Sephadex compound precipitated with ice cold acetone solution of $NaClO_4$ and isolated as an sodium salt (19.6 mg, 0.08 mmol, 12%).

^{1}H NMR (400 MHz, D_2O): δ 8.52 (s, 1 H, H8$_A$), 8.24 (s, 1 H, H2$_A$), 7.79 (d, $J_{H,H}$ 7.8 Hz, 1 H, H5$_U$), 6.12 (d, $J_{H,H}$ 6.3 Hz, 1 H, H1'$_A$), 5.86 (d, $J_{H,H}$ 4.3 Hz, 1 H, H1'$_U$), 5.72 (d, $J_{H,H}$ 7.8 Hz, 1 H, H6$_U$), 4.76 – 4.73 (m, 1 H, H2'$_A$), 4.55 (dd, $J_{H,H}$ 4.9, 3.3 Hz, 1 H, H3'$_A$), 4.42 – 4.37 (m, 1 H, H4'$_A$), 4.33 – 4.19 (m, 7 H, H3'$_U$, H4'$_U$, H5'$_U$, H5"$_U$, H5'$_A$, H5"$_A$) ppm, **^{31}P NMR (162 MHz, D_2O):** δ -10.73 (2 P, Pα_{γ}), -22.27 (1 P, P$_{\beta}$) ppm.

2.7. Compound 7 (Up_4U)

Compound **7** was obtained according to the general procedure starting from uridine 5'-monophosphate (110 mg, 0.26 mmol, 1.5 equiv.), pyrophosphate diimidazolide (58 mg, 0.18 mmol, 1 equiv.) and zinc chloride (8 equiv.) in DMF (2.5 mL). After purification by DEAE Sephadex compound precipitated with ice cold acetone solution of $NaClO_4$ and isolated as an sodium salt (108 mg, 0.12 mmol, 95%).

^{1}H NMR (400 MHz, D_2O): δ 7.98 (d, $J_{H,H}$ 8.2 Hz, 2 H, H5$_{U1}$, H5$_{U2}$), 6.01 – 5.98 (m, 4 H, H1'$_{U1}$, H6$_{U1}$, H1'$_{U2}$, H6$_{U2}$), 4.45 – 4.42 (m, 2 H, H2'$_{U1}$, H2'$_{U2}$), 4.39 (t, $J_{H,H}$ 5.3 Hz, 2 H, H3'$_{U1}$, H3'$_{U2}$), 4.30 – 4.24 (m, 6 H, H5'$_{U1}$, H5"$_{U1}$, H5'$_{U2}$, H5"$_{U2}$) ppm, **^{31}P NMR (162 MHz, D_2O):** δ -9.64 (2 P,Pα_{δ}), -21.38 (2 P, P$_{\beta(\chi)}$) ppm.

2.8. Compound 8 (Ap_3A)

Compound **8** was obtained according to the general procedure starting from adenosine 5'-diphosphate triethylammonium salt (60 mg, 0.08 mmol, 1 equiv.), adenosine 5'-monophosphate imidazolide sodium salt (60 mg, 0.12 mmol, 1 equiv.), zinc chloride (130 mg, 0.96 mmol, 8 equiv.), and DMSO (3 mL). The product was purified by DEAE Sephadex followed by additional purification by RP HPLC. Compound **8** was isolated as an ammonium salt (46

mg, 1180 mOD, 52%). After liophylization the final product was found as a white powder. Stored at -20°C.

^{1}H NMR (500 MHz, D_2O, 25°C): δ 8.34 (s, 2 H), 8.14 (s, 2 H), 6.01 (d, $J_{H,H}$ 4.8 Hz, 2 H), 4.62 (t, $J_{H,H}$ 4.8 Hz, 2 H), 4.49 (t, $J_{H,H}$ 4.8 Hz, 2 H), 4.38–4.25 (m, 6 H) ppm; **^{31}P NMR (202 MHz, D_2O, 25°C):** δ -10.62 (d, $J_{P,P}$ 19.2 Hz, 2P), -22.11 (t, 19.2, 1 P) ppm.

3. References

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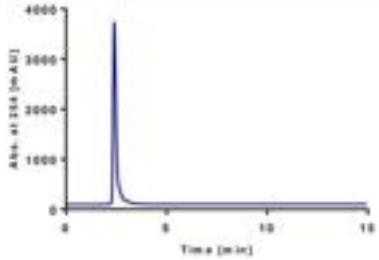
4. **Raw data: HPLC profiles, NMR and HRMS spectra**

Compound 1: Cp_5C

Chemical structure

HPLC



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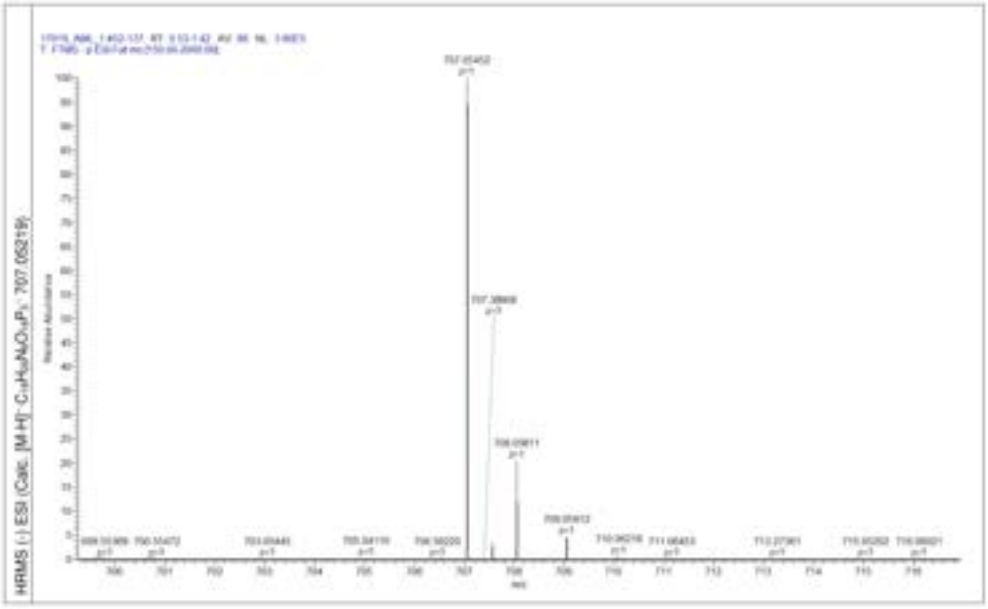

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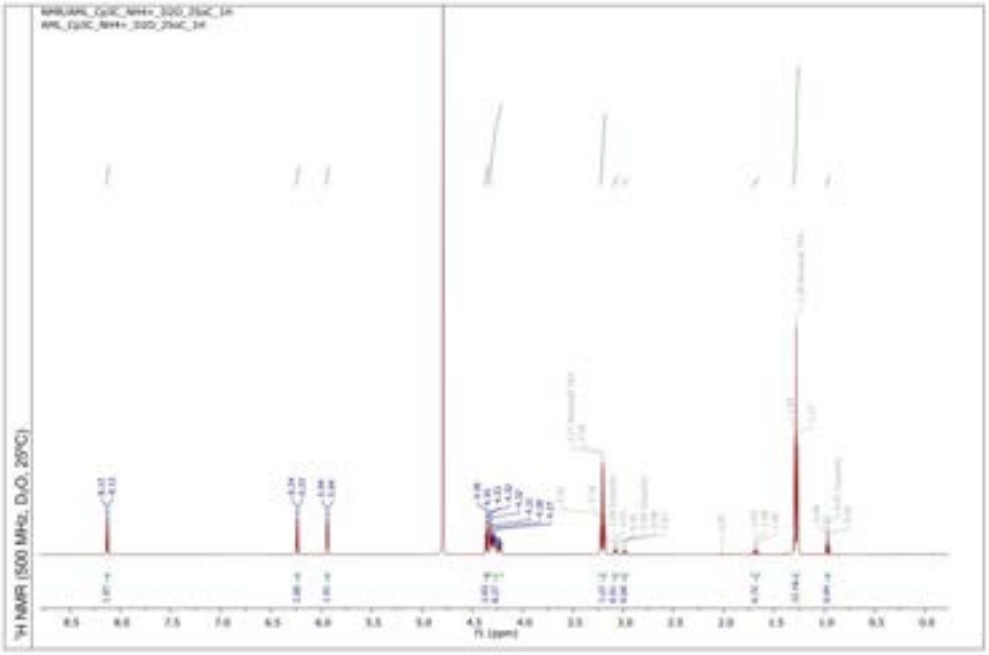

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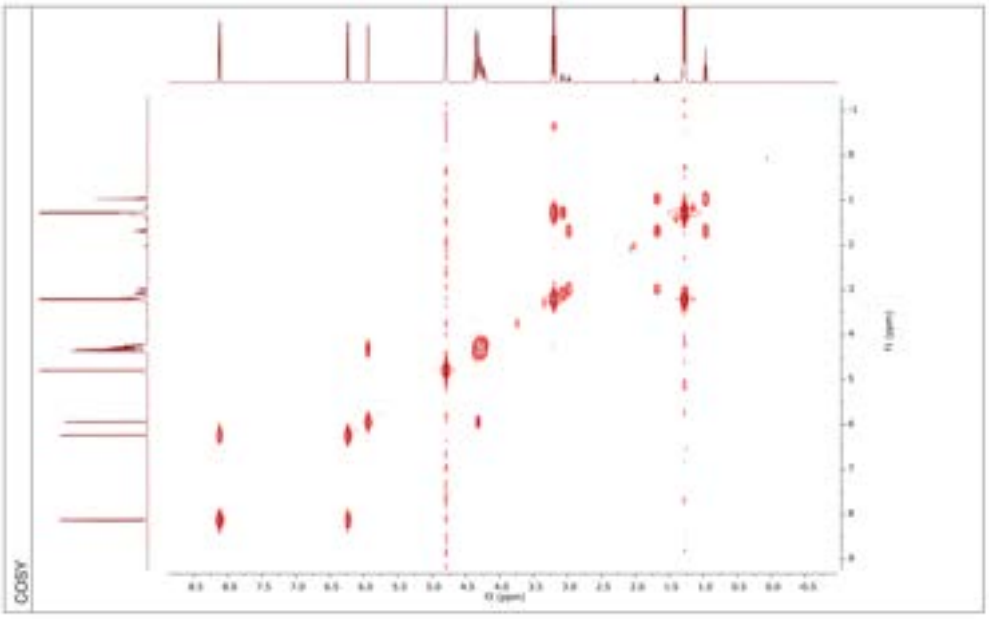

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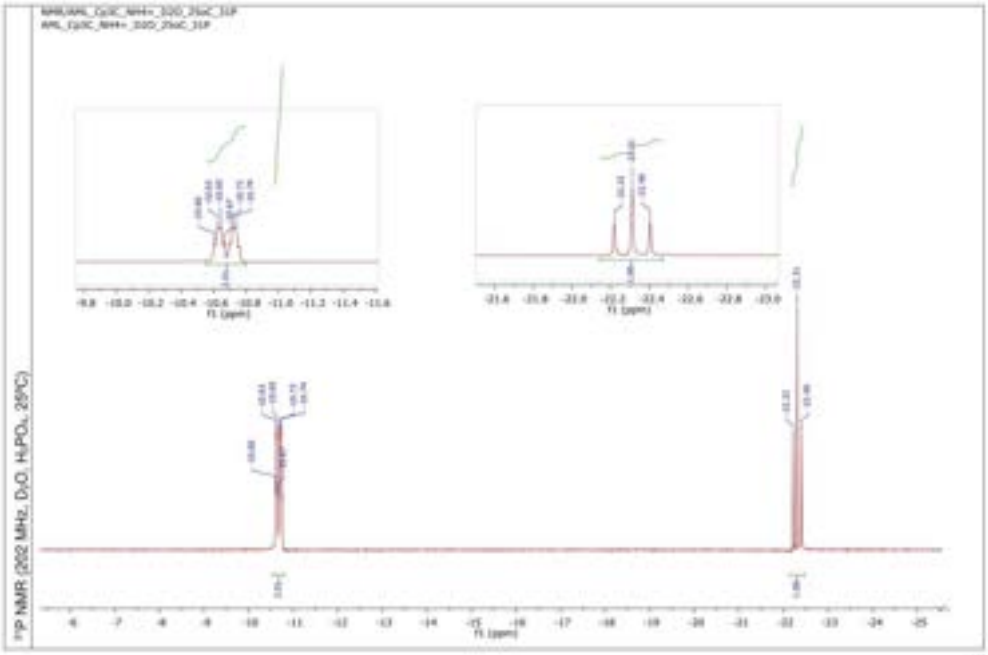

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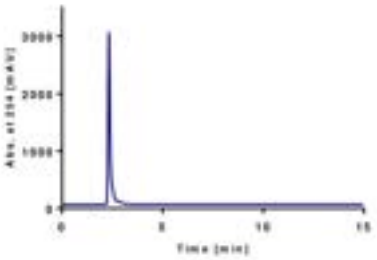
Compound 2: CpuC

Chemical structure

HPLC



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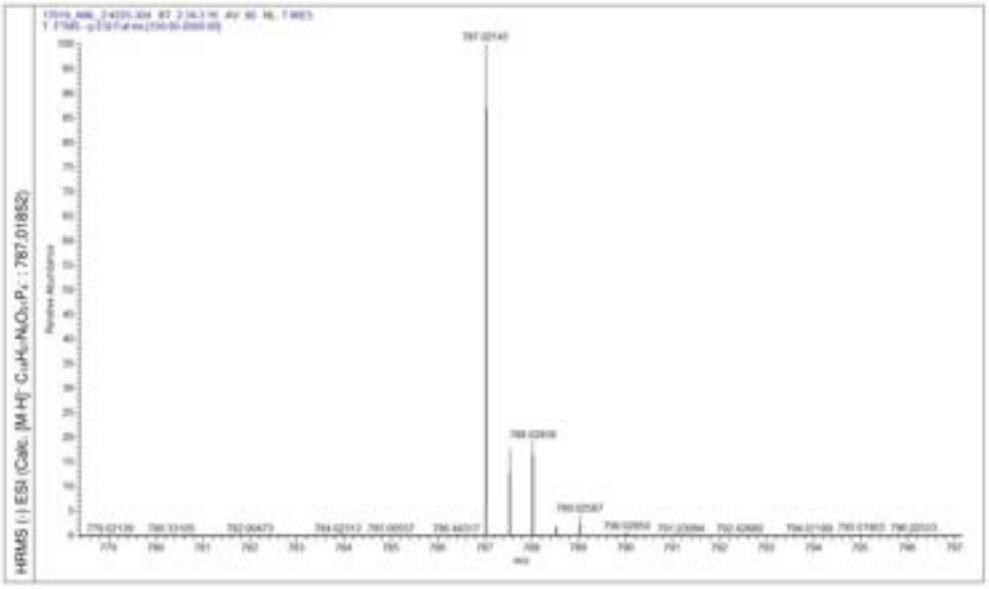
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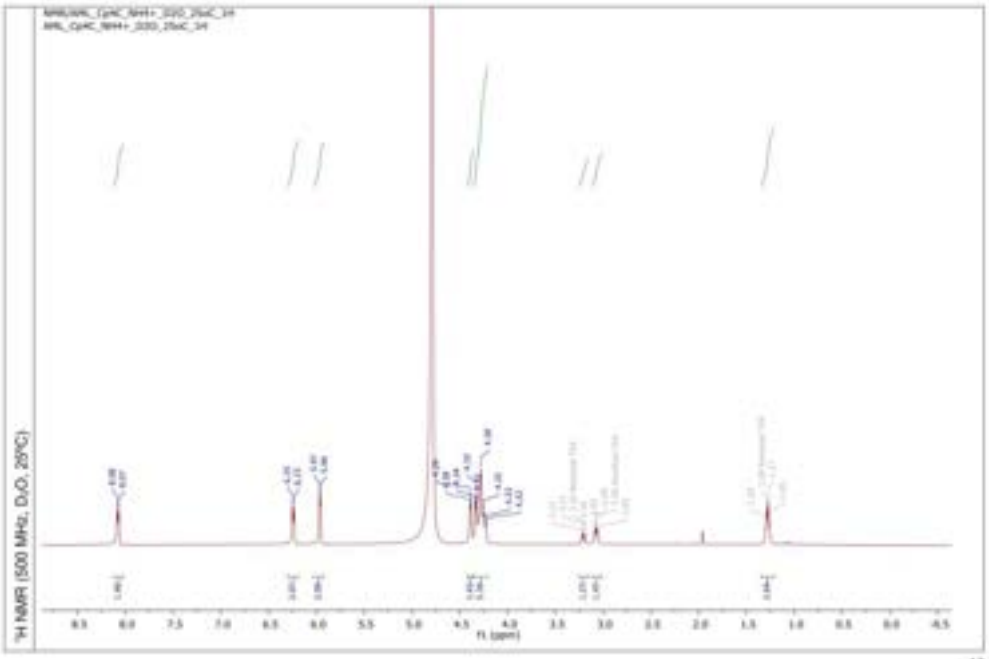
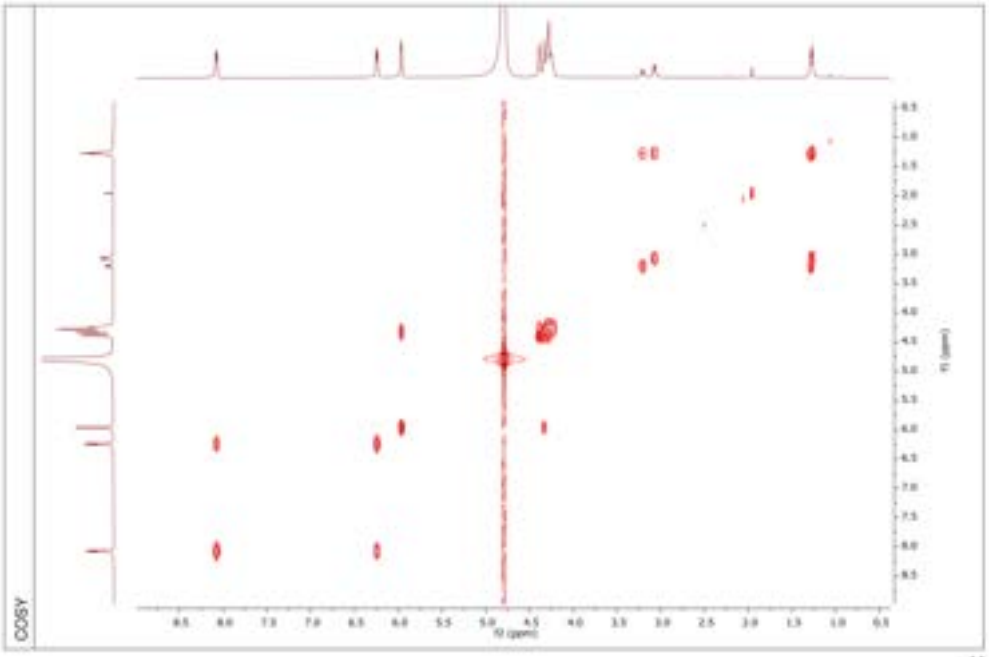
787.02142
789.02839
789.02587
m/z

12


¹H NMR (500 MHz, D₂O, 25°C)
f1 (ppm)

13



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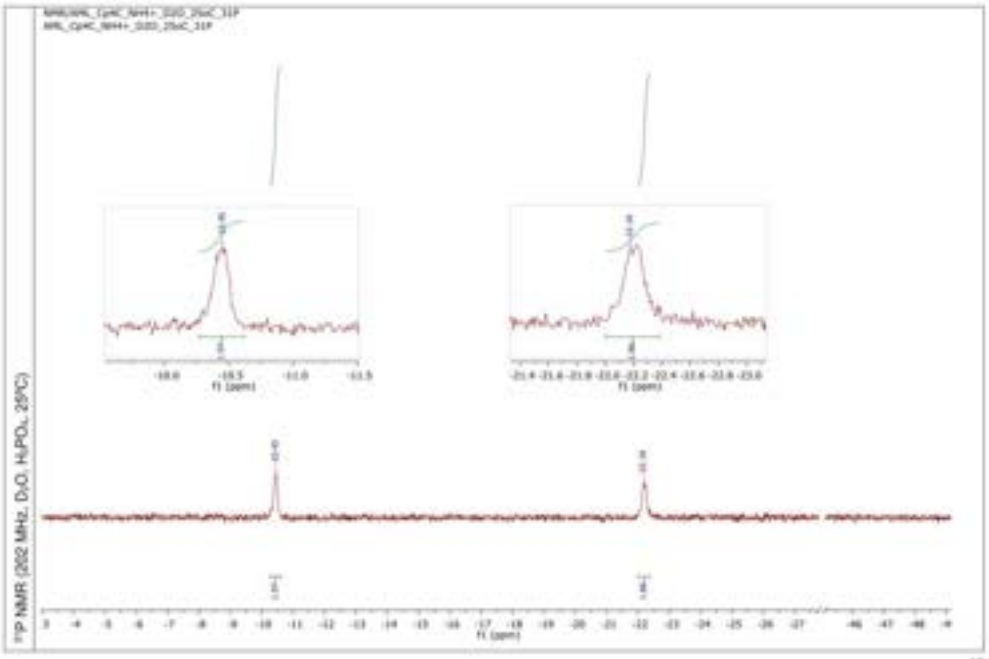
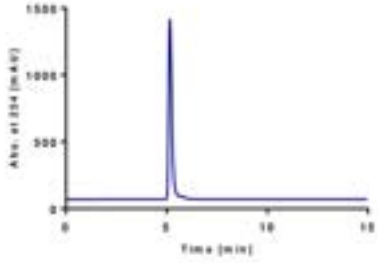


15

Compound 3: Ap_2C

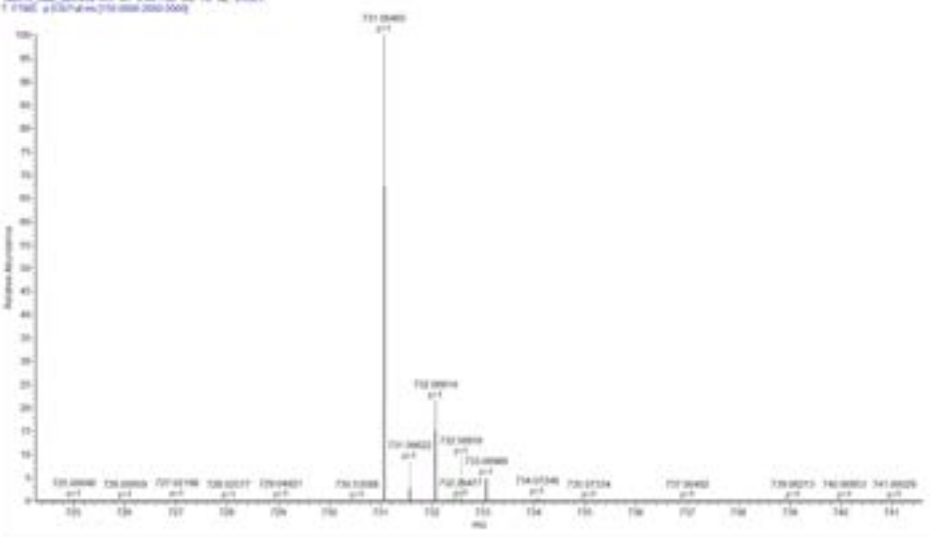
Chemical structure

HPLC



16

HRMS (-) ESI (Calc. [M-H]⁻ $C_{19}H_{26}N_8O_{17}P_2^-$: 731.06342)



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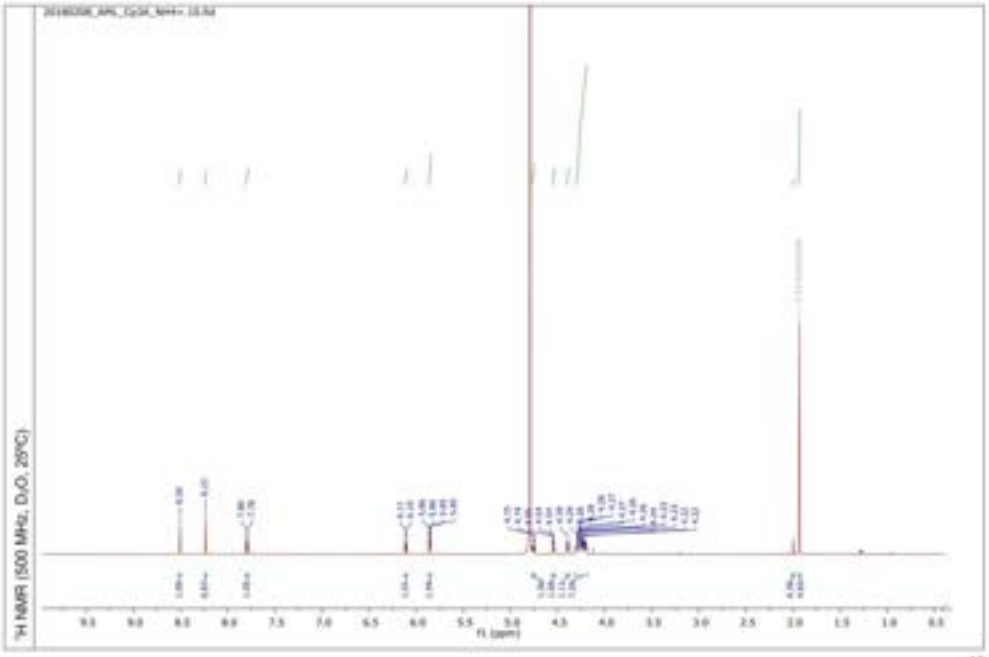

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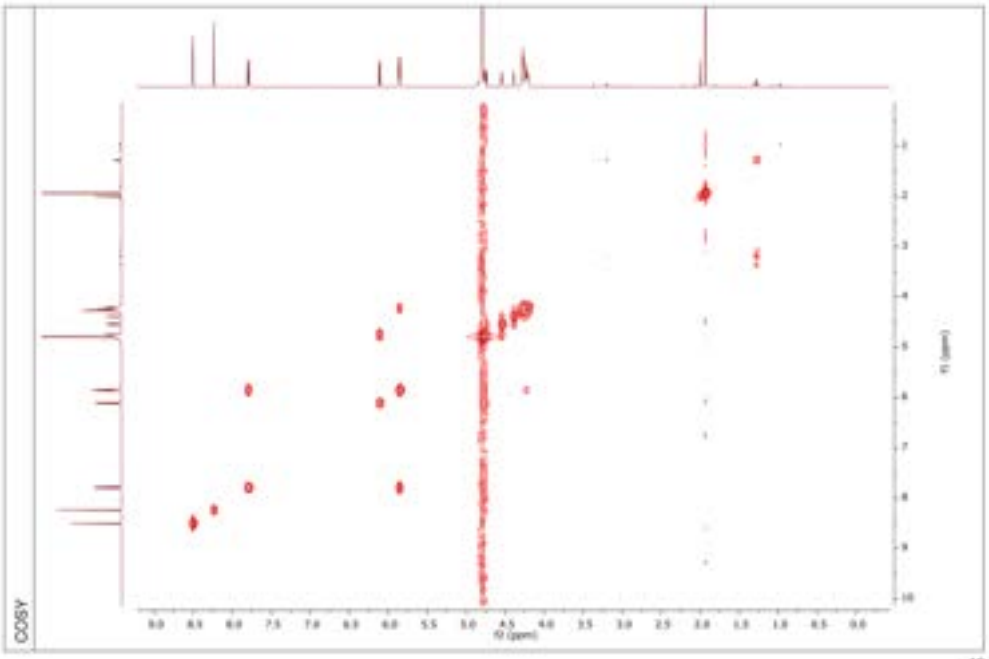

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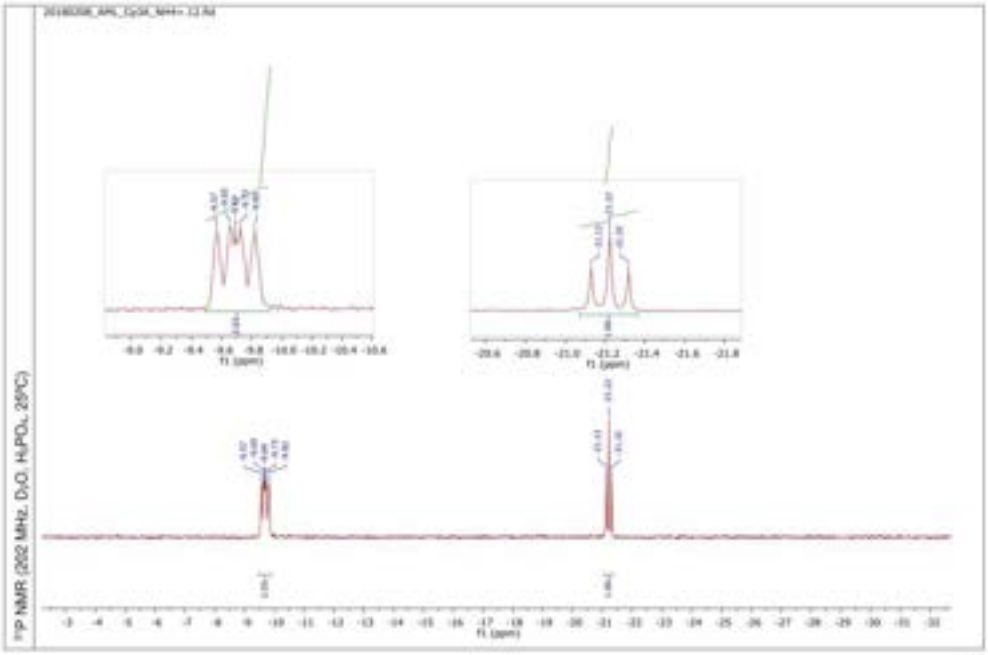
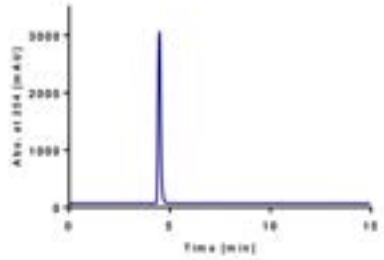


20

Compound 4: Ap$_4$C

Chemical structure

HPLC



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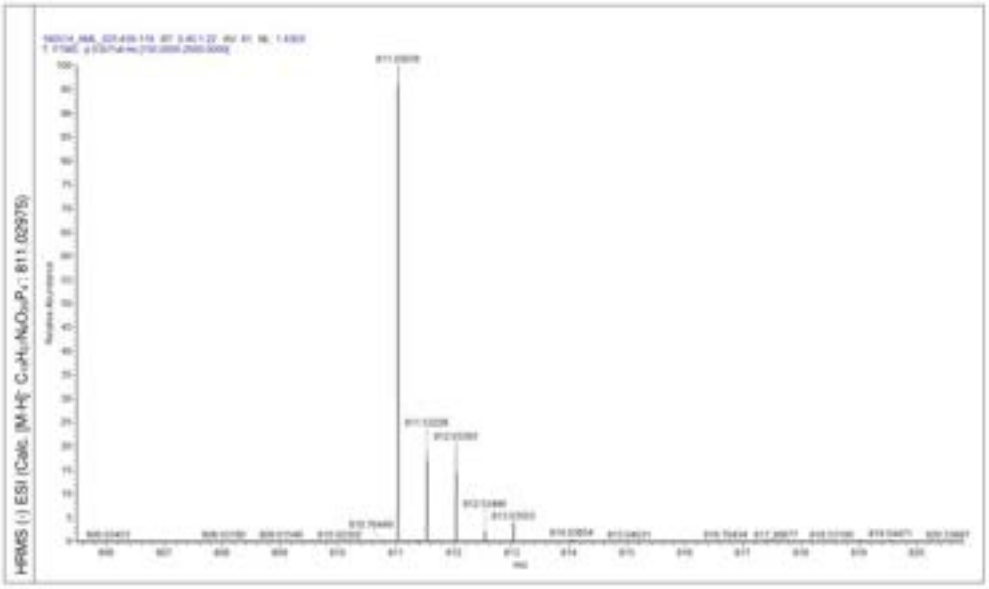
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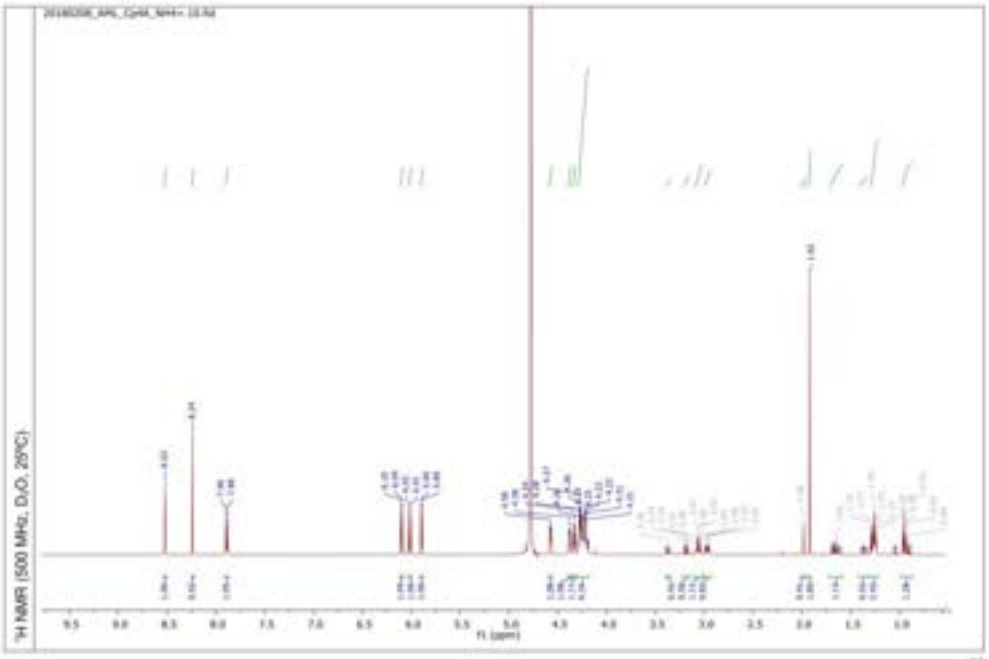
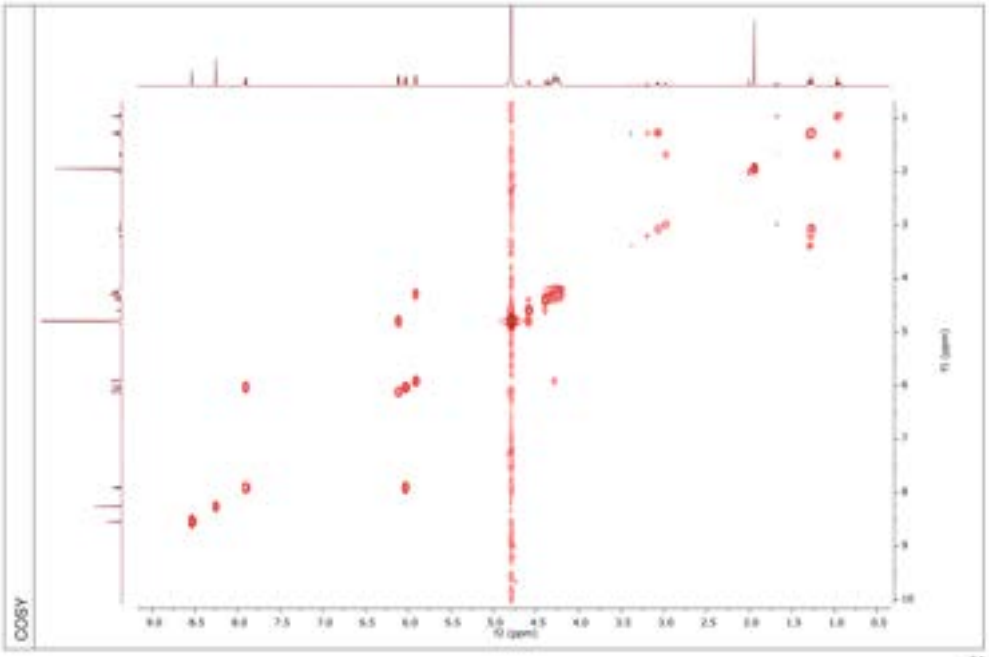
22


^{1}H NMR (500 MHz, D_2O, 25°C)

23



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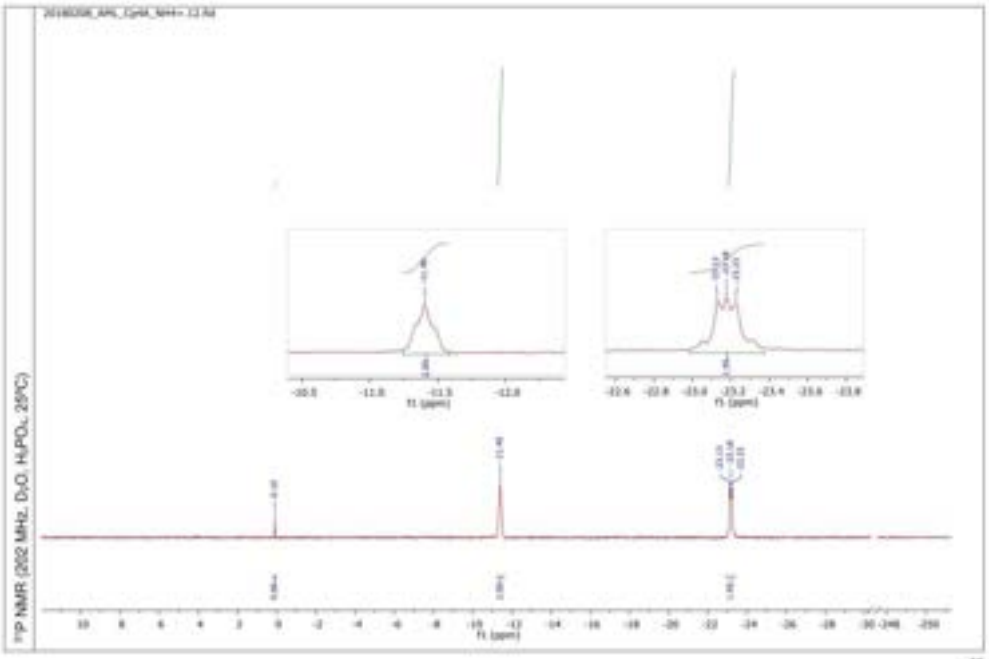
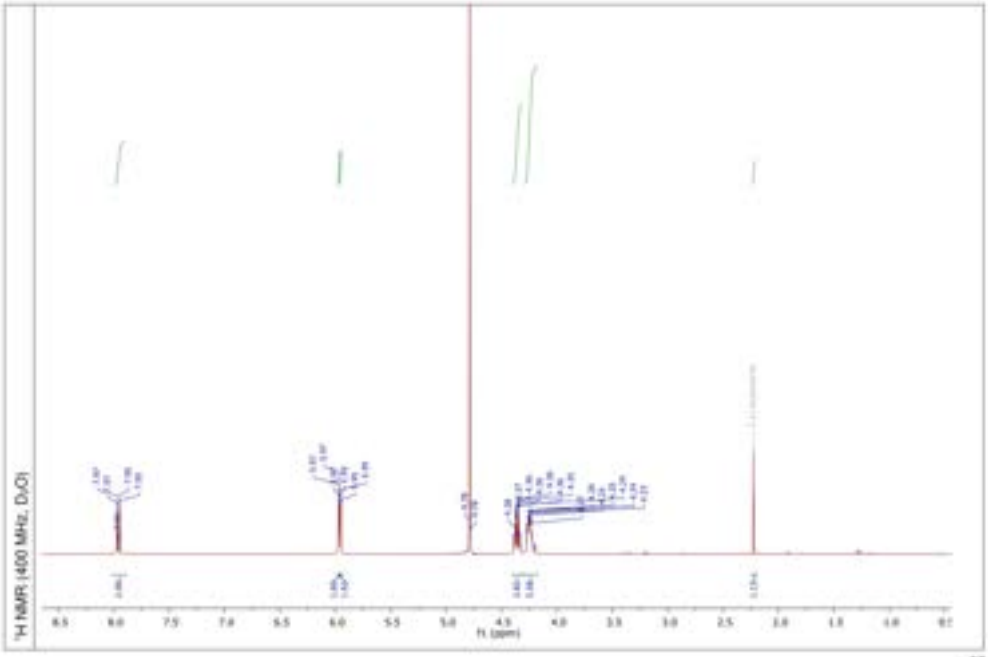


25

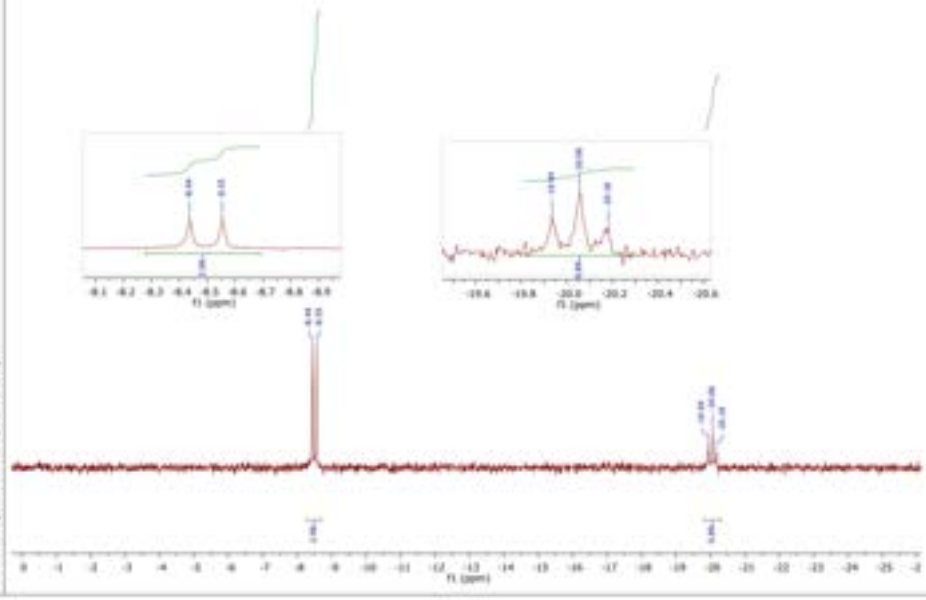
Compound 5: Up_3U

Chemical structure

26



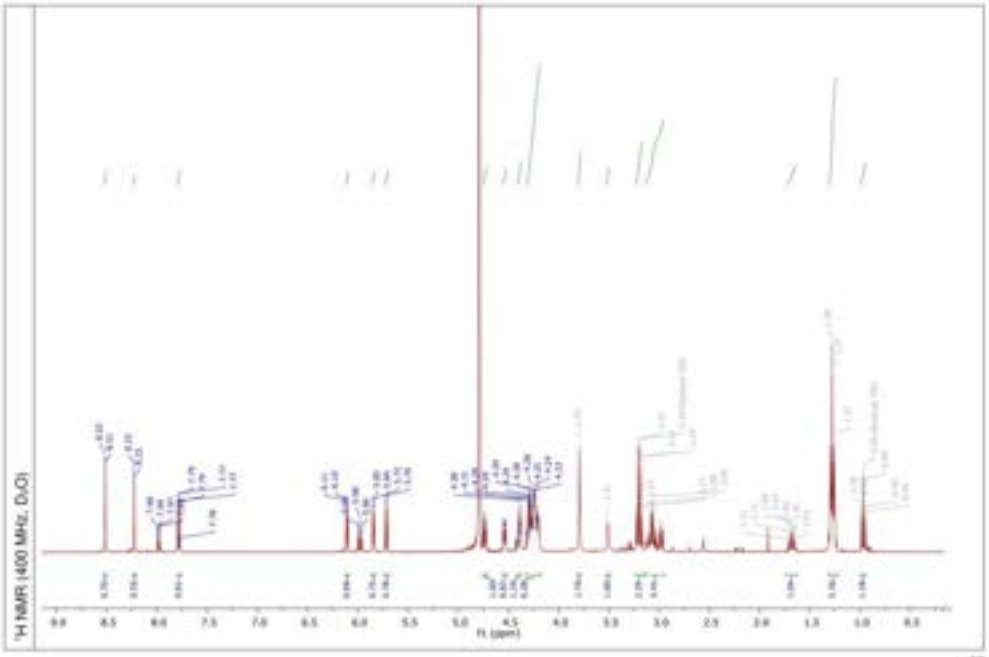
27

^{31}P NMR (162 MHz, D_2O, H_3PO_4)	

28

Compound 6: Ap_3U	
Chemical structure	

29



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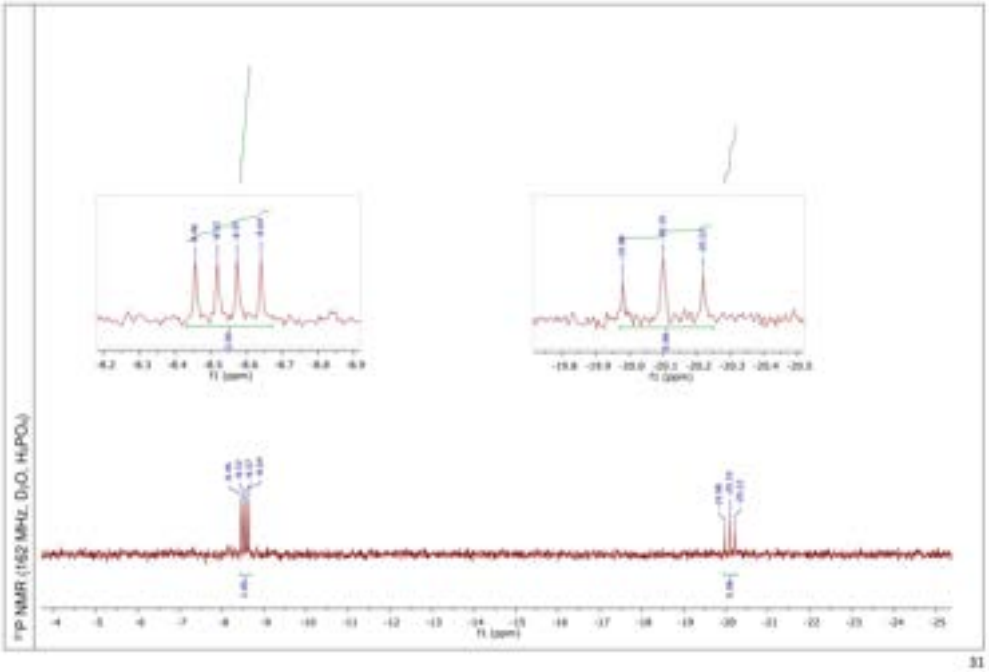
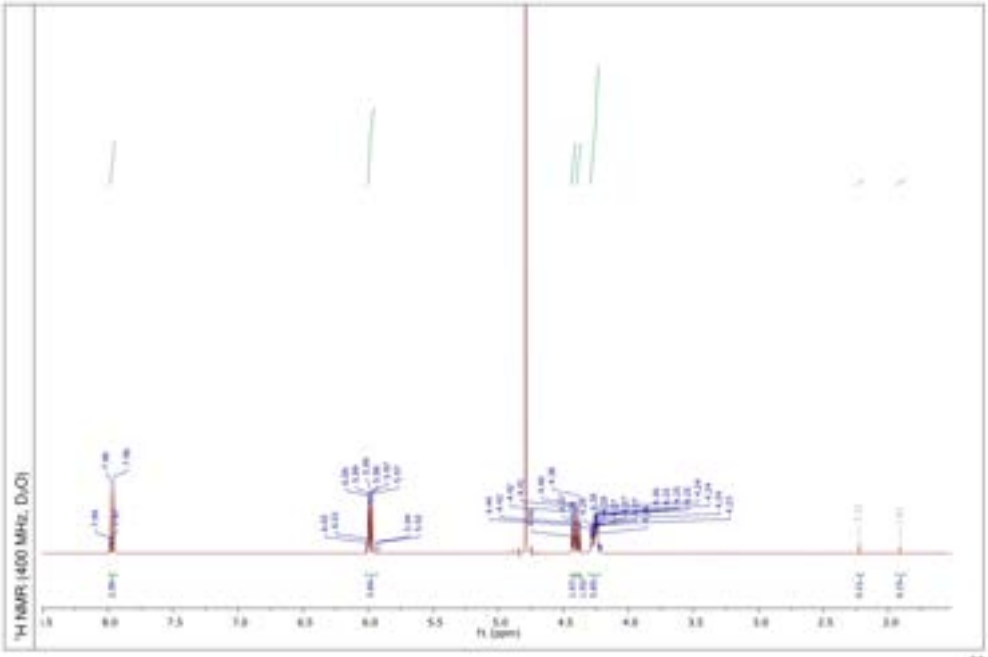


31

Compound 7: Up_5U

Chemical structure

32



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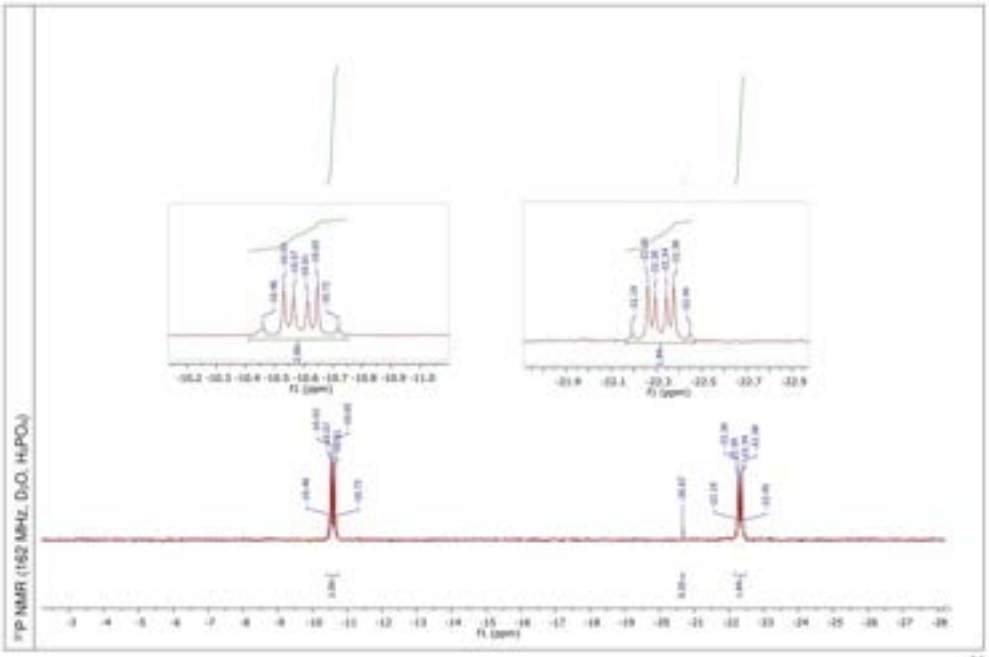
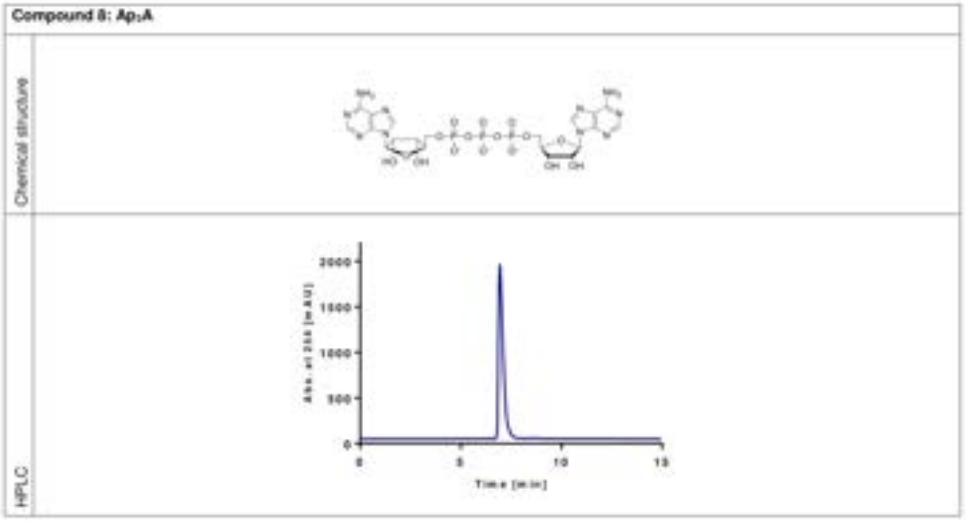
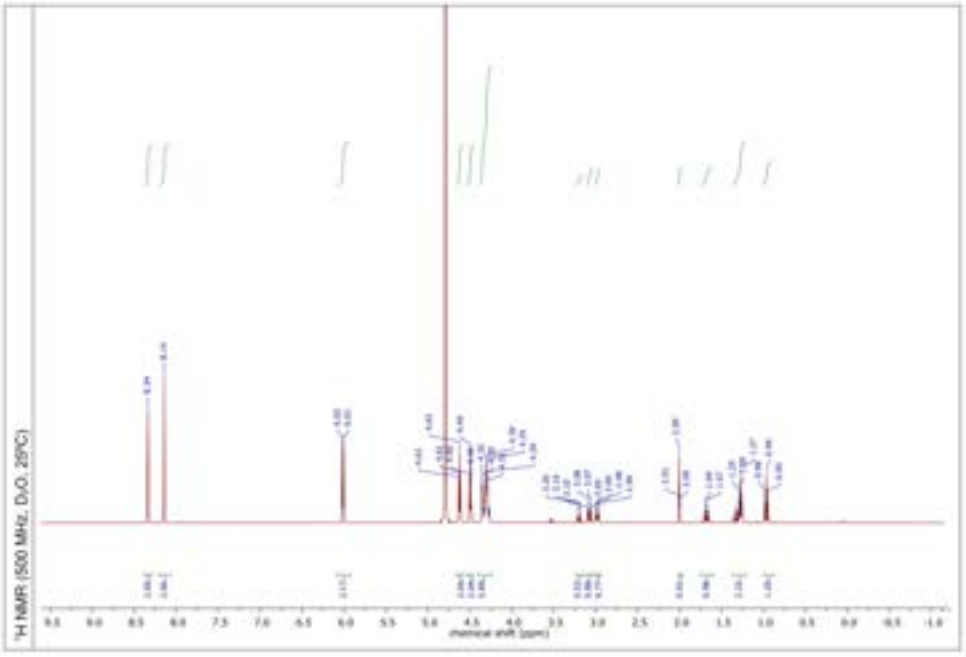

^{31}P NMR (162 MHz, D_2O, H_3PO_4)
F1 (ppm)

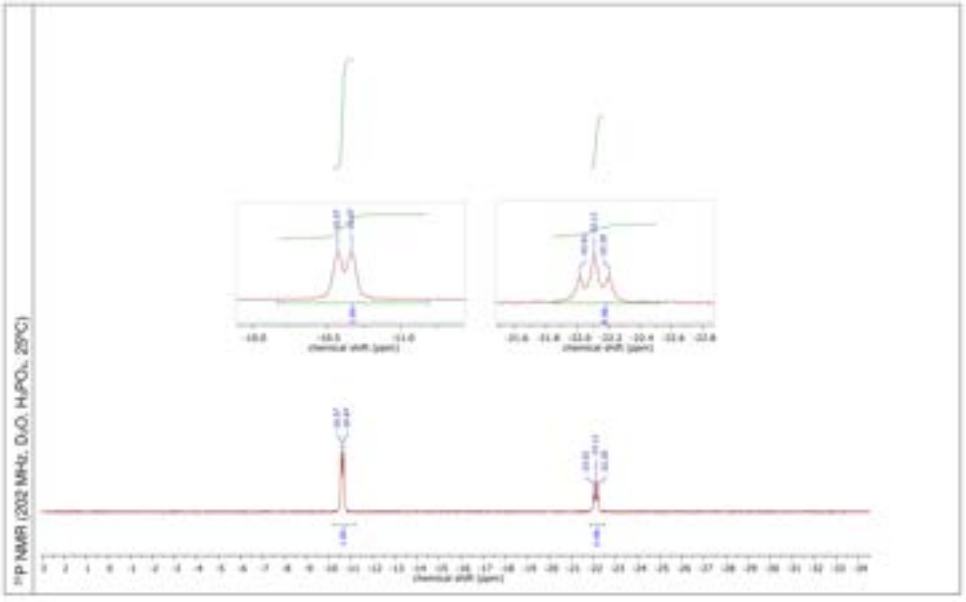
34


Compound 8: Ap$_3$A
Chemical structure
HPLC
Time [min]

35


^{1}H NMR (500 MHz, D$_2$O, 25°C)
chemical shift (ppm)

36


^{31}P NMR (202 MHz, D$_2$O, H$_3$PO$_4$, 25°C)
chemical shift (ppm)

37

Publikacja 3

Pietrowska-Borek M., **Dobrogojski J**., Wojdyła-Mamoń AM., Romanowska J., Gołębiewska J., Borek S., Murata K., Ishihara A., Pedreño MÁ., Guranowski A. 2021. Nucleoside 5′-phosphoramidates control the phenylpropanoid pathway in *Vitis vinifera* suspension-cultured cells. *International Journal of Molecular Sciences*, 22, 13567.

International Journal of
Molecular Sciences

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Article

Nucleoside 5′-Phosphoramidates Control the Phenylpropanoid Pathway in *Vitis vinifera* Suspension-Cultured Cells

Małgorzata Pietrowska-Borek [1,*], Jędrzej Dobrogojski [1], Anna Maria Wojdyła-Mamoń [1], Joanna Romanowska [2], Justyna Gołębiewska [2], Sławomir Borek [3], Koichi Murata [4], Atsushi Ishihara [5], Maria Ángeles Pedreño [6] and Andrzej Guranowski [1]

[1] Department of Biochemistry and Biotechnology, Poznań University of Life Sciences, 60-632 Poznań, Poland; jedrzej.dobrogojski@up.poznan.pl (J.D.); anna.wojdyla@up.poznan.pl (A.M.W.-M.); guranow@up.poznan.pl (A.G.)
[2] Department of Nucleoside and Nucleotide Chemistry, Institute of Bioorganic Chemistry, Polish Academy of Sciences, 61-704 Poznań, Poland; joarom@ibch.poznan.pl (J.R.); jgolebiewska@ibch.poznan.pl (J.G.)
[3] Department of Plant Physiology, Adam Mickiewicz University Poznań, 61-614 Poznań, Poland; borek@amu.edu.pl
[4] Graduate School of Agriculture, Tottori University, Tottori 680-8553, Japan; voba.scan24@gmail.com
[5] Department of Life and Environmental Agricultural Sciences, Tottori University, Tottori 680-8553, Japan; aishihara@tottori-u.ac.jp
[6] Department of Plant Biology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain; mpedreno@um.es
* Correspondence: malgorzata.pietrowska-borek@up.poznan.pl

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Abstract: It is known that cells contain various uncommon nucleotides such as dinucleoside polyphosphates (Np_nN's) and adenosine 5′-phosphoramidate (NH_2-pA) belonging to nucleoside 5′-phosphoramidates (NH_2-pNs). Their cellular levels are enzymatically controlled. Some of them are accumulated in cells under stress, and therefore, they could act as signal molecules. Our previous research carried out in *Arabidopsis thaliana* and grape (*Vitis vinifera*) showed that Np_nN's induced the expression of genes in the phenylpropanoid pathway and favored the accumulation of their products, which protect plants against stress. Moreover, we found that NH_2-pA could play a signaling role in *Arabidopsis* seedlings. Data presented in this paper show that exogenously applied purine (NH_2-pA, NH_2-pG) and pyrimidine (NH_2-pU, NH_2-pC) nucleoside 5′-phosphoramidates can modify the expression of genes that control the biosynthesis of both stilbenes and lignin in *Vitis vinifera* cv. Monastrell suspension-cultured cells. We investigated the expression of genes encoding for phenylalanine ammonia-lyase (*PAL1*), cinnamate-4-hydroxylase (*C4H1*), 4-coumarate:coenzyme A ligase (*4CL1*), chalcone synthase (*CHS1*), stilbene synthase (*STS1*), cinnamoyl-coenzyme A:NADP oxidoreductase (*CCR2*), and cinnamyl alcohol dehydrogenase (*CAD1*). Each of the tested NH_2-pNs also induced the expression of the *trans*-resveratrol cell membrane transporter *VoABCG44* gene and caused the accumulation of *trans*-resveratrol and *trans*-piceid in grape cells as well as in the culture medium. NH_2-pC, however, evoked the most effective induction of phenylpropanoid pathway genes such as *PAL1*, *C4H1*, *4CL1*, and *STS1*. Moreover, this nucleotide also induced at short times the accumulation of *N*-benzoylputrescine (BenPut), one of the phenylamides that are derivatives of phenylpropanoid and polyamines. The investigated nucleotides did not change either the lignin content or the cell dry weight, nor did they affect the cell viability throughout the experiment. The results suggest that nucleoside 5′-phosphoramidates could be considered as new signaling molecules.

Keywords: grape; uncommon nucleotides; signaling molecules; stilbenes; lignin

1. Introduction

Continuing our long-lasting studies on uncommon nucleotides, over a decade ago [1–6], we began to investigate the biochemistry of a rather neglected nucleotide, adenosine 5′-phosphoramidate (NH_2-pA), since it appeared to be a very good substrate of Fhit (for

fragile histidine triad) proteins [7]. Much earlier, this compound was detected among cellular nucleotides purified from the alga *Chlorella pyrenoidosa* [8]. It is known that in various organisms including plants, NH_2-pA can be synthesized [5,9] and degraded [7,10,11] by various enzymes, and it is considered as an enzymatic mechanism controlling the concentration of this nucleotide in cells. Its synthesis proceeds according to the reaction SO_4-pA + NH_4^+ → NH_2-pA + SO_4^{2-} + $2H^+$ catalyzed by adenylyl sulfate:ammonia adenylyltransferase (EC 2.7.7.51) (Figure 1). This activity was found in the algae *Chlorella pyrenoidosa* and *Euglena gracilis*, the amoeba *Dictyostelium discoideum*, the bacterium *Escherichia coli*, and the higher plants *Hordeum vulgare*, *Spinacia oleracea* [8], and *Lupinus luteus* [5]. In the latter organism, this transferase activity proved to be an inherent property of dinucleoside triphosphatase, the Fhit protein [5]. So far, various enzymes have been shown to catalyze the degradation of NH_2-pA, in most cases by hydrolysis to ammonia and AMP [10–15], and in a few cases to ammonia and ADP by phosphorolysis [14]. Interestingly, Fhits, regardless of their origin, are able to catalyze both the synthesis and cleavage of NH_2-pA [5].

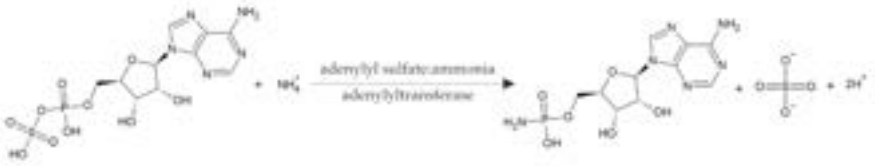

Figure 1. Scheme of the reaction catalyzed by adenylyl sulfate:ammonia adenylyltransferase (EC 2.7.7.51).

Our recent studies on in vitro cultivated *Arabidopsis* seedlings showed that exogenous NH_2-pA induced the expression of genes of the general phenylpropanoid pathway such as *PAL1*, *PAL2*, *PAL3*, *PAL4*, *C4H*, *4CL1*, *4CL2*, and *4CL3*. Moreover, it was also observed that induction of *CCR2*, *CHS*, and *ICS2* expression caused the accumulation of lignins, anthocyanins, and salicylic acid, respectively [4], which protect cells against various types of stresses. Other compounds that are involved in plant defenses against abiotic and biotic stresses are phenylamides, also termed as phenolamides or hydroxycinnamic acid amides [16]. The phenylamides arise from phenolic moieties, hydrocinnamic and benzoic acids, covalently linked through amide bonds to an aromatic monoamine or an aliphatic polyamine. Their synthesis is positioned at the crossroads of the phenylpropanoid pathway and the metabolism of amines [16] and can be used in the cross-linking of cell wall components in plants (Figure 2) [17,18]. An elevated concentration of phenylamides has been reported in a wide range of plant species, and it can play a protective role against biotic stresses [19–21]. This is why we decided to check whether the NH_2-pNs also affect the metabolism of those compounds.

Although it is not known whether any of the NH_2-pNs accumulates in response to environmental stresses, according to our earlier observation of the induction of the phenylpropanoid pathway in *Arabidopsis thaliana* seedlings by NH_2-pA [4], it seems plausible that biotic and abiotic environmental factors do affect the accumulation of this nucleotide, the putative regulatory molecule. The signaling transduction pathways underlying both abiotic and biotic stresses mediating the regulation of cellular responses are still intensively studied by many researchers. One of the defense strategies in higher plants under abiotic and biotic stresses is an activation of the phenylpropanoid pathway [22]. This pathway occurs widely in plant species, conferring adaptive advantages to diverse ecosystems. Its activation leads to the enhanced production of various phenylpropanoid compounds such as flavonoids [23,24], lignins [25], anthocyanins [26], salicylic acid [27], and stilbenes [28]. These metabolites reduce the adverse effects caused by stress-induced oxidative damage. One of the most studied stilbenes is *trans*-resveratrol. This compound is especially involved in plant–pathogen interactions [28] and plays an important role in plant responses to cadmium [29]. Besides the phenylpropanoid-based mechanism of plant responses to various

biotic and abiotic stresses, another mechanism is the regulation of the ratio of S-containing compounds such as methionine, glutathione, phytochelatins, and glucosinolates by the activity of ATP-sulfurylase [30]. Through these S-compounds, that enzyme is involved in the plant tolerance of several biotic and abiotic stresses. For example, glutathione can control the gene expression of antioxidant enzymes such as superoxide dismutase or glutathione reductase as well as enzymes of the phenylpropanoid pathway (e.g., chalcone synthase and phenylalanine ammonia-lyase) under cadmium stress [31]. ATP-sulfurylase catalyzes the activation of SO_4^{2-}, yielding high-energy adenosine-5′-phosphosulfate (APS) [30]. It is known that in plants, APS can be converted into NH_2-pNs by ammonolysis catalyzed by adenylylsulfate-ammonia adenylyltransferase [9] and Fhit proteins [5]. Moreover, Fhit can degrade NH_2-pA, releasing AMP and NH_3 [7,10,15].

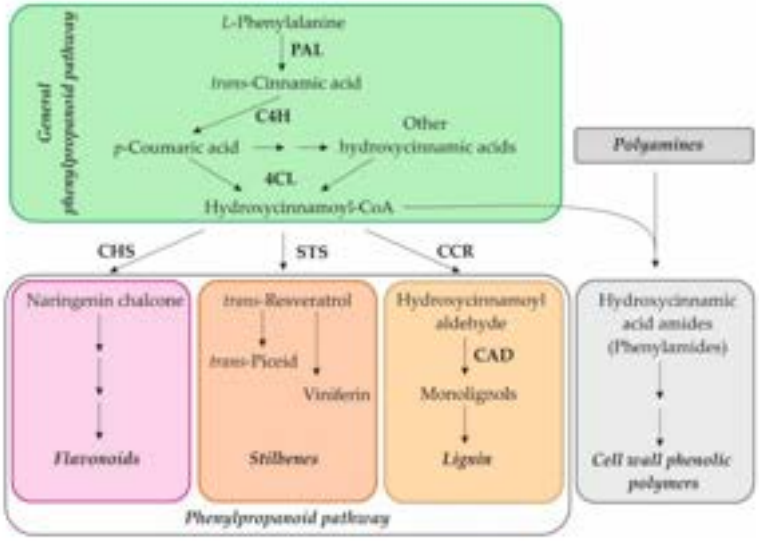

Figure 2. Scheme of the phenylpropanoid pathway and connection to the phenylamide metabolism. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; STS, stilbene synthase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase.

The main goal of our research was to learn how NH_2-pA as well as other NH_2-pNs including NH_2-pG (guanosine 5′-phosphoramidate), NH_2-pC (cytidine 5′-phosphoramidate), and NH_2-pU (uridine 5′-phosphoramidate) regulate the metabolism of phenylpropanoids and biosynthesis of phenylamides in grape cells. Moreover, we wanted to determine how these uncommon nucleotides impact the expression of the gene coding for the VvABCG44 transporter, which was proven to be involved in the transport (export) of *trans*-resveratrol in *Vitis vinifera*. This paper describes the results of experiments conducted on a *Vitis vinifera* cv. Monastrell suspension cell culture and presents a hypothesis concerning links between NH_2-pNs and the production of *trans*-resveratrol.

2. Results

In this study, we used a suspension cell culture (SCC) of the grape cell cultivar Monastrell, which is a very convenient model. First, because of the equal distribution of molecules studied as effectors among the cells, and second, this particular variety of the grape effectively synthesizes the phenylpropanoid molecule *trans*-resveratrol. The

analysis of gene expression and accumulation of different products of the phenylpropanoid pathway was carried out as described in our earlier studies [3,4,6]. For details, see Section 4. In our earlier studies on the effect of NH_2-pA on the expression of the genes coding for phenylalanine ammonia-lyase (PAL) and 4-coumarate:coenzyme A ligase (4CL) in *Arabidopsis* seedlings, we found that of the concentrations tested in the 0.05–25 µM range, 5 µM NH_2-pA appeared to be the most effective [4]. In addition, in the experiments on the grape suspension cells described here, this relatively low concentration of NH_2-pA evoked marked effects. Therefore, each of the investigated NH_2-pNs was applied to the cells at a fixed 5 µM concentration. Based on previous studies [3,4,6,32–34], we chose the following genes: *PAL1*, *C4H1*, *4CL1*, *STS1*, *CAD1*, and *CCR2*. In addition, we selected the time points of the experiment based on our previous works [3,6].

2.1. Do the NH_2-pNs Affect Chalcone Synthase (*CHS1*) Gene Expression?

The expression of the *CHS1*, the branch point of flavonoid biosynthesis, was evaluated at the same time points as other gene expressions were analyzed in this study, but it was not detected. The same results were observed in the grape suspension cell culture in our previous studies [3,6] and by Lijavetzky and coworkers [32]. These data strongly suggest that flavonoids are not synthesized in the dark in cells of this plant species.

2.2. Effect of Exogenous NH_2-pNs on the Expression of Genes of the General Phenylpropanoid Pathway

Expression of the three genes *PAL1*, *C4H1*, and *4CL1* was analyzed in the cells collected between 6 and 72 h of growth after elicitation. The results of these experiments are summarized in Figure 3a–c. A marked increase in the expression of the studied genes was observed in the grape cells collected after 72 h. Interestingly, NH_2-pC evoked the most significant effect of the analyzed compounds, with an approximately 8-fold increase in *PAL1*. It was over 2-fold higher in comparison to the effect exerted by NH_2-pG and NH_2-pU and about 4-fold higher than that caused by NH_2-pA. Additionally, the expression of *4CL1* was induced much more effectively by NH_2-pC than by the other tested NH_2-pN, and it reached about a 10-fold increase with respect to the controls. Effects evoked by 5 µM NH_2-pG, NH_2-pA, or NH_2-pU were less spectacular, with only 4-, 3.2-, and 3-fold increases compared to the control, respectively. The expression of *PAL1* and *4CL1* after 6 and 24 h of elicitation by each of the tested nucleotides did not change. In the case of *C4H1* expression, we observed an inhibitory effect evoked by NH_2-pU, NH_2-pA, and NH_2-pG at 72 h. It was about 2- to 3-fold lower than in the control. In cells treated with NH_2-pC, the expression of *C4H1* increased up to 2.5-fold. However, it was 5- to 8-fold higher than in cells treated with other nucleotides (Figure 3a–c).

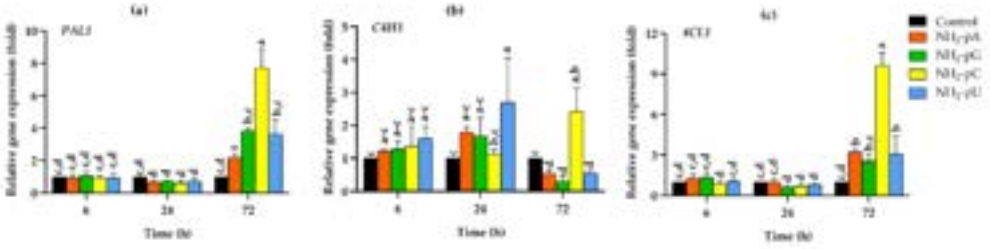

Figure 3. Expression of general phenylpropanoid pathway genes in cells of *Vitis vinifera* cv. Monastrell treated with 5 µM nucleoside 5′-phosphoramidates. (**a**) *PAL1*, phenylalanine ammonia-lyase; (**b**) *C4H1*, cinnamate-4-hydroxylase; (**c**) *4CL1*, 4-coumarate:CoA ligase. Total RNA was reverse-transcribed into cDNA and used as a template for real-time quantification PCR reaction as described in the Section 4. Specific primers were designed for *PAL1*, *C4H1*, *4CL1*, and *EFa1* (elongation factor

1-alpha, which was used as an endogenous control). The expression level of *PAL1*, *C4H1*, and *4CL1* in the control cells (no nucleotide added) was set to 1. Values represent the mean ± standard deviation of the three replicates. Values without a common letter were significantly different according to the analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) multiple range test ($p \leq 0.05$).

2.3. Effect of Exogenous NH_2-pNs on Stilbene Synthase Gene (*STS1*) Expression and Stilbene Accumulation in Grape Cells

All of the tested NH_2-pNs increased the expression of *STS1* about 2-fold after 24 h, but only NH_2-pG, NH_2-pC, and NH_2-pU also increased the expression of *STS1* in 72 h. At this time point, the most effective was NH_2-pC, even causing a 13-fold higher expression than in the control (Figure 4a). Such a spectacular effect inspired us to investigate how this induction of *STS1* expression affects the accumulation of the related stilbene compounds (i.e., *trans*-resveratrol and its glycoside - *trans*-piceid). In the cells collected after 6 and 12 h, no significant effect of the tested nucleotides on *trans*-resveratrol content was found. However, after 24 h of elicitation with any of the investigated NH_2-pNs, a dramatic increase in the level of this secondary metabolite was observed (Figure 4b). The most significant effect was evoked by NH_2-pA and NH_2-pU at 24 h. In their presence, the accumulation of *trans*-resveratrol in the grape cells reached 961 μg g^{-1} dry weight (DW) and 821 μg g^{-1} DW, respectively, and it was about 1.7- and 1.5-fold higher than in the control cells (Figure 4b). We did not observe statistically significant changes in *trans*-resveratrol accumulation in the presence of NH_2-pG compared with the control cells. However, in grape cells treated with the pyrimidine nucleotide, NH_2-pC, the accumulation of *trans*-resveratrol only reached 76 μg g^{-1} DW, and it was 7-fold lower than in the control cells. In the cells collected after 48 h, the *trans*-resveratrol content was much lower than that at 24 h of the experiment, and in those collected after 72 h, it was as low a level as in the cells collected after 6 and 12 h of elicitation (Figure 4b). The *trans*-piceid content, similar to *trans*-resveratrol, was low at 6 and 12 h, irrespective of the nucleotide treatment (Figure 4c). The content of *trans*-piceid was clearly elevated after 24 h of elicitation including in the control cells, but it was dramatically decreased in the cells treated with NH_2-pG, being 13-fold lower than in the control cells and reached only 183 μg g^{-1} DW (Figure 4c). Interestingly, however, after 48 h, the accumulation of *trans*-piceid reached a maximum. At this time, in cells treated with NH_2-pA or NH_2-pC, the level of this stilbene was over 2-fold higher than in the control cells. Similar to the *trans*-resveratrol content, the level of *trans*-piceid decreased dramatically after 72 h of elicitation and reached a level comparable to that observed after 6 and 12 h of the experiment (Figure 4c).

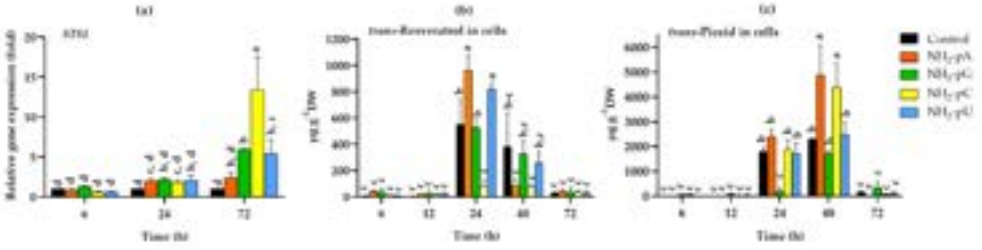

Figure 4. Expression of stilbene synthase gene (*STS1*) and accumulation of stilbenes in cells of *Vitis vinifera* cv. Monastrell treated with 5 μM nucleoside 5′-phosphoramidates. (**a**) *STS1*, stilbene synthase; (**b**) accumulation of *trans*-resveratrol; (**c**) accumulation of *trans*-piceid. Total RNA was reverse-transcribed into cDNA and used as a template for real-time quantification PCR reaction as described in the Section 4. Specific primers were designed for *STS1* and *EFa1* (elongation factor 1-alpha, which was used as an endogenous control). The expression level of *STS1* in the control cells (no nucleotide added) was set to 1. Values represent the mean ± standard deviation of the three replicates. Accumulation of *trans*-resveratrol and *trans*-piceid was determined using the HPLC method as described in the Section 4. Values without a common letter are significantly different according to ANOVA and Tukey's HSD multiple range test ($p \leq 0.05$).

2.4. Expression of the Gene Coding for the Resveratrol Transporter VvABCG44 (ATP-Binding Cassette Transporter) and Stilbene Content in the Spent Media

It is known that treatment of cultured grape cells with an elicitor, cyclodextrin, causes the accumulation of *trans*-resveratrol and induction of gene expression of the full-size ABCG transporter, which is associated with the transport of this stilbene compound in plants [35]. Therefore, we also analyzed the effects of NH_2-pNs on the expression of the *VvABCG44* gene. As shown in Figure 5a, each of the investigated nucleoside phosphoramidates evoked around a 3-fold increase of the gene expression, and it was already observed after 6 h of elicitation. Then, this gene expression declined at 24 and 72 h. An exception was observed in cells treated with NH_2-pG at 72 h, since the increase in the gene expression was still 2-fold higher than in the controls. The time-course of *trans*-resveratrol and *trans*-piceid accumulation in the spent medium in response to NH_2-pNs are shown in Figure 5b,c, respectively. Intensive export of these compounds from the cells to the media occurred, and it was observed just after 6 h of elicitation. The concentration of *trans*-resveratrol after 6 h of NH_2-pG application really reached 9.5 µM (Figure 4b), but the concentration of *trans*-piceid after 6 h of NH_2-pC reached less than 2 µM (Figure 5c). In the spent medium in which the cells were treated with NH_2-pA, we observed a gradual increase in *trans*-resveratrol content up to 48 h. Then, the level of this stilbene drastically decreased (Figure 5b). At 72 h of the experiment, the concentration of *trans*-resveratrol was at the same level as in the control media. The highest accumulation of *trans*-piceid in the spent media caused by nucleotides was observed at 24 h, and for NH_2-pA, NH_2-pC, NH_2-pG, and NH_2-pU, it was 12-, 9-, 7-, and 5-fold higher than in the control media, respectively; however, only the 12-fold increase was statistically significant (Figure 5c).

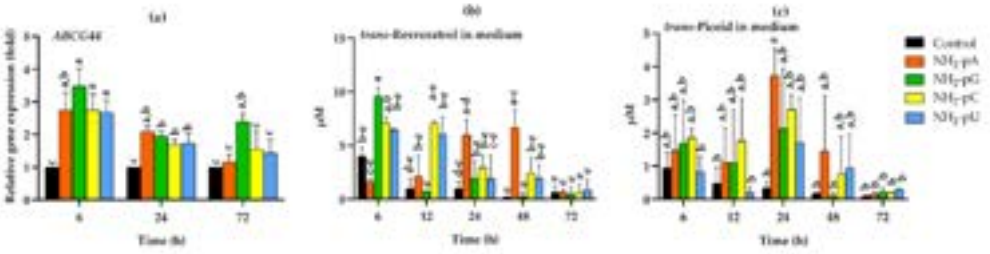

Figure 5. Expression of the *VvABCG44* resveratrol transporter gene in the cells of *Vitis vinifera* cv. Monastrell treated with 5 µM nucleoside 5′-phosphoramidates, and accumulation of stilbenes in the spent medium. (**a**) *VvABCG44*, resveratrol transporter gene; (**b**) accumulation of *trans*-resveratrol; (**c**) accumulation of *trans*-piceid. Total RNA was reverse-transcribed into cDNA and used as a template for real-time quantification PCR reaction as described in the Section 4. Specific primers were designed for *VvABCG44* and *EFa1* (elongation factor 1-alpha, which was used as an endogenous control). The expression level of *VvABCG44* in the control cells (no nucleotide added) was set to 1. Accumulation of *trans*-resveratrol and *trans*-piceid was determined using the HPLC method as described in the Section 4. Values are the mean ± standard deviation of the three replicates. Values without a common letter were significantly different according to ANOVA and Tukey's HSD multiple range test ($p \leq 0.05$).

2.5. Cell Viability

Because we observed, both in the cells and in the spent media, a considerable decrease in the content of *trans*-resveratrol and *trans*-piceid at 72 h of the experiment, and due to the fact that at this time there was no effect of nucleotides on the content of these two stilbenes, we assessed the cell viability and cell growth (expressed as dry weight content) to exclude the possibility of cell death caused by the exogenous application of nucleotides. As shown in Figure 6, no losses in cell viability were observed by fluorescent microscopy at 72 h of treatment with the nucleotides.

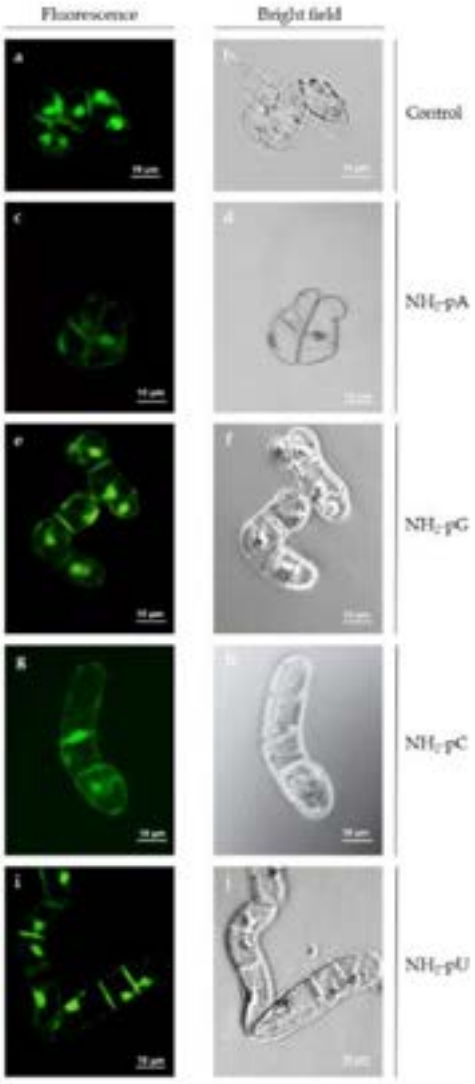

Figure 6. Viability of *Vitis vinifera* cell suspension culture treated with 5 μM NH_2-gNs at 72 h. It was evaluated by incubating cells in the presence or absence of the indicated nucleotide for 1–2 min in fresh Gamborg medium using the Plant Cell Viability Assay Kit (Sigma-Aldrich, Burlington, MA, USA) as described in the Section 4. Fluorescence was observed with an AxioVert 200 Carl Zeiss microscope using a Zeiss filter (FS09 exc = 495 nm, emi = 517 nm). (**a**,**b**) control cells; (**c**,**d**) cells treated with NH_2-pA; (**e**) and (**f**) NH_2-pG; (**g**,**h**) NH_2-pC; (i,j) NH_2-pU. The left-hand column shows cells under the fluorescence microscope, and the right-hand column shows cells under the bright field microscope. No red fluorescence, which indicates cell damage or mortality (FS15 exc = 538 nm, emi = 617 nm) was found (data not shown).

We also analyzed changes in the cell dry weight throughout the experiment. As shown in Figure 7, the nucleotide-treated cells displayed a similar biomass increase (from 10 to over 29 g DW L^{-1}), and therefore cell growth as the control (i.e., untreated cells).

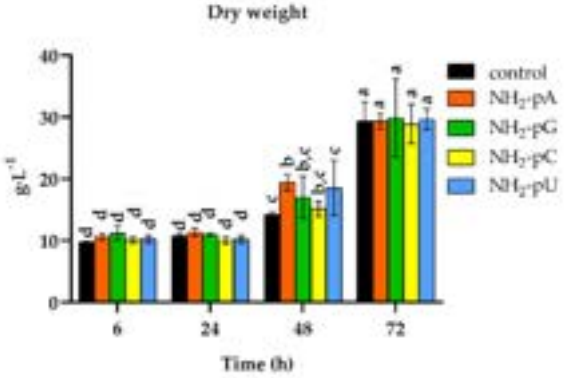

Figure 7. Time course of cell growth (expressed as cell dry weight per liter) of *Vitis vinifera* treated with 5 µM nucleoside 5′-phosphoramidates. Cell samples were air-dried for 48 h at 70 °C. Values represent the mean ± standard deviation of the three replicates. Values without a common letter were significantly different according to ANOVA and Tukey's HSD multiple range test ($p \leq 0.05$).

2.6. Expression of Genes Encoding for Enzymes of Monolignol Biosynthesis and Lignin Content

We tested both the expression of genes involved in lignin biosynthesis and the accumulation of lignin, one of the main products of the phenylpropanoid pathway. *CCR2* gene encodes cinnamoyl-CoA reductase, which is the first step in monolignol biosynthesis. As can be seen in Figure 8a, the expression of *CCR2* was induced by nucleotides only at 6 h of treatment, and for NH_2-pU, NH_2-pA, NH_2-pC, and NH_2-pG, it was 1.8-, 2-, 2.2- and 2.9-fold higher than in the control, respectively. Then, a reduced expression of *CCR2* was observed, being at 72 h 2-fold lower in all nucleotide treatments than in the control (i.e., untreated cells) (Figure 8a).

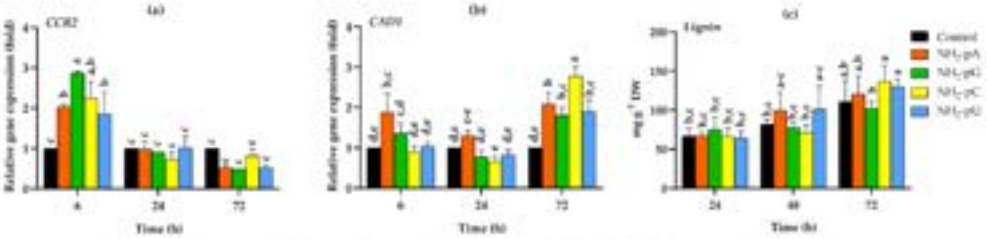

Figure 8. Expression of cinnamoyl-CoA reductase (*CCR2*), cinnamyl alcohol dehydrogenase (*CAD1*), and lignin content in cells of *Vitis vinifera* cv. Monastrell treated with 5 µM nucleoside 5′-phosphoramidates. (**a**) *CCR2*, cinnamoyl-CoA reductase (**b**) *CAD1*, cinnamyl alcohol dehydrogenase (**c**) lignin content. Total RNA was reverse-transcribed into cDNA and used as a template for real-time quantification PCR reaction as described in the Section 4. Specific primers were designed for *CCR2*, *CAD1*, and *EFa1* (elongation factor 1-alpha, which was used as an endogenous control). The expression level of *CCR2* and *CAD1* in the control cells (no nucleotide added) was set to 1. The lignin content was determined as described in the Section 4. Values represent the mean ± standard deviation of the three replicates. Values without a common letter were significantly different according to ANOVA and Tukey's HSD multiple range test ($p \leq 0.05$).

CAD1 gene expression, encoding cinnamyl alcohol dehydrogenase, was also assessed. Expression of this gene increased up to 2.7-fold in cells treated with 5 µM NH_2-pC at 72 h. At this time point, all other tested nucleotides evoked only a 2-fold higher effect than in the control cells (Figure 8b).

On the other hand, the nucleotides investigated in this study during the experiments had no effect on the lignin content, and its level was similar to that found in control cells (Figure 8c).

2.7. Effect of Exogenous NH_2-pNs on the Content of Phenylamides in Both Cells and the Spent Media

Among the twenty-five phenylamides tested (Supplementary Materials (Methods S2)), only *N*-benzoylputrescine (BenPut) was accumulated in grape cells. The content of this phenylamide depended on the NH_2-pN nature and treatment time (Figure 9). Interestingly, at 6 h of treatment, NH_2-pC evoked induction of accumulation of BenPut. Its content reached 0.32 µg g^{-1} DW, and its level hardly changed throughout the experiment. In the case of other NH_2-pNs, BenPut was not detected at 6 h. At 24 h, an accumulation of BenPut was observed in cells treated with NH_2-pA, NH_2-pG, and NH_2-pC. At the further time points of the experiment, both in the controls and cells treated with nucleotides, the level of BenPut was similar and remained stable. To our knowledge, this is the first time detecting the accumulation of phenylamides in *Vitis vinifera*.

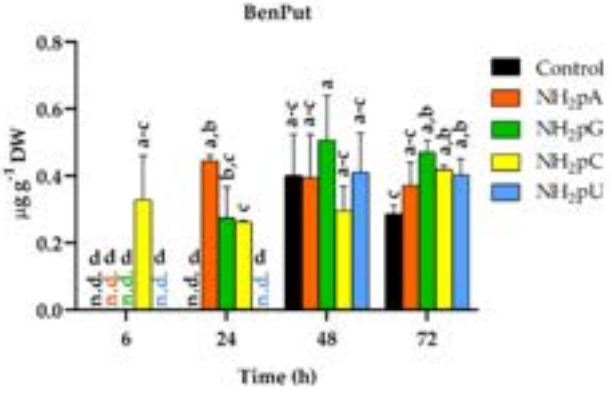

Figure 9. BenPut accumulation in cells of *Vitis vinifera* treated with 5 µM nucleoside 5′-phosphoramidates. Values represent the mean ± standard deviation of the three replicates. Values without a common letter were significantly different according to ANOVA and Tukey's HSD multiple range test ($p \leq 0.05$). n.d., not detected.

On the other hand, we did not observe the accumulation of any of the twenty-five tested phenylamides in the spent media.

3. Discussion

This study demonstrated that the uncommon nucleotide NH_2-pA, naturally occurring in organisms, applied to a cell suspension of *Vitis vinifera*, induced the expression of genes that control both the biosynthesis of stilbenes (Figures 4a and 5a) and lignins (Figure 8a,b). This induction caused a transient accumulation of *trans*-resveratrol and *trans*-piceid, both in the cells and spent media (Figures 4b,c and 5b,c), respectively. Another purine nucleotide, NH_2-pG, also induced the gene expression of resveratrol-cell-membrane-transporter throughout the experiment (Figure 5a). In fact, three canonical congeners of NH_2-pA: NH_2-pG, NH_2-pC, and NH_2-pU, which have not been so far identified as natural metabolites in any organism, also affected the expression of the aforementioned genes and

accumulation of stilbene compounds. Although all the tested nucleoside phosphoramidates acted as elicitors, some differences in effectiveness were observed among them. In fact, NH_2-pA (purine nucleotide) proved to be the most effective in inducing *VvABCG44* gene expression as well as in *trans*-resveratrol and *trans*-piceid accumulation. Additionally, NH_2-pC (pyrimidine nucleotide) turned out to be quite effective in inducing genes of the phenylpropanoid pathway in *Vitis vinifera*. However, during a short exposure time (6 h), NH_2-pG evoked the most significant effect on the expression of *VvABCG44* (Figure 5a) and *trans*-resveratrol accumulation in the spent medium (Figure 5b) among all investigated NH_2-pNs. The level of *trans*-resveratrol and *trans*-piceid decreased at 72 h of the experiment both in the medium and cells (Figure 4b,c and Figure 5b,c, respectively). As the cell suspension of *Vitis vinifera* remains alive at the end of the treatment with these nucleotides (Figure 6), it is plausible to think that *trans*-resveratrol and its glucoside, *trans*-piceid could be transformed by the action of cellular or extracellular peroxidases into other more complex stilbenes (such as viniferins) [36]. Results obtained from this study together with those previously carried out in *Arabidopsis* seedlings treated with NH_2-pA [4] suggests that the investigated nucleotides can act as signal molecules in plants. Moreover, our earlier studies showed that any common nucleotides such as AMP, GMP, UMP, and CMP that could be a product of degradation of NH_2-pNs did not evoke the accumulation of stilbenes in *Vitis vinifera* suspension cell culture [6].

We also investigated whether exogenously applied NH_2-pNs affected in grape cells the biosynthesis of lignin - other compounds derived from the phenylpropanoid pathway, known to be accumulated in plant tissues in response to abiotic or biotic stresses [25]. It was found, however, that the nucleotides used substantially modified neither the lignin content nor the cell growth (assessed as cell dry weight). Still, it should be kept in mind that both lignin biosynthesis and dry weight accumulation are long-term processes, and 72 h of treatment might not be sufficient to observe this effect on the accumulation of lignin and cell dry weight. These results nevertheless suggest that NH_2-pNs would be involved in the early signaling stages in response to environmental stimuli.

Considering the signaling role of the investigated nucleotides, the question is: what is the target of NH_2-pA, or generally, all NH_2-pNs? To answer this question, we postulate that in the control of gene expression by NH_2-pNs, the HIT proteins, which catalyze the cleavage of the phosphoramide bond in these nucleotides, are involved. As mentioned earlier, hydrolysis of the P–N bond liberates more energy than splitting the phosphate anhydride (P-O) bond; −38 kJ/mol versus −34 kJ/mol, respectively [9]. Whether the postulated signal transduction mediated by NH_2-pNs causes the adenylation, or generally the nucleotidylation, of the hypothetical target molecule, or causes only its conformational changes is another intriguing question awaiting elucidation. Speculating further, we suggest that, in plant cells, there is a link between the metabolism of sulfur and NH_2-pAs: first, by the double role of Fhit protein, which can act as an adenylylsulfate:ammonia adenylyltransferase, and as nucleoside phosphoramidase [5]; and second, by the known activation of sulfate metabolic pathways under biotic and abiotic stresses in plants [30].

Based on our previous studies on the effect of NH_2-pA on phenylpropanoid metabolism in *Arabidopsis* seedlings [4], a fact considered in our literature review [37], and the results presented here, we postulate that NH_2-pNs are involved in the plant response to environmental stresses via induction of the phenylpropanoid pathway. In Figure 10, we summarize the knowledge about the pathway of NH_2-pA metabolism and its effect on the phenylpropanoid pathway. Although our results indicate that another NH_2-pN (i.e., NH_2-pC) exerts impressive effects on the same genes of the phenylpropanoid pathway (Figures 3 and 4), we do not know if this compound occurs in nature and what enzymatic reaction might be responsible for its biosynthesis. We trust that our findings open new avenues that will be followed by different 'omic' studies that will shed more light on physiological functions of these nucleotides.

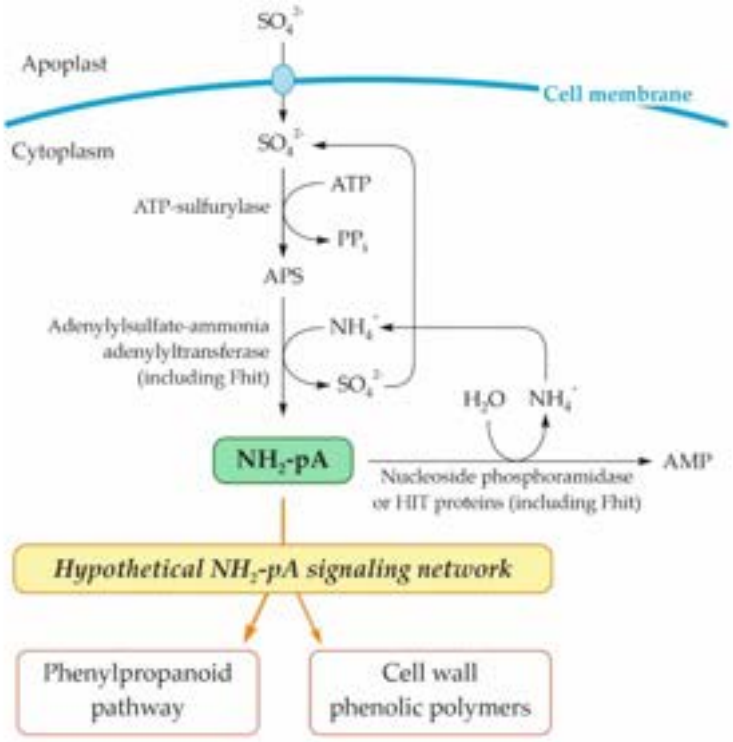

Figure 10. Schematic representation of metabolism of NH_2-pA in plant cells and its effect on the phenylpropanoid pathway. APS, adenosine 5'-phosphosulfate.

4. Materials and Methods

4.1. Plant Materials

Vitis vinifera L. cv. Monastrell calli were established as described by Calderon et al. [38] and maintained at 25 °C in darkness in 250 mL flasks containing 100 mL of fresh culture medium (Gamborg B_5, Duchefa, The Netherlands). Monastrell SCC was initiated by inoculating friable callus pieces in 250 mL Erlenmeyer flasks containing 100 mL of liquid Gamborg B_5 medium (pH 6.0) at 25 °C in the dark and were routinely maintained by periodic subcultures every 14–16 days as described by Belchi-Navarro et al. [39] and Almagro et al. [40].

4.2. Elicitor Treatment

Elicitation experiments were carried out in triplicate using 10-day-old Monastrell SCC. At that stage of cell development, 3 g of fresh weight of cells was washed with cold distilled water, transferred into 50 mL flasks, suspended in 15 mL of fresh Gamborg B_5 medium supplemented with 5 µM NH_2-pN (NH_2-pA, NH_2-pG, NH_2-pU, or NH_2-pC), and incubated for 72 h at 25 °C in the dark on a rotary shaker (110 rpm). Control samples, without elicitors, were always run in parallel. The cells were harvested after 6, 24, 48, and 72 h, separated from the culture medium by filtration under a gentle vacuum, rapidly washed with cold distilled water, frozen in liquid nitrogen, and kept at −80 °C until use. The spent culture media were also frozen and stored at −20 °C until use.

4.3. NH_2-pNs Chemical Synthesis

Details of the chemical synthesis of NH_2-pA, NH_2-pG, NH_2-pU, and NH_2-pC, and their characterization by HRMS, ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR are given in the Supplementary Materials (Methods S2).

4.4. Quantification of Trans-Resveratrol and Trans-Piceid

Extracellular content of *trans*-resveratrol and *trans*-piceid was determined as described by Pietrowska-Borek et al. [3,6]. For this, 20 µL of diluted and filtered (Anopore 0.2 µm) samples were analyzed by HPLC in the UV–VIS range using a LiChrospher 100 RP-18 column (250 × 4 mm, 5 µm; Merck, Darmstadt, Germany). Gradient elutions were performed with 0.05% TFA (solvent A) and 0.05% TFA in methanol:acetonitrile (60:40 *v*/*v*; solvent B): 0 min, 10% B; 5 min, 15% B; 40 min, 35% B; 45 min, 65% B; 50 min, 65% B; and 55 min, 10% B, setting the flow rate at 1 mL min^{-1}. To determine the intracellular content of *trans*-resveratrol and *trans*-piceid, 200 mg of freeze-dried cells were extracted overnight with 4 mL of methanol at 4 °C with continuous shaking, and then 20 µL of each sample was analyzed on a LiChrospher 100 RP-18 column as described above. *trans*-Resveratrol and *trans*-piceid were identified (at 308 nm) and quantified by comparison with standards of commercial *trans*-resveratrol (Sigma-Aldrich, St. Louis, MO, USA) and *trans*-piceid (ChromaDex, Los Angeles, CA, USA) using respective calibration curves.

4.5. Lignin Determination

Lignin content was measured based on the method described by Syros et al. [41]. The harvested cells were air-dried at 70 °C, and 0.1 g dry mass was subjected to triple ethanol extraction at 80 °C. Each time, 3 mL of 80% (*v*/*v*) ethanol was added, and after the incubation, it was precisely discarded. The first extraction lasted for 1.5 h, the second, and the third for 1 h. Subsequently, 3 mL of chloroform was added, and the samples were heated to 62 °C. After 1 h, chloroform extract was removed, and samples were air-dried in an oven at 50 °C. Dried cells were digested at 70 °C in 2.6 mL of a solution of 25% (*v*/*v*) acetyl bromide in acetic acid containing 2.7% (*v*/*v*) perchloric acid. After 1 h of incubation, 100 µL of each sample was added to 580 µL of a solution of 2 N sodium hydroxide and acetic acid. The reaction was terminated by adding 20 µL of 7.5 M hydroxylamine hydrochloride. Then, the samples were filled up to 2 mL with acetic acid, and the absorbance at 280 nm was measured. Lignin content was expressed as mg g^{-1} DW, using a linear calibration curve with a commercial lignin alkali standard (Sigma, St. Louis, MO, USA).

4.6. Determination of Phenylamide Content in Cells and Spent Media

Phenylamide analysis was performed according to Morimoto et al. [19]. The grape cells were air-dried at 70 °C, then phenylamides were extracted with 10 mL of 80% methanol. For concentration, the samples were dried on a SpeedVac, and suspended in 300 µL of methanol. To extract phenylamides from the spent media, solid-phase extraction (SPE) (Superclean ENVI-18 SPE Tubes, Supelco, Bellefonte, PA, USA) was applied. The compounds from the SPE columns were eluted with 80% methanol and concentrated by drying on a SpeedVac, and suspending in 300 µL of methanol. Then, the samples were subjected to LC-MS/MS analysis. More details are given in the Supplementary Materials (Methods S2).

4.7. Cell Viability

Cell viability was evaluated using the Plant Cell Viability Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. The cells were incubated for 1–2 min in fresh Gamborg medium, then 10 µL of the assay kit diluted in 1 M PBS pH 7.4 was added to 90 µL of cell suspension and mixed by gently tapping the tube. Fluorescence was monitored with an AxioVert 200 Carl Zeiss microscope using a Zeiss filter (FS09 exc = 495 nm, emi = 517 nm and PS15exc = 538 nm, emi = 617 nm).

4.8. Genes Expression Analyses

Total RNA was extracted from 200 mg of Monastrell frozen cells using the RNeasy Plant Minikit (Qiagen, Hilden, Germany) according to the supplier's recommendations as previously described [3,6]. The concentration of each RNA sample was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Only the RNA samples with a 260/280 ratio between 1.9 and 2.1 were used for the analysis. The integrity of RNA samples was also assessed by agarose gel electrophoresis and purity was confirmed by PCR using *EFa1*-specific primers. Then, 3 µg of total RNA was used for cDNA synthesis with oligo(dT)$_{20}$ (50 µM) primers and the Superscript III Reverse Transcriptase Kit (Invitrogen). A quantitative real-time PCR reaction was carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad) and iTaq Universal SYBR Green Supermix (Bio-Rad), and the specific primers for Monastrell genes (*PAL1*, *C4H1*, *4CL1*, *CHS1*, *STS1*, *VtABCG44*, *CCR2*, *CAD1*, and *EFa1*). The comparative C_T method for relative quantification was used with *EFa1* as an endogenous control. The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta CT}$ [42]. Primer sequences and GenBank accession numbers are presented in the Supplementary Materials (Table S1).

4.9. Statistical Analysis

Data concerning mRNA level and concentrations of stilbenes, lignin, phenylamide and dry weight are the means of three independent replicates ± standard deviation. The statistical significance of the differences between averages was determined by ANOVA using Tukey's HSD multiple range test at $p \leq 0.05$.

Supplementary Materials: Supplementary Materials are available online at https://www.mdpi.com/article/10.3390/ijms222413567/s1.

Author Contributions: M.P.-B. and A.G. conceived and designed the study; M.P.-B. performed the experiments, analyzed the results, and wrote the bulk of the manuscript; J.D. carried out part of the experiments and prepared the figures; A.M.W.-M. carried out part of the experiments; J.R. developed a method for the chemical synthesis of nucleoside 5′-phosphoramidates as well as performing their synthesis and purification; J.G. performed synthesis and purification of nucleoside 5′-phosphoramidates; S.B. performed the statistical analysis and was involved in the writing of the manuscript; K.M. and A.I. performed experiments on the determination of phenylamide concentrations; M.Á.P. provided the plant material and was involved in writing the manuscript; A.G. was involved in the discussion and writing of the manuscript. All authors have read and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials

Nucleoside 5'-phosphoramidates control phenylpropanoid pathway in *Vitis vinifera* suspension-cultured cells

Methods S1: NH_2-pNs chemical synthesis

S1.1. Experimental

All reactions were performed in anhydrous solvents. Pyridine was dried over P_2O_5, distilled and kept over molecular sieves 4 Å until the amount of water was less than 20 ppm. The amount of water in solvents was determined with Karl Fisher coulometric titration. Solvents and volatile by-products were removed in a rotary evaporator under reduced pressure using water bath temperature not exceeding 40°C. NMR spectra were obtained on Bruker Avance II 400 MHz instrument in 5 mm tubes. For ^{31}P NMR experiments, 0.1 M solutions of the starting H-phosphonates were used. ^{31}PNMR chemical shifts are reported relative to 85% H_3PO_4 in water used as an external standard. Mass spectra were recorded on Thermo Fisher Scientific Q-Exactive Orbitrap mass spectrometer Brukermicro Q-TOF mass spectrometer with the ESI technique with negative ionization. For TLC analysis, the pre-coated plates (Merck silica gel F254) were used, and for column chromatography silica gel Si 60, 35-70 mesh (Merck) was used. Nucleoside 5'-H-phosphonates were obtained using a method developed by Romanowska et al.[1] Chemical reagents and solvents were commercial grade from Aldrich. Natural ribonucleosides (A, C, G, U) protected with the 2',3'-di-*O*-acetyl group were obtained from ChemGenes. All synthesized compounds were of purity higher than 98% as judged from ^{1}H NMR spectroscopy.

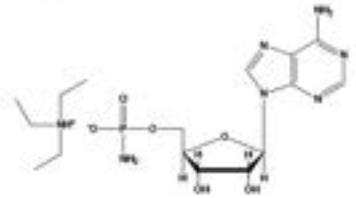
S1.2. General procedure for the synthesis and purification of nucleoside 5'-phosphoramidates (NH_2-pNs)

Prior to the reaction, lithium amide (3 equiv. 153 mg) and the appropriate nucleoside 5'-H-phosphonate $TEAH^+$ salt (1 mmol, 1 equiv.) were separately dried overnight under vacuum. Nucleoside 5'-H-phosphonate was dissolved in pyridine (10 mL) and TMSCl (3 equiv., 381 µL) was added[2,3]. After 5 min, a lithium amide and then solution of I_2 in pyridine (1.5 equiv., 381 mg) were quickly injected into the reaction mixture. The mixture was stirred for 5 min at room temperature. After the reaction was complete (ca. 5 min, ^{31}P NMR), the mixture was quenched with addition of water and the excess of iodine was decomposed with ethanethiol. The solution was concentrated *in vacuo* and the residue was treated with aqueous ammonia (25%, 10 mL) for 1 hour. The products - NH_2-pNs were isolated by a silica gel column chromatography using a stepwise gradient of water (0–10% v/v) in acetonitrile containing Et_3N (5% v/v). Fractions containing pure products, appropriately NH_2-pA, NH_2-pG, NH_2-pC and NH_2-pU were collected and solvents evaporated to furnish colorless, crispy foams.

S1.3. General procedure for a direct conversion of nucleosides into nucleoside 5'-H-phosphonates

Ammonium (9H-fluoren-9-yl)methyl-H-phosphonate (0.5 mmol, 1.1 equiv.) was dissolved in a mixture of pyridine and triethylamine (in a ratio 4:1) and evaporated to convert it to the triethylammonium salt. To this, appropriate nucleoside (0.5 mmol, 1 equiv.) was added and both compounds were rendered anhydrous by repeated evaporation of the added pyridine. The residue was dissolved in 5 mL of CH_2Cl_2/pyridine (95:5, v/v) and PvCl (1.5 equiv.) was added. Phosphonylation was complete (^{31}P NMR, TLC) after ca 20 min. The reaction mixture was diluted with the same volume of methylene chloride, and washed with water (one third of the total volume) to afford H-phosphonate diesters. The organic layer was evaporated and the remaining oily residue was dissolved in CH_3CN/Et_3N [2 : 1, v/v; 5 mL per 0.5 mmol of diester] and kept for 20 min at room temperature to effect a quantitative elimination of the (9H-fluoren-9-yl)methyl group. The reaction mixture was evaporated and products were isolated by a silica gel filtration using a stepwise gradient of methanol (0 – 20% v/v) in methylene chloride containing Et_3N (3% v/v). Fractions containing pure product were collected and evaporated, to furnish colourless, crispy foams[1].

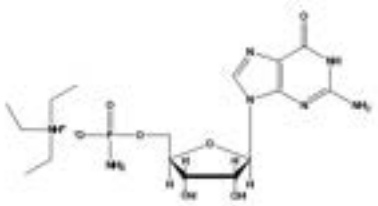
S1.4. Synthesis of adenosin-5'-yl phosphoramidate, TEAH$^+$ salt (NH_2-pA)



Adenosin-5'-yl phosphoramidate was obtained according to the general procedure starting from 2',3'-di-O-acetyladenosine 5'-H-phosphonate TEAH$^+$ salt (mg, 1 mmol, 1 equiv.), and after purification by silica gel column chromatography compound was isolated as an triethylamonium salt (357 mg, 0.8 mmol, 80%).

Adenosin-5'-yl phosphoramidate, TEAH$^+$ salt. Yield 80%. **HRMS** *m/z*: [M-Et_3NH^+]$^-$ calcd for [$C_{10}H_{14}N_6O_6P$]$^-$, 345.0718; found 345.0714. **^{1}H NMR** (D_2O, 400 MHz, δ): 8.43 (1H, s), 8.11 (1H, s), 6.06 (1H, d, $^3J_{H1H}$ = 5.6 Hz), 4.73 (1H, t, $^3J_{H2H}$ = 5.3 Hz), 4.50 (1H, m), 4.38 (1H, m), 4.10 (2H, m), 3.18 (6H, q, $^3J_{HH}$ = 7.3 Hz), 1.26 (9H, t, $^3J_{HH}$ = 7.3Hz). **^{13}C NMR** (D_2O, 100.6 MHz, δ): 154.84, 152.10, 148.62, 139.80, 118.23, 87.04, 83.94 (d, $J_{P,C}$ = 8.9 Hz), 74.30, 70.35, 63.85 (d, $J_{P,C}$ = 4.84 Hz), 46.59, 8.17. ^{31}P NMR{^{1}H} (CH_3CN, 121.4 MHz, δ): 9.3. **^{31}P NMR** (CH_3CN, 121.4 MHz, δ): 9.3 (m).

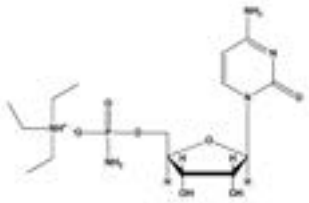
S1.5. Synthesis of guanosin-5'-yl phosphoramidate, TEAH$^+$ salt (NH_2-pG)



Guanosin-5'-yl phosphoramidate was obtained according to the general procedure starting from 2',3'-di-*O*-acetylguanosine 5'-H-phosphonate TEAH$^+$ salt (mg, 1 mmol, 1 equiv.), and after purification by silica gel column chromatography compound was isolated as an triethylamonium salt (231 mg, 0.6 mmol, 50%).

Guanosin-5'-yl phosphoramidate, TEAH$^+$ salt. Yield 50%. **HRMS *m/z*:** [M-Et$_3$NH$^+$]$^-$ calcd for [$C_{10}H_{14}N_5O_7P$]$^-$, 361.0667; found 361.0660. **^{1}H NMR** (D_2O, 400 MHz, δ): 8.07 (1H, s), 5.88 (1H, d, $^3J_{H,H}$ = 5.6 Hz), 4.46 (1H, m), 4.31 (1H, m), 4.07 (1H, m), 3.17 (6H, q, $^3J_{H,H}$ = 7.3 Hz), 1.25 (9H, t, $^3J_{H,H}$ = 7.3 Hz). **^{13}C NMR** (D_2O, 100.6 MHz, δ): 157.12, 149.37, 147.90, 139.86, 119.86, 87.60, 84.16 (d, $J_{P,C}$ = 9.02 Hz), 73.50, 70.41, 63.95 (d, $J_{P,C}$ = 4.78 Hz), 46.63, 8.20. ^{31}P NMR{^{1}H} (CH_3CN, 121,4 MHz, δ): 9.4. ^{31}P NMR (CH_3CN, 121,4 MHz, δ): 9.4 (t, $^3J_{P,H}$ = 4.8 Hz).

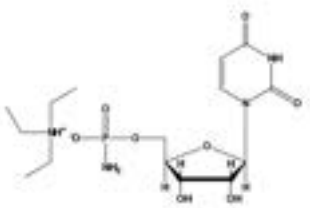
S1.6. Synthesis of cytidine-5'-yl phosphoramidate, TEAH$^+$ salt (NH_2 pC)



Cytidine-5'-yl phosphoramidate was obtained according to the general procedure starting from 2',3'-di-*O*-acetylcytidine 5'-H-phosphonate TEAH$^+$ salt (mg, 1 mmol, 1 equiv.), and after purification by silica gel column chromatography compound was isolated as an triethylamonium salt (330 mg, 0.78 mmol, 78%).

Cytidine-5'-yl phosphoramidate, TEAH$^+$ salt. Yield 78%. **HRMS *m/z*:** [M-Et$_3$NH$^+$]$^-$ calcd for [$C_9H_{14}N_4O_7P$]$^-$, 321.0606; found 321.0614. ^{1}H NMR (D_2O, 400 MHz, δ): 8.00 (1H, d, $^4J_{H,H}$ = 7.6 Hz), 6.12 (1H, d, $^3J_{H,H}$ = 7.6 Hz), 5.98 (1H, d, $^3J_{H,H}$ = 2.7 Hz), 4.32 (2H, m), 4.18, 4.27 (1H, m), 4.17 (1H, m), 4.05 (1H, m), 3.20 (6H, q, $^3J_{H,H}$ = 7.3 Hz), 1.29 (9H, t, $^3J_{H,H}$ = 7.2 Hz). **^{13}C NMR** (D_2O, 100,6 MHz, δ): 165.48, 156.82, 141.66, 96.30, 89.38, 82.85 (d, $J_{P,C}$ = 8.9 Hz), 74.18, 69.35, 63.31 (d, $J_{P,C}$ = 4.7 Hz), 58.72, 46.61, 8.21. **^{31}P NMR**{^{1}H} (CH_3CN, 121,4 MHz, δ): 9.2. ^{31}P NMR (CH_3CN, 121,4 MHz, δ): 9.2.

S1.7. Synthesis of uridin-5'-yl phosphoramidate, TEAH$^+$ salt (NH_2 pC)



Uridin-5'-yl phosphoramidate was obtained according to the general procedure starting from 2',3'-di-O-acetyluridine 5'-H-phosphonate $TEAH^+$ salt (mg, 1 mmol, 1 equiv.), and after purification by silica gel column chromatography compound was isolated as an triethylamonium salt (296 mg, 0.7 mmol, 70%).

Uridin-5'-yl phosphoramidate, $TEAH^+$ salt. Yield 70%. **HRMS m/z:** $[M\text{-}Et_3NH^+]^-$ calcd for $[C_9H_{13}N_2O_9P]^-$: 322.0446; found 322.0448. **^{1}H NMR** (D_2O, 400 MHz, δ): 8.00 (1H, d, $^4J_{HH}$ = 8.2 Hz), 5.98 (1H, d, $^3J_{HH}$ = 4.8 Hz), 5.95 (1H, d, $^3J_{HH}$ = 8.1 Hz), 4.37 (1H, m), 4.34 (1H, m), 4.28 (1H, m), 4.12 (1H, m), 4.08 (1H, m), 3.21 (6H, q, $^3J_{HH}$ = 7.3 Hz), 1.30 (9H, t, $^3J_{HH}$ = 7.34 Hz). ^{13}C NMR (D_2O, 100.6 MHz, δ): 166.08, 151.67, 141.68, 102.46, 88.56, 83.40 (d, $J_{P,C}$ = 8.9 Hz), 73.82, 69.75, 63.54 (d, $J_{P,C}$ = 4.7), 46.62, 8.24. ^{31}P NMR{^{1}H} (CH_3CN, 121.4 MHz, δ): 9.2. **^{31}P NMR** (CH_3CN, 121.4 MHz, δ): 9.2.

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Supplementary Materials

Nucleoside 5′-phosphoramidates control phenylpropanoid pathway in *Vitis vinifera* suspension-cultured cells

Methods S2: Identification and determination of phenylamides content

The extracts of freeze dried cells and spent media were subjected to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) in the multiple reaction monitoring (MRM) mode for the determination of phenylamide concentrations. Analysis was performed using a Quattro Micro API mass spectrometer (Waters, Milford, MA, USA) connected to an Acquity UPLC system (Waters). The LC conditions were as follows: column: ACQUITY UPLC BEH C18 (2.1 mm ID, 50 mm length, 1.7 µm particle size) (Waters); column temperature: 40°C; solvents: 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B); gradient: 5%–70% B/(A + B) within 10 min; flow rate: 0.2 mL/min. The MRM conditions were optimized using authentic compounds previously synthesized [1]. The ion transition, cone voltage and collision energy are summarized in Table 1. A mixture of authentic compounds in methanol at 10 µg/mL each were used for generation of standard curves.

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Table. MRM conditions for the detection of phenylamides in cultured cells and spent media by liquid chromatography coupled with tandem mass spectrometry.

Compound*	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
CinAgm	261.19	130.92	25.00	32.00
CouAgm	277.11	146.89	25.00	26.00
CafAgm	293.09	162.83	30.00	30.00
FerAgm	307.05	176.89	34.00	24.00
BenAgm	265.21	175.93	25.00	20.00
CinTyr	268.12	131.00	20.00	14.00
CouTyr	284.10	146.87	25.00	22.00

CafTyr	300.09	162.82	20.00	22.00
FerTyr	313.99	176.91	28.00	18.00
BenTyr	242.20	120.98	20.00	26.00
CinTry	291.16	130.92	20.00	18.00
CouTry	307.08	146.93	20.00	22.00
CafTry	323.13	162.96	20.00	26.00
FerTry	337.04	176.90	30.00	14.00
BenTry	265.18	143.96	20.00	18.00
CinSer	307.14	130.91	20.00	22.00
CouSer	323.04	146.88	22.00	16.00
CafSer	338.97	176.95	22.00	12.00
FerSer	353.03	176.89	24.00	14.00
BenSer	281.16	160.04	20.00	16.00
FerPut	265.24	176.96	20.00	18.00
CafPut	251.19	162.97	20.00	22.00
CouPut	235.21	146.95	20.00	22.00
CinPut	219.23	102.93	20.00	34.00
BenPut	193.20	104.90	20.00	26.00

Abbreviations: CinAgm: *N*-cinnamoylagmatine; CouAgm: *N*-*p*-coumaroylagmatine; CafAgm: *N*-caffeoylagmatine; FerAgm: *N*-feruloylagmatine; BenAgm: *N*-benzoylagmatine; CinTyr: *N*-cinnamoyltyramine; CouTyr: *N*-*p*-coumaroyltyramine; CafTyr: *N*-caffeoyltyramine; FerTyr: *N*-feruloyltyramine; BenTyr: *N*-beyzoyltyramine; CinTry: *N*-cinnamoyltryptamine; CouTry: *N*-*p*-coumaroyltryptamine; CafTry: *N*-caffeoyltryptamine; FerTry: *N*-feruloyltryptamine; BenTry: *N*-benzoyltryptamine; CinSer: *N*-cinnamoylserotonin; CouSer: *N*-*p*-coumaroylserotonin; CafSer: *N*-caffeoylserotonin; FerSer: *N*-feruloylserotonin; BenSer: *N*-benzoylserotonin; FerPut: *N*-feruloylputresscine; CafPut: *N*-caffeoylputresscine; CouPut: *N*-*p*-coumaroylputresscine; CinPut: *N*-cinnamoylputresscine; BenPut: *N*-benzoylputresscine

Table S1. Primer sequences used for quantitative real-time polymerase chain reaction.

Gene Symbol	GeneBank ID	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Length (bp)
PAL3	XM_002281763.2	CCGAACCGAATCAAGGACTG	GTTCCAGCCACTGAGACAATC	183
C4H1	XM_002266202.1	TCCAAGTCACCGAGCCTGAT	GCAGGAATGTCATAGCCACC	109
4CL3	XM_002272746.2	CTGATGCCGCTGTTGTTTCG	GCAGGATTTTACCCGATGGA	198
CHS1	EC996578.1	GTCCCAGGGTTGATTTCCAA	TCTCTTCCTTCAGACCCAGTT	157
STS1	XM_002264419.2	CGCCAGGAGATAATCACTGCT	GCACCAGGCATTTCTACACC	134
CCR2	XM_002273418.3	ACAGCATGACGACTCTCTTCG	AGTGACAAGGGGTGGATTGA	182
CAD1	100247381	GGAGGGATGAAGGAGACACA	CTTTCAGCGTGTTGCCAATG	166
VvABCG44	AB910387.1	TAGGAGTGGTTGCAGCTGTG	TTTTGCTCCGTGTGACTTCTT	114
EFa1	XP_002284964.1	GAACTGGGTGCTTGATAGGC	AACCAAAATATCCGGAGTAAAAGA	164

Publikacja 4

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The Plasma Membrane Purinoreceptor P2K1/DORN1 Is Essential in Stomatal Closure Evoked by Extracellular Diadenosine Tetraphosphate (Ap_4A) in *Arabidopsis thaliana*

Jędrzej Dobrogojski [1], Van Hai Nguyen [2], Joanna Kowalska [2], Sławomir Borek [3] and Małgorzata Pietrowska-Borek [1,*]

[1] Department of Biochemistry and Biotechnology, Faculty of Agriculture, Horticulture and Bioengineering, Poznań University of Life Sciences, Dojazd 11, 60-632 Poznań, Poland; jedrzej.dobrogojski@up.poznan.pl
[2] Division of Biophysics, Institute of Experimental Physics, Faculty of Physics, University of Warsaw, Pasteura 5, 02-093 Warsaw, Poland; nguyen.van-hai@fuw.edu.pl (V.H.N.); joanna.kowalska@fuw.edu.pl (J.K.)
[3] Department of Plant Physiology, Faculty of Biology, Adam Mickiewicz University Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland; borek@amu.edu.pl
* Correspondence: malgorzata.pietrowska-borek@up.poznan.pl

Abstract: Dinucleoside polyphosphates (Np_nNs) are considered novel signalling molecules involved in the induction of plant defence mechanisms. However, Np_nN signal recognition and transduction are still enigmatic. Therefore, the aim of our research was the identification of the Np_nN receptor and signal transduction pathways evoked by these nucleotides. Earlier, we proved that purine and pyrimidine Np_nNs differentially affect the phenylpropanoid pathway in *Vitis vinifera* suspension-cultured cells. Here, we report, for the first time, that both diadenosine tetraphosphate (Ap_4A) and dicytidine tetraphosphate (Cp_4C)-induced stomatal closure in *Arabidopsis thaliana*. Moreover, we showed that plasma membrane purinoreceptor P2K1/DORN1 (does not respond to nucleotide I) is essential for Ap_4A-induced stomata movements but not for Cp_4C. 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 is a part of the signal transduction pathway in stomatal closure evoked by extracellular Ap_4A but not by Cp_4C. Reactive oxygen species (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. The interaction between P2K1/DORN1 and Ap_4A leads to changes in the transcription of signalling hubs in signal transduction pathways.

Keywords: abscisic acid; diadenosine tetraphosphate (Ap_4A); dicytidine tetraphosphate (Cp_4C); dinucleoside polyphosphates (Np_nNs); extracellular ATP (eATP); plant signalling; reactive oxygen species (ROS); uncommon nucleotides

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1. 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 [1]. 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 [2]. Among them, extracellular ATP (eATP) plays an essential role in plant growth [3–8] and development [9,10]. Extracellular ATP regulates responses to biotic stress [11–14] and abiotic stress [15–18]. One of the reactions that eATP can control is

stomatal movements [12,19,20]. In this reaction, the cytoplasmic Ca^{2+} ions ($[Ca^{2+}]_{cyt}$) and the complex signalling cross-talk between second messengers, such as nitric oxide (NO) [7,21,22], and reactive oxygen species (ROS) [12,23–25] 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 [12,26,27].

We have a longstanding interest in the function of dinucleoside polyphosphates (Np_nNs) 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_nNs. We postulated the participation of Np_nNs in the plant defence responses since they induce synthesis of the phenylpropanoid pathway-delivered secondary metabolites [28–30]. The phenylpropanoid pathway participates in plant defence responses [31,32]. Identification of Ap_4A and other Np_nNs across prokaryotic and eukaryotic cells testifies to their universality [33]. Due to the dramatic increase in levels of various Np_nNs observed in cells subjected to abiotic stress factors [34–38], 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_nNs. Moreover, we observed that diadenosine polyphosphates (Ap_nA) induced stilbene biosynthesis. In contrast, dicytidine polyphosphates (Cp_nC) strongly inhibited this reaction but markedly induced the expression of the cinnamoyl-CoA reductase gene that controls lignin biosynthesis [30]. Nonetheless, the underlying mechanism of Np_nN signal recognition and transduction in plants remains elusive. The growing number of plant enzymes found to be involved in Np_nN biosynthesis and degradation strengthens the hypothesis of their signalling function [2,33].

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) [26] and P2K2/DORN2, which belong to the L-type lectin receptor kinase (LecRK) protein family [39,40]. LecRK proteins activate the processes controlling stress responses, development, growth, and disease resistance [41]. Although eATP sensing and action in plants have been elucidated, the mechanisms of signal perception and transduction evoked by Np_nNs, such as Ap_4A and Cp_4C, remain enigmatic. In animal cells, among the different nucleotides and nucleosides, eATP, together with Ap_4A, 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 [42–46]. Therefore, we hypothesise that the purinoreceptor P2K1/DORN1, a receptor of eATP, is also necessary for sensing Ap_4A in plant cells. Moreover, we wondered whether P2K1/DORN1 is also engaged in the effects evoked by the pyrimidine nucleotide Cp_4C.

Here, we present, for the first time, evidence for the involvement of the P2K1/DORN1 receptor in the sensing of Ap_4A 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 Ap_4A and Cp_4C evoked stomatal closure in Col-0 plants. This effect was abolished in the *dorn1*-3 mutant by Ap_4A but not Cp_4C. This result confirms the requirement of P2K1/DORN1 for Ap_4A-induced stomatal closure. Nevertheless, our research indicates the involvement of superoxide ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2) in the signal transduction evoked by Ap_4A and Cp_4C, 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, Ap_4A induced the expression of the tested genes. Moreover, the gene expression in *dorn1*-3 was almost abolished by the Ap_4A effect.

2. Results

2.1. Ap_4A and Cp_4C Induce Stomatal Closure

Our previous research showed that exogenous Np_nNs induce the biosynthesis of secondary metabolites that play an essential role in the plant defence strategy [28–30]. We wondered how the signal evoked by Np_nNs 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 the involvement of the purinoreceptor P2K1/DORN1 in *Arabidopsis thaliana* [12,26]. Therefore, based on similarities in the ATP and Ap_4A structures, we tested the effect of these nucleotides on stomatal movements. Moreover, we also included cytidine nucleotides in our research because of the different effects of purine and pyrimidine Np_nNs on the phenylpropanoid pathway in *Vitis vinifera* cells [30]. To trace stomatal movement under the nucleotide treatment, we examined the ability of purine Np_nNs such as Ap_3A and Ap_4A to stimulate stomatal closure. Additionally, for the positive control, we tested the effects of ADP and ATP, as described earlier [12,26], as well as ABA—a well-known molecule controlling stomatal movements [47,48]. Exogenous Ap_4A significantly reduced the stomatal aperture in the light. It was at a similar level compared to the effect evoked by ATP and ADP. However, Ap_3A did not evoke such an effect (Figure 1). We also examined stomatal movement under the treatment of cytidine mono- and dinucleotides (CDP, CTP, Cp_3C, Cp_4C). Interestingly, only Cp_4C triggered significant stomatal closure among tested cytidine nucleotides. As expected, ABA closed stomata [49] (Figure 1).

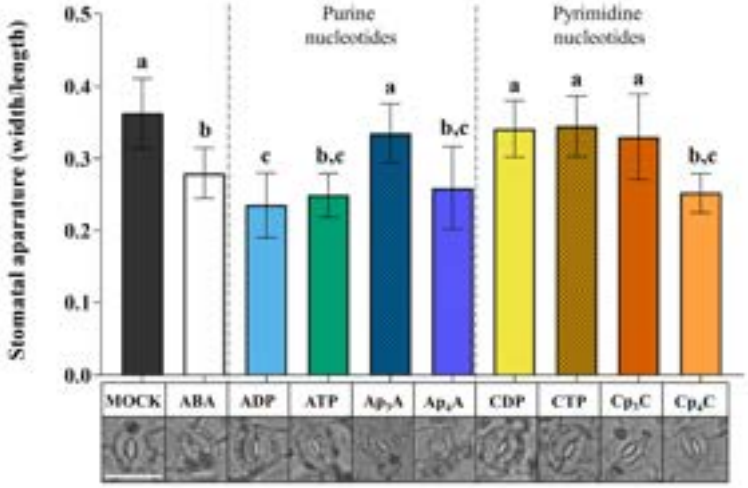

Figure 1. Diadenosine tetraphosphate (Ap_4A) and dicytidine tetraphosphate (Cp_4C), similar to adenosine diphosphate (ADP) and adenosine triphosphate (ATP), induce stomatal closure in *Arabidopsis thaliana* Col-0 plants. Images represent stomata in the abaxial epidermis of a leaf treated for 2 h with the MOCK solution MES/KOH opening buffer, 10 µM abscisic acid (ABA), and 2 mM purine and pyrimidine nucleotides. White bar = 25 µm. Bars represent mean values ± SD, $n \geq 20$, 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 Np_nNs to mononucleotides [50]. To confirm that Ap_4A and Cp_4C 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 Ap_4A was not degraded to the corresponding mononucleotide. Only a trace amount of CTP was detected in a solution of Cp_4C after the investigation (Figure S1).

2.2. P2K1/DORN1 Is Involved in Signal Perception Evoked by Ap_4A but Not Cp_4C

Plants respond to eATP by the induction of a complex signalling network after signal recognition by the P2K1/DORN1 and P2K2 receptors [26,39]. Similarities in stomatal movements evoked by eATP, Ap_4A, and Cp_4C led us to hypothesise that those nucleotides could interact with P2K1/DORN1. Based on the results presented in Figure 1, Ap_4A and Cp_4C 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 [12,26]. We found that Ap_4A and eATP did not close stomata in *dorn1-3* mutant leaves. Contrary to this, Cp_4C significantly closed stomata in *dorn1-3* mutant leaves. As expected, ABA-treated mutant leaves also showed closed stomata [12] (Figure 2). Thus, the results strongly suggest that besides eATP, P2K1/DORN1 may also be involved in signal perception elicited by Ap_4A but not Cp_4C.

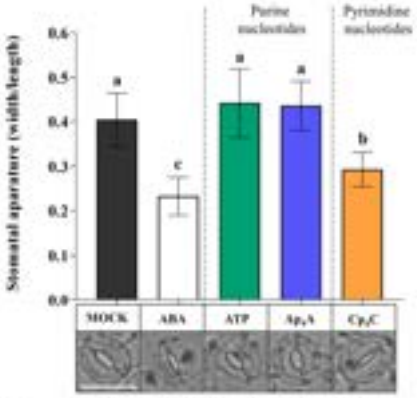

Figure 2. Diadenosine tetraphosphate (Ap_4A), similar to extracellular (eATP), did not induce stomatal closure in the *dorn1-3 Arabidopsis thaliana* mutant. However, dicytidine tetraphosphate (Cp_4C) and abscisic acid (ABA) evoked stomatal closing. Images represent stomata in the abaxial epidermis of *dorn1-3* leaf treated for 2 h with MOCK solution opening buffer, 10 µM ABA and 2 mM adenosine triphosphate (ATP), Ap_4A, and Cp_4C. White bar = 25 µm. Bars represent mean values ± SD, $n \geq 20$, 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 ($p < 0.05$).

2.3. ROS Are Produced in Leaves under Nucleotide Treatment

It was previously found that the 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 [12]. This phosphorylation causes an increase in the generation of extracellular ROS, such as $^{\bullet}O_2^-$, which is then converted into H_2O_2 in the extracellular environment [51,52]. Notably, the apoplastic production of ROS is one of the fastest physiologically common responses to external stimuli observed in plants [53,54]. Considering all the above-described information, we decided to investigate the accumulation of $^{\bullet}O_2^-$ and H_2O_2 in *Arabidopsis thaliana* leaves in response to 2 mM ATP, CTP, Ap_4A, and Cp_4C. Our experiments revealed that the NBT staining of leaves, indicating $^{\bullet}O_2^-$ 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 an accumulation

of $^{\bullet}O_2^-$ (Figure 3a). DAB 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 slight DAB staining. In the *dorn1*-3 mutant, only CTP and Cp_4C evoked an accumulation of H_2O_2 in the leaves. Nevertheless, only weak DAB staining was caused by CTP (Figure 3b).

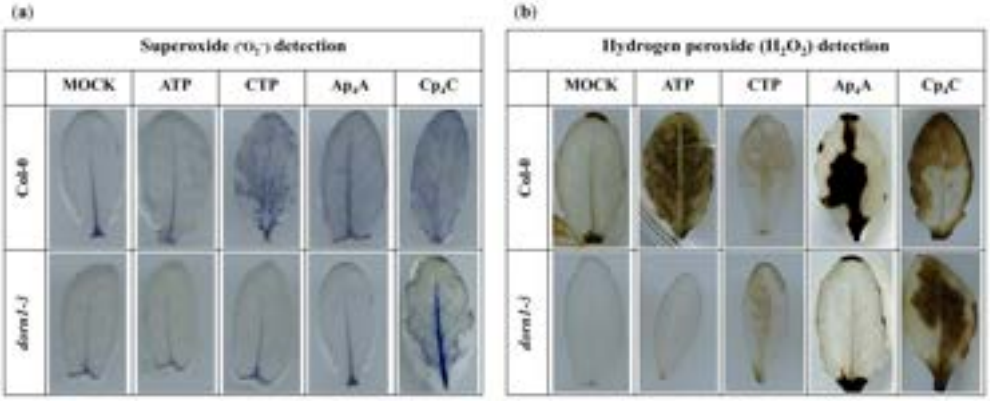

Figure 3. Histochemical detection of $^{\bullet}O_2^-$ (**a**) and H_2O_2 (**b**) in leaves of *Arabidopsis thaliana* Col-0 and the *dorn1*-3 mutant triggered by 2 mM adenosine triphosphate (ATP), cytidine triphosphate (CTP), diadenosine tetraphosphate (Ap_4A), dicytidine tetraphosphate (Cp_4C) after 2 h treatment. Leaves were stained with nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) for $^{\bullet}O_2^-$ and H_2O_2 detection, respectively. The experiment was repeated six times, and representative leaves were chosen.

2.4. ROS Are Involved in Signal Transduction Evoked by eATP, Ap_4A and Cp_4C, Leading to Stomatal Closure

Based on the results indicating that Ap_4A and Cp_4C induced the production of ROS (Figure 3a,b), we wondered whether these key signalling molecules are components of signal transduction pathways evoked by Np_nNs leading to stomatal closure. We simultaneously applied superoxide dismutase (SOD) and catalase (CAT), enzymes scavenging ROS [54,55], and thereby sought to confirm the role of $^{\bullet}O_2^-$ and H_2O_2 in the transduction pathway of the signal generated by Ap_4A and Cp_4C. Interestingly, CAT and SOD eliminated the effect of stomatal closure under simultaneous nucleotide treatment, so our observations showed the direct involvement of $^{\bullet}O_2^-$ and H_2O_2 in stomatal closure evoked by eATP, Ap_4A, and Cp_4C. However, the plants did close their stomata upon adding ABA (Figure 4).

2.5. P2K1/DORN1 Is Implicated in Ap_4A- and eATP-Responsive Gene Expression

It is known that transcriptional upregulation of defence-related and wound-response genes by eATP is P2K1/DORN1-dependent [26,56]. Thus, we decided to investigate whether Ap_4A 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 Ap_4A, 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 oxidase respiratory burst oxidase homologs (RBOHs), RBOHD, and RBOHF, which generate ROS [54]. We found that Ap_4A up-regulated *RBOHF* but not by eATP in Col-0 plants. Interestingly, both eATP and Ap_4A downregulated *RBOHF* expression in the *dorn1*-3 mutant (Figure 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. Ap_4A evoked slight changes in expression levels of *RBOHD* in Col-0 and *dorn1*-3 plants (Figure 5a).

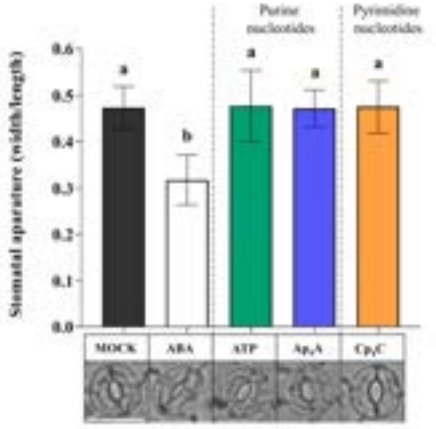

Figure 4. Reactive oxygen species (ROS) enzyme scavengers, catalase (CAT) and superoxide dismutase (SOD), eliminate the effect of stomatal closure after the 2 mM adenosine triphosphate (ATP), diadenosine tetraphosphate (Ap_4A), dicytidine tetraphosphate (Cp_4C) treatment in *Arabidopsis thaliana* Col-0 leaves. White bar = 25 µm. Bars represent mean values ± SD, $n \geq 20$, 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 ($p < 0.05$).

Other components involved in a variety of signalling pathways, ranging from development to stress responses, are cyclic nucleotide-gated channels (CNGCs) [57,58]. Moreover, AtCNGC2 mediates eATP signal transduction in cells of the root epidermis [20]. We found that Ap_4A 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 (Figure 5b). We also focused on essential protein kinases, such as SnRKs, that regulate cellular energy homeostasis, stress response, and growth [59]. 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 [60]. Our experiments showed that eATP strongly induced the expression of *SnRK1.1*, *SnRK1.2*, and *PV42a* in Col-0 plants. Although Ap_4A 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 Ap_4A treatment caused induction of the expression (Figure 5c). Extracellular ATP and Ap_4A increased the expression of *SnRK2.2*, *SnRK2.3*, and *SnRK2.6* in Col-0 plants. In the *dorn1-3* mutant plants, Ap_4A 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 (Figure 5c). The strong relationships between secondary messengers, such as ROS and MAPKs, are often highlighted in the literature [61,62]. MAPK6, among its roles in various metabolic processes in plants, can regulate the activities of diverse targets, including transcription factors [63]. We observed an up-regulation of *MAPK6* expression by both eATP and Ap_4A in Col-0 plants and down-regulation in the *dorn1-3* mutant (Figure 5d). Among the transcription factors that MAPKs regulate, we tested the regulation of expression of the zinc-finger transcription factors (*ZAT6* and *ZAT12*) and found that eATP and Ap_4A up-regulated 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 Ap_4A downregulated the expression of both genes in the mutant plants (Figure 5e).

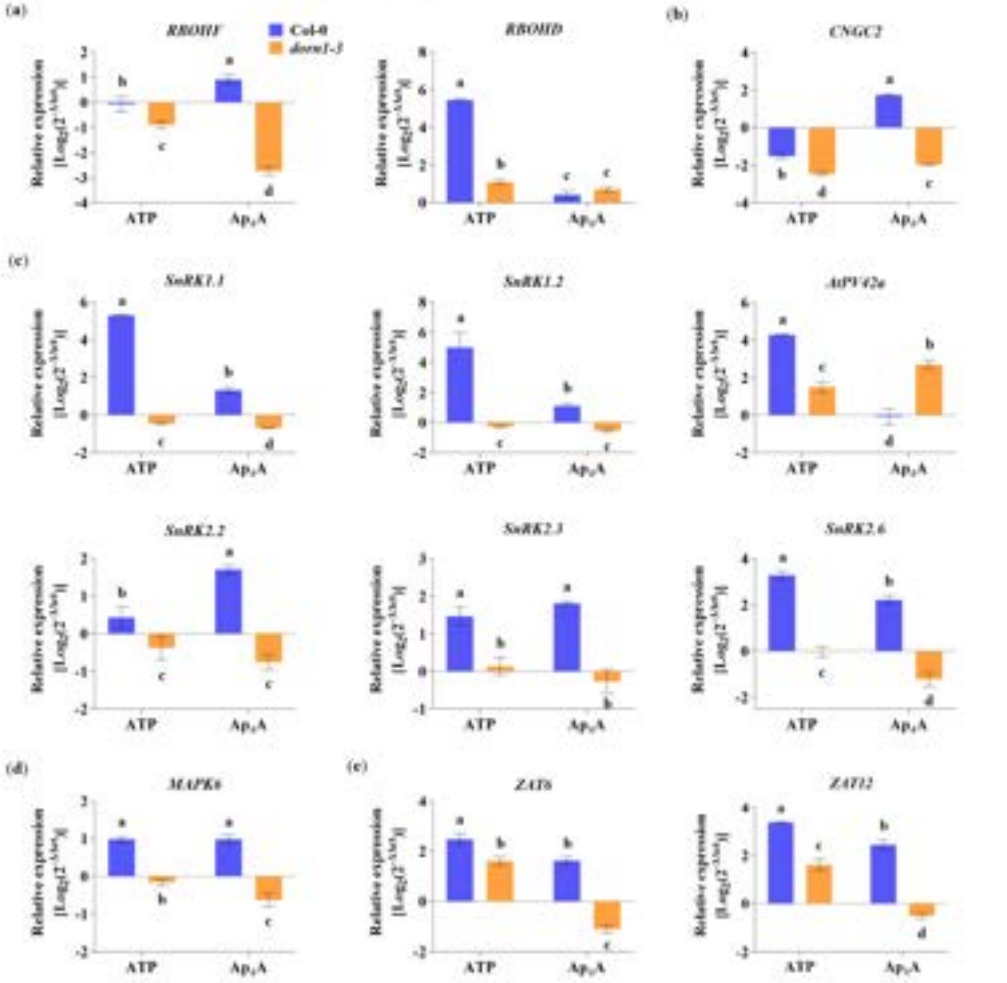

Figure 5. The purinoceptor P2K1/DORN1 is involved in the Ap_4A-induced transcriptional response in *Arabidopsis thaliana* Col-0 leaves. Graphs present the changes in the gene expression level for NADPH oxidase respiratory burst homologs (*RBOHD* and *RBOHF*) (**a**), cyclic nucleotide-gated channel 2 (*CNGC2*) (**b**), SNF1/AMPK-related protein kinases (*SnRKs*) (**c**), mitogen-activated protein kinase 6 (*MAPK6*) (**d**), and transcription factors (*ZAT6* and *ZAT12*) (**e**). Leaves taken from Col-0 and the *dorn1-3* mutant were treated for 2 h with 2 mM adenosine triphosphate (ATP) and diadenosine tetraphosphate (Ap_4A). Transcript levels are represented as $Log_2(2^{-\Delta\Delta Ct})$ 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 ($p < 0.05$).

3. 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, Np_nNs 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 [33,64]. Although there are identified Ap_4A-binding protein targets in cells [33], the signalling pathways are still unclear. We reported previously that extracellular Np_nNs regulate the phenylpropanoid pathway, producing secondary metabolites—key molecules in response to abiotic stress in *Arabidopsis thaliana* and *Vitis vinifera* [28–30]. Notably, one of the phenylpropanoid pathway enzymes, 4-coumarate:CoA ligase, is known to catalyse the synthesis of Ap_4A [65], and its activity was increased by Ap_4A [28]. It is known that some extracellular Ap_nN may become internalised and operate intracellularly [33]. 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 Ap_4A and Cp_4C evoked stomatal closure in *Arabidopsis thaliana* leaves (Figure 1). We did not observe such an effect in *dorn1*-3 plants under the Ap_4A effect (Figure 2). Thus, we can conclude that plasma membrane purinoreceptor P2K1/DORN1 is essential in Ap_4A perception. However, our research also indicates that P2K1/DORN1 is not involved in signal perception elicited by Cp_4C (Figure 2). Such results suggest that in plants, P2K1/DORN1 is not Cp_4C-binding, or there are other protein(s) interacting with this nucleotide. After Ap_4A signal recognition, P2K1/DORN1 stimulates ROS burst and the defence-related response. Our data indicating ROS involvement in the plant response to Ap_4A and Cp_4C support the hypothesis concerning the signalling function of Np_4Ns (Figures 3 and 4). Moreover, the HPLC assay proved that Ap_4A was not degraded to corresponding mononucleotides, which could evoke stomatal closure during the experiment (Figure S1). Only a tiny amount of CTP was detected in a solution of Cp_4C after the investigation. Still, as we proved, CTP did not evoke stomatal closure (Figure 1). Therefore, it confirms that the observed stomatal closure and ROS accumulation were caused by Ap_4A and Cp_4C but not by their decomposition products.

The upregulation of defence-related genes encoding proteins involved in signalling hubs was reported [59]. The expression of the genes described in this research was mostly abolished or down-regulated in the *dorn1*-3 mutant (Figure 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 [54]. Our transcriptional analysis focused on elements that integrate various signals and included cyclic nucleotide-gated channels (CNGCs) 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) (Figure 5). The transcript level of *CGNC2* increased only under Ap_4A in Col-0 plant leaves (Figure 5b). Involving CGNC2 in another purine nucleotide, eATP, signal transduction in the root epidermis and eATP-induced Ca^{2+} influx were described by Wang [20]. This result suggests that CNGC channels can be a part of signal transduction evoked by Ap_4A.

Rapid systemic signalling in response to stress can be stimulated by RBOHD and RBOHF, producing apoplastic ROS [66]. 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 [12], while *RBOHD* expression was significantly reduced in *dorn1-3* mutant plants. Our studies showed that transcriptomic changes in both *RBOHD* and *RBOHF* evoked by Ap_4A are similar, but in the *dorn1-3* plants, the expression of *RBOHF* was also strongly inhibited (Figure 5a). This observation correlated with the accumulation of ROS in *Arabidopsis thaliana* leaves (Figure 3). Stress signalling in plants also involves different families of kinases, including the MAPK module, that can be activated by ROS [67]. Moreover, it was previously shown that MAPKs are activated by eATP [26,68–70]. We observed the induction of *MAPK6* expression evoked by eATP and Ap_4A (Figure 5d), and it is known that MPK6 modulates actin remodelling to activate stomatal defence in *Arabidopsis thaliana* [71]. MAPK pathways are necessary for several ABA responses in many plant species, including antioxidant defence and guard cell signalling [47,48]. Protein complexes SNF1-related protein kinase 1s (SnRK1s) and SnRK2s play a prominent role in ABA signalling [72,73]. Numerous studies indicate SnRK1s and SnRK2s as regulators of the target of rapamycin (TOR) kinase activity in controlling autophagy [74,75]. We observed that Ap_4A induced the expression of both *SnRK1*s and *SnRK2*s at a similar level in Col-0 plants. However, induction evoked by eATP was much higher for *SnRK1*s than *SnRK2*s in wild-type plants. In the *dorn1-3* mutant, the expression of *SnRK1*s and *SnRK2*s was decreased (Figure 5c). Also, neither of the tested pyrimidine nucleotides, CTP and Cp_4C, affected the expression of *SnRK*s in Col-0 plants (Figure S2). It is known that SnRKs can regulate RBOH, which is engaged in ROS production [54]. The SnRK1s and SnRK2s were identified as critical nodes for stress and growth signalling pathways [59]. 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 [76]. Studies on AKINβ1, subunit SnRK1, showed its regulatory effect on secondary metabolic processes (e.g., flavonoid metabolism) [77]. Another SnRK1 subunit is PV42a, which is the CBS domain protein. Ap_4A did not change the expression of the gene encoding AtPV42a in Col-0 plants (Figure 5c). It is known that enzymes containing CBS domains can be regulated by Ap_4A binding [33]. Therefore, we postulate that AtPV42a regulates SnRK1s in response to Ap_4A. Moreover, SnRK1, SnRK2, and MAPK interact with transcriptional factors [78,79]. The induction of ZAT12 and ZAT6 transcription factors in which MAPK6 is involved in an abiotic stress marker was described [63]. In the present research, we found that Ap_4A 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 (Figure 5e). It is known that the transcript level of *ZAT6* positively affected the concentrations of phenylpropanoids, including anthocyanin and total flavonoids [80]. Moreover, it was proved that ZAT6 and ZAT12 are involved in the response to cadmium stress and abiotic stress in plants [81–84], and the expression of *ZAT12* was strictly dependent on the ROS wave [85,86].

The results of our research presented here shed more light on the signalling function of Ap_4A, its perception and signal transduction pathway in plants. We had previously proposed a hypothetical Np_nN signalling network in a plant cell [2]. 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 [2,28–30]. Here, we fill a few gaps in this network (Figure 6).

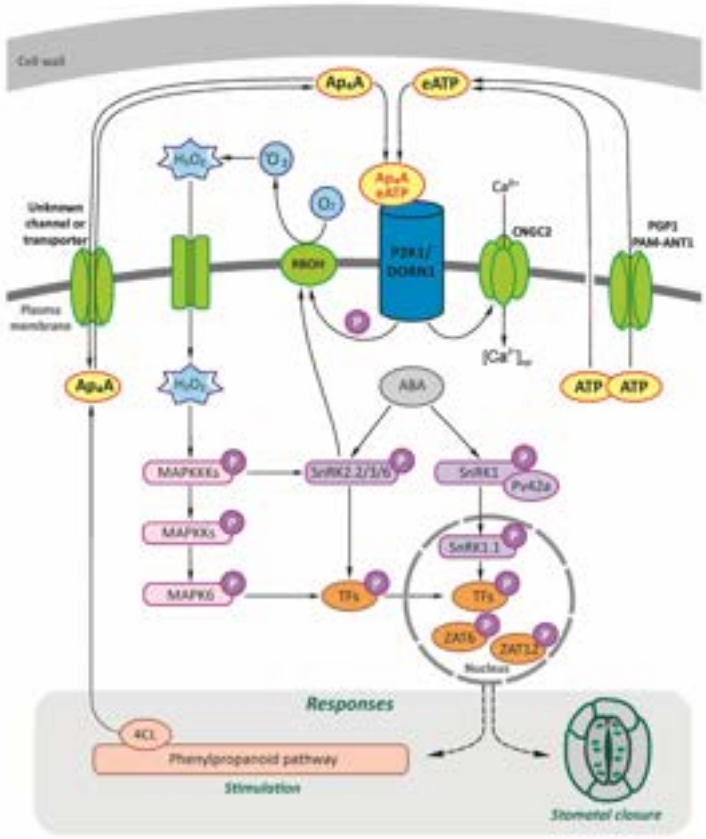

Figure 6. Hypothetical working model of diadenosine tetraphosphate (Ap_4A) signalling network in a plant cell. Ap_4A, similar to extracellular adenosine triphosphate (eATP) [26], can be recognised by the purinoreceptor P2K1/DORN1 and lead to stomatal closure. As our study showed, Ap_4A triggered the reactive oxygen species (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-localised nucleotide transporters [15,87]. Extracellular ATP recognition by P2K1/DORN1 evoked phosphorylation of RBOHD [12]. Also, CNGC2 [20] and MAPK cascades are involved in eATP signal transduction [26,68–70]. We previously described that 4-coumarate:CoA ligase (4CL), the branch point of the phenylpropanoid pathway, can synthesise Ap_4A [65], and its activity is induced by Ap_4A [28]. As yet, no channel or transporter for Ap_4A in plants is known. P, phosphate.

4. Materials and Methods

4.1. Nucleotides

Ap_4A and Cp_4C were synthesised following previously reported procedures, purified by reverse-phase HPLC, and isolated as ammonium (NH_4^+) salts. The purities (>95%) were confirmed by analytical HPLC, ^1H NMR and ^{31}P NMR [30].

4.2. 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.

4.3. 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 $CaCl_2$ (control), (ii) 10 µM abscisic acid (ABA, Sigma Aldrich, St. Louis, MO, USA, A1049) dissolved in the MOCK solution MES/KOH buffer, and (iii) 2 mM ADP (Sigma, A2754), ATP (Sigma, AA8937), Ap_3A, Ap_4A, and CDP (Sigma, C9755), CTP, Cp_3C, Cp_4C dissolved in the MOCK solution MES/KOH buffer. We chose 2 mM concentration of nucleotides based on literature data concerning the effect of eATP on regulation of stomatal aperture [12].

CTP and Np_nNs were synthesised as described previously [30]. Stomata were observed using the ZOE Fluorescent Cell Imager (Bio-Rad, Hercules, CA, USA, 1450031EDU). Measurements, including stomatal aperture width and length, were performed with ImageJ 1.54g 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.

4.4. 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 $^{\bullet}O_2^-$ detection was composed of 0.5% nitroblue tetrazolium (NBT, Sigma-Aldrich, N6876) dissolved in 10 mM potassium phosphate buffer, pH 7.8 [88], and the staining solution for H_2O_2 synthesis was composed of 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, D5905) (1 mg ml^{-1} DAB) dissolved in 10 mM potassium phosphate buffer, pH 7.4, and 0.05% Tween [89]. 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.

4.5. Gene Expression Analyses

According to the manufacturer's instructions, total RNA was extracted from leaves using the RNeasy Plant Mini Kit (Qiagen, Germantown MD, USA). Evaluation of RNA purity, cDNA synthesis, reverse transcription, and RT-qPCR were performed as described previously by Pietrowska-Borek and co-workers [28,90,91]. 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 [92] 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.

4.6. 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 by the ANOVA with the post-hoc Tukey's HSD multiple comparisons test ($p < 0.05$) using Statistica, Version 13 (TIBCO Software Inc., Palo Alto, CA, USA).

5. Conclusions

In the present work, we confirmed that in plants, an Ap_4A receptor exists, and we found that it is purine receptor P2K1/DORN1. Moreover, we indicated ROSs as second messengers, kinases, and transcription factors engaged in the Ap_4A signal transduction pathway. Nevertheless, further studies, both in silico and in vitro, on the binding of Ap4A to the P2K1/DORN1, including key residues that modulate Ap4A affinity, are required. We believe that the presented results in this paper contribute to the description of the role of Np_nNs in signalling hubs and can help better understand the function of uncommon nucleotides in plants.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms242316688/s1. Refs. [93–100] are cited in the supplementary materials.

Author Contributions: J.D. 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; V.H.N. and J.K. synthesised the dinucleoside polyphosphates and participated in reviewing and editing of the manuscript; S.B. participated in writing and critically reviewing the manuscript and co-designed and prepared Figure 6; M.P.-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 have read and agreed to the published version of the manuscript.

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Supplementary Materials

The plasma membrane purinoreceptor P2K1/DORN1 is essential in stomatal closure evoked by extracellular diadenosine tetraphosphate (Ap_4A) but not by dicytidine tetraphosphate (Cp_4C) in *Arabidopsis thaliana*

Jędrzej Dobrogojski, Van Hai Nguyen, Joanna Kowalska, Sławomir Borek and Małgorzata Pietrowska-Borek

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

A T-DNA insertion line of LecRK-I.9 (Salk_042209; *dorn1-3*) was obtained from the Nottingham Arabidopsis Stock Centre (NASC, UK). Genomic DNA was extracted from 4-week-old *Arabidopsis thaliana* leaves using a slightly modified hexadecyltrimethylammonium bromide (CTAB) protocol [1]. Homozygosity for the T-DNA insertion was confirmed by PCR-based genotyping using the specific primers listed in Table **S1**. PCR conditions were as follows: 35 cycles at 95°C for 30 s, 50°C for 15 s, and 72°C for 1:40 min. Then, gel electrophoresis (1% (w/v) agarose in 1X TBE buffer) was conducted for gene product visualisation and final homozygosity confirmation.

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

Purpose	Gene ID (TAIR)	Primers	Sequence (5′→3′)	Product length	Source
Genotype					
p2k1-3 mutant	**AT5G60300**	*mlcat5g60300-s*	TCCATGCAACAGTTGCGTTGTCT		[2]
		P2K1_R	CTGCAATACCCAAACAGTGGTA		
		LB01	GCGTGGACCGCTTGCTGCAACT		
qPCR					
	AT3G03090	*SnRK1.1_F*	CCGCTCCAGAGGTAATTTCG	68 bp	[3]
		SnRK1.1_R	CACACCACAGCTCCAGACATCT		
	AT3G29160	*SnRK1.2_F*	CACCATTCCTGAGATCCGTCA	66 pb	
		SnRK1.2_R	GAGACAGCAAGATAACGAGGGAG		
	AT3G50500	*SnRK2.2_F*	ATATGCCATCGGGATCTGAA	115 bp	[4]
		SnRK2.2_R	TTGCTTGGGAATGAAGAACAG		
	AT5G66880	*SnRK2.3_F*	GTTGGATGGAAGTCCTGCTC	146 bp	
		SnRK2.3_R	TGCCATCATATTCCTGACGA		
	AT4G33950	*SnRK2.6_F*	CACAGGAAGCTTGGACATAGAT	94 pb	[5]
		SnRK2.6_R	GTACACAATCTCTCCGCTACTG		
	AT1G15330	*AtPV42a_F*	GGGATTCTCACGATGCTTGAC	135 bp	[6]
		AtPV42a_R	TGTCCAGAGACTGAGTCCTTCG		
	AT2G43790	*MAPK6_F*	ACGATGCCATAAGCACCCTTGC	161 bp	[7]
		MAPK6_R	GCGGCTCCATCGCCTCAGAT		
	AT5G04340	*ZAT6_F*	AAACCGTGACCTTGACCTGC	300 bp	[8]
		ZAT6_R	CTCCGTTCTTTCCTTCGTAGTG		
	AT5G59820	*ZAT12_F*	GAGTCACAAGAAGCCTAACAACGA	242 bp	[9]
		ZAT12_R	AAGCCACTCTCTTCCCACTGCTA		
	AT5G15410	*CNGC2_F*	TCTTCAGGTGGATTGGACTGT	89 bp	[10]
		CNGC2_R	TCCACCGTTGATTTGGAGGT		
	AT5G47910	*RBOHD_F*	CATGCGGGTGCCCATTT	51 bp	[2]
		RBOHD_R	ATCCGCGGCAATTAAACG		
	AT5G64060	*RBOHF_F*	CTTGGCATTGGTGCAACTCC	151 bp	[11]
		RBOHF_R	TCTTTCGTCTTGGCGTGTCA		
	AT3G18780	*ACT2_F*	ACTTTCATCAGCCGTTTTGA	190 bp	[12]
		ACT2_R	ACGATTGGTTGAATATCATCAG		

2

Method S2. High-performance liquid chromatography (HPLC) analysis of 2 mM solutions of Ap4A and Cp4C after 2 h leaf epidermal peel treatment.

Potential degradation of Ap4A and Cp4C after 2 h incubation on a slide with a leaf was tested. Samples were analysed for purine nucleotides according to [13] with minor modifications. Samples of Ap4A solution (20 µl) were diluted three times with K_2/KH_2PO_4 buffer and filtered (Anopore 0.2 µm), and analysed by HPLC in the UV–VIS range using a Discovery C18 column (4.6 x 250 mm, 5 µm; Supelco); flow rate 1 ml·min^{-1}. Gradient elution was performed with 0.1 M KH_2PO_4, pH 6.0 (solvent A); solvent A/methanol (9: 1, v/v) (solvent B): 0–9 min, 0% B; 9–15 min, 25% B; 15–17.5 min, 90% B; 17.5–19 min, 100% B; 19–23 min, 100% B and 23–30 min, 0% B. Solutions of pyrimidine nucleotides, Cp4C, were analysed according to [14] with minor modifications. The samples were chilled, diluted three times with 50 mM TEAB (triethylamine buffer, pH 7.4), and filtered (Anopore 0.2 µm). Then, samples were analysed by HPLC in the UV–VIS range using a Discovery C18 column (4.6 x 250 mm, 5 µm; Supelco); flow rate 1 ml·min^{-1}. The column was eluted with a linear gradient of 50 mM TEAB (pH 7.4) (solvent A) and solvent A:acetonitrile (60:40, v/v) (solvent B); 0–19 min, 40% B. Nucleotides were identified (purines at 260 nm and pyrimidines at 271 nm) and quantified by comparison with respective standards.

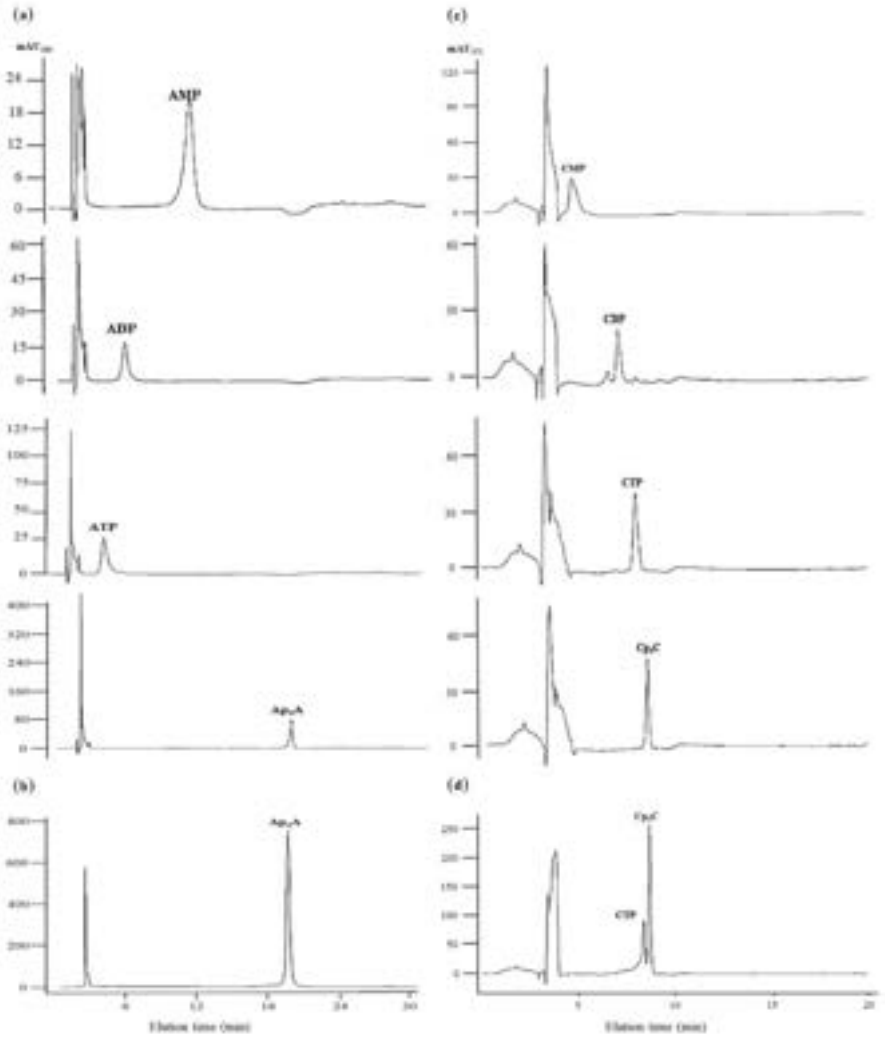

Figure S1. High-performance liquid chromatography analysis of ApcA and CpC solutions after 2 h of leaf epidermal peel treatment. High-performance liquid chromatography (HPLC) analysis was carried out to elucidate ApcA and CpC potential degradation during 2 h of treatment. Samples were analysed for purine nucleotides according to [13], and samples were analysed for pyrimidine nucleotides according to [14]. (**a**) Chromatography of adenine (AMP, ADP, ATP and ApcA) and (**c**) cytidine (CMP, CDP, CTP and CpC) nucleotide standards, and (**b**) ApcA and (**d**) CpC solution from microscope slide after 2 h of epidermal peel treatment. As we observed, a small amount of CTP was detected only in the sample containing CpC.

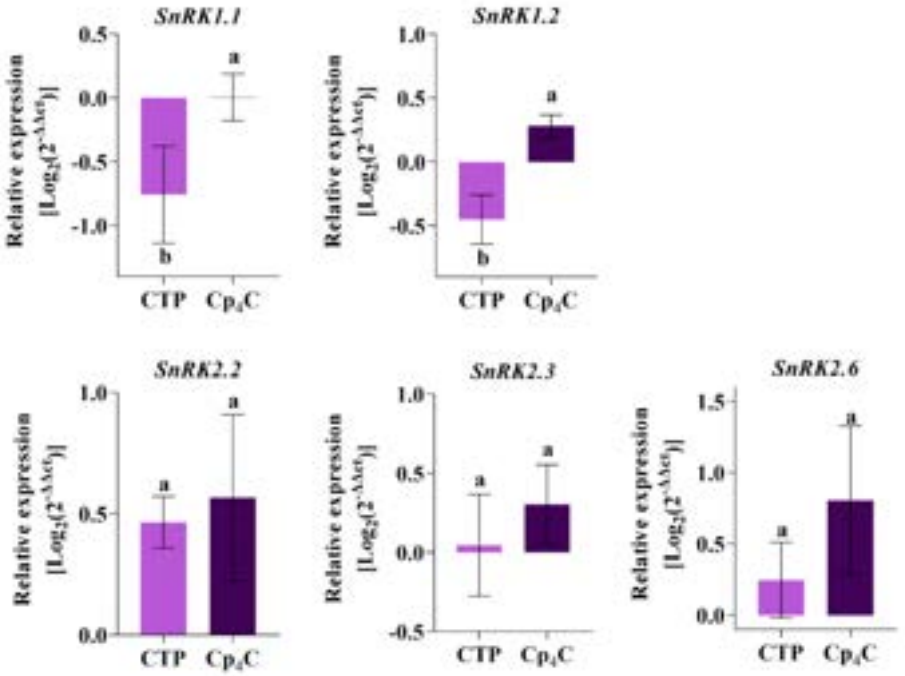

Figure S2. CTP and Cp4C do not up-regulate expression of *SnRK* genes. Relative gene expression in 4-week-old leaves of Col-0 and *dorn1*-3 treated with a 2 mM solution of CTP and Cp4C for 2 h. Afterwards, the total RNA was isolated from leaves and transcribed into cDNA, which was used as a template for quantitative real-time PCR, according to the description in the Material and Methods. Transcript expression is represented as the $Log_2(2^{-\Delta\Delta Ct})$ compared to MOCK-treated plants. The housekeeping gene *AtACT2* was used for data normalisation as an endogenous control. Data are mean ± SD from three independent trials within >3 biological replicates. According to the ANOVA statistical analysis and Tukey's HSD multiple range test ($p < 0.05$), values with different letters above the error bars are statistically significant.

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