

journal homepage: www.elsevier.com/locate/febsopenbio

Diadenosine polyphosphates (Ap₃A and Ap₄A) behave as alarmones triggering the synthesis of enzymes of the phenylpropanoid pathway in *Arabidopsis thaliana*



Małgorzata Pietrowska-Borek^{a,*}, Katarzyna Nuc^b, Małgorzata Zielezińska^a, Andrzej Guranowski^{b,*}

^a Department of Plant Physiology, Poznań University of Life Sciences, 60-637 Poznań, Poland

^b Department of Biochemistry and Biotechnology, Poznań University of Life Sciences, 60-637 Poznań, Poland

ARTICLE INFO

Article history:

Received 23 September 2011

Revised 30 September 2011

Accepted 1 October 2011

Keywords:

Diadenosine tetraphosphate
Diadenosine triphosphate
Phenylalanine ammonia-lyase
4-Coumarate:CoA ligase
Phenylpropanoid pathways
Alarmones

ABSTRACT

It is known that cells under stress accumulate various dinucleoside polyphosphates, compounds suggested to function as alarmones. In plants, the phenylpropanoid pathways yield metabolites protecting these organisms against various types of stress. Observations reported in this communication link these two phenomena and provide an example of a metabolic “addressee” for an “alarm” signaled by diadenosine triphosphate (Ap₃A) or diadenosine tetraphosphate (Ap₄A). In response to added Ap₃A or Ap₄A, seedlings of *Arabidopsis thaliana* incubated in full nutrition medium increased both the expression of the genes for and the specific activity of phenylalanine ammonia-lyase and 4-coumarate:coenzyme A ligase, enzymes that control the beginning of the phenylpropanoid pathway. Neither adenine mononucleotides (AMP, ADP or ATP) nor adenosine evoked such effects. Reactions catalyzed *in vitro* by these enzymes were not affected by Ap₃A or Ap₄A.

© 2011 Federation of European Biochemical Societies. Published by Elsevier B.V.

Open access under [CC BY-NC-ND license](http://creativecommons.org/licenses/by-nc-nd/3.0/).

1. Introduction

Dinucleoside 5',5'''-P¹,Pⁿ-polyphosphates, N_p_nN's (where N and N' are 5'-O-nucleosides and n is the number of phosphate residues in the polyphosphate chain that links the two 5'-esterified nucleosides), are naturally occurring compounds. They can be synthesized by some ligases [1–6], firefly luciferase [7] and certain transferases [8–10], and have been identified in bacteria [11,12], yeast [13,14] and animals, including sea urchin [15], *Artemia salina* [16], *Drosophila* [17] and mammals [18–20]. The presence of N_p_nN's has been demonstrated in yellow lupin seedlings (Guranowski, unpublished observation) but no detailed report of the occurrence of these compounds in plants has appeared yet. Three plant ligases, phenylalanyl- and seryl-tRNA synthetases [4] and 4-coumarate:CoA ligase [6], have been shown to catalyze the synthesis of diadenosine 5',5'''-P¹,P⁴-tetraphosphate (Ap₄A) and some other adenylyl derivatives. Since it has been shown that cells subjected to stresses such as elevated temperature, ethanol or cadmium, accumulate various N_p_nN's [11–14], these compounds have

been termed *alarmones*. However, no clear metabolic or molecular target (“addressee”) of the postulated “alarm” signalled by the N_p_nN's has been experimentally demonstrated. In higher plants, heavy metals including cadmium (II) stimulate the production of many compounds that protect plant tissues against these harmful agents. Among such compounds are products of the phenylpropanoid pathway, including flavonoids and lignins [21–24]. We wondered, therefore, whether exogenously applied Ap₃A and Ap₄A, the most predominant N_p_nN's that probably also accumulate in plant cells subjected to stress-inducing agents like Cd(II), could affect the activities and/or synthesis of any enzymes of the phenylpropanoid pathway. This communication reports that 7-day-old seedlings of *Arabidopsis thaliana* incubated in medium containing micromolar concentration of Ap₃A or Ap₄A increased the specific activities of phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL) as well as the expression of the genes encoding these enzymes. To the best of our knowledge, this is the first evidence in a plant system that exogenously applied N_p_nN's can signal stress conditions by triggering a cascade of reactions to yield various protective compounds.

2. Materials and methods

2.1. Plant growth conditions

Wild-type *A. thaliana*, ecotype Col-0 (Lehle Seeds, USA) were grown in sterile full nutrition medium prepared as described by

Abbreviations: PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate:coenzyme A ligase; CHS, chalcone synthase; Ap₃A, diadenosine 5',5'''-P¹,P³-triphosphate; Ap₄A, diadenosine 5',5'''-P¹,P⁴-tetraphosphate; HPLC, high performance liquid chromatography

* Corresponding authors. Fax: +48 61 8487179 (M. Pietrowska-Borek), fax: +48 61 8487146 (A. Guranowski).

E-mail addresses: gosp@up.poznan.pl (M. Pietrowska-Borek), guranow@up.poznan.pl (A. Guranowski).

Scheible and co-workers [25]. The *Arabidopsis* seedlings (100–120) were kept in 250-ml glass Erlenmeyer flasks containing 30 ml of the above medium in orbital shakers with constant (24 h), uniform fluorescent light ($150 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 22 °C. During the first 3 days the shaker's speed was low (30 rpm) and then it was set at 80 rpm. After 5 days, the old medium was removed and replaced with a fresh portion of the same medium. After a further 2 days each flask was supplemented with a 30 μl aliquot of the compound under study at an appropriate concentration, or water (control), and the growth continued. The plants were harvested after 5, 10, 30, 60, 120 or 180 min, depending on the experiment. A group of plants from each flask was quickly blotted on tissue paper, washed twice with an excess of distilled water, blotted on tissue paper again, frozen in liquid nitrogen, and kept at -80°C for analysis.

2.2. Determination of *Ap₃A* and *Ap₄A* in the growth medium

To monitor uptake of the dinucleotides by *A. thaliana* seedlings, samples of the growth medium (2 ml) were collected at the same time as the plants and analyzed by HPLC as described earlier [26].

2.3. mRNA level determination

Total RNA was extracted from *A. thaliana* seedlings using an RNeasy Plant Kit (Qiagen). DNA was removed with RNase-free DNase (Qiagen). RNA purity was confirmed by PCR using actin-specific primers. RNA concentration was determined with a Qubit fluorometer (Invitrogen) and 4 μg of total RNA was used for cDNA synthesis. RNA and oligo(dT)₁₉ (50 μM) primers were mixed in a total volume of 42 μl and incubated for 5 min at 65 °C followed by 1 min on ice. SuperScript III reverse transcriptase (Invitrogen), dNTP mix, 5 \times first strand buffer, DTT and RNase inhibitor (RNase-OUT Invitrogen) were mixed at 4 °C and dispensed into the tubes with RNA. The reaction was carried out in 60 μl at 50 °C for 60 min. Reverse transcriptase was inactivated by heating at 70 °C for 15 min. A real-time quantitative PCR reaction was performed (Mastercycler[®] ep realplex, Eppendorf) on the synthesized cDNA (20 ng) using HotStar-IT SYBR Green qPCR Master Mix (USB) and the following primers specific for *A. thaliana* (*PAL1*, *PAL2*, *4CL*, common to *4CL1*, *4CL2* and *4CL3*, and *CHS*, respectively): *PAL1F* 5'-CCAAATGATTGTCTGTGAAGTGG-3', *PAL1R* 5'-CCGATGTTTGTATG-GATATTGAG-3', *PAL2F* 5'-CAATGGATCAAATCGAAGCA-3', *PAL2R* 5'-TATTCGGCGTTCAAATC-3' and *4CL* (*4CL1*, *4CL2*, and *4CL3*); *4CLF* 5'-CATCCCTAACCACTCCCACTC-3' i *4CLR* 5'-GGAGGAGGAT-CATTACAACGTC-3', *CHSF* 5'-GGCAAAGAAGCGGCAGTGAAGG-3' and *CHSR* 5'-GACGGAAGGACGGAGACCAAG-3'. Standard cycling conditions were: 2 min at 50 °C, 10 min at 95 °C and 40 cycles alternating between 15 s at 95 °C and 55 °C for 15 s and 1 min at 60 °C, then the melting curve profiles were determined. The comparative C_T (cycle threshold) method for relative quantification was used with *actin* ACTF 5'-ACTTTCATCAGCCGTTTGA-3' and ACTR 5'-ACGATTGGTTGAATATCATCAG-3' as the endogenous control. The amount of target, normalized to an endogenous reference and relative to the calibrator, was determined using the 2^{- $\Delta\Delta\text{C}_T$} method [27]. The GenBank Accession Nos. for the sequences used in this work are: NM_129260 (*PAL1*), NM_115186 (*PAL2*), NM_179462 (*4CL1*), NM_113019 (*4CL2*), NM_179513 (*4CL3*), NM_121396 (*CHS*), NM_114519 (*actin*).

2.4. Enzyme extraction and assays

2.4.1. PAL activity

Frozen *A. thaliana* seedlings (0.5 g) were ground in a mortar in liquid nitrogen and mixed with 5 ml of extraction buffer (150 mM

Tris-HCl, pH 8.8, 12 mM 2-mercaptoethanol, 0.1 g ml⁻¹ Dowex[®] 1X4-200). The homogenate was centrifuged at 23,000g for 30 min at 4 °C. The supernatant, referred to as the enzyme extract, was used for the determination of phenylalanine ammonia-lyase (EC 4.3.1.5) activity according to Alokam and co-workers [28] by measuring the increase in A₂₉₀ for 10 min at 30 °C due to the accumulation of *trans*-cinnamic acid ($\epsilon_{290} = 9.5 \text{ mM}^{-1} \text{ cm}^{-1}$). The PAL assay mixture (0.2 ml) contained 50 mM Tris-HCl, pH 8.8, 5 mM L-phenylalanine and 10 μl enzyme extract.

2.4.2. 4CL activity

Frozen *A. thaliana* seedlings (0.5 g) were ground in a mortar with liquid nitrogen and 5 ml of extraction buffer (100 mM Tris-HCl, pH 7.8, 5 mM 2-mercaptoethanol, 5% glycerol). Next, 0.5 g Dowex[®] 1X4-200 was added and the sample stirred for 15 min at 4 °C. The homogenate was centrifuged at 23,000g for 30 min. The 4-coumarate-CoA ligase (EC 6.2.1.12) was assayed according to Knobloch and Hahlbrock [29]. The reaction mixture (0.2 ml) contained 100 mM Tris-HCl (pH 7.8), 0.1 mM *p*-coumaric acid, 0.5 mM ATP, 0.3 mM CoA, 5 mM MgCl₂ and 10 μl enzyme extract (4–6 μg of protein). The activity of 4CL was determined at 30 °C. Formation of coumaroyl-CoA was measured by monitoring the A₃₃₃ ($\epsilon_{333} = 21 \text{ mM}^{-1} \text{ cm}^{-1}$) [30]. Assays with *Ap₃A* or *Ap₄A* were performed with pure recombinant At4CL2 kindly donated by Dr. Erich Kombrink (Max Planck Institute for Plant Breeding Research, Cologne, Germany).

2.4.3. CHS activity

The extraction and assay of chalcone synthase (EC 2.3.1.74) was performed according to a modification of the method of Fischer and co-workers [31]. Frozen *A. thaliana* seedlings (0.5 g) were ground in a mortar in liquid nitrogen and mixed with extraction buffer (100 mM KH₂PO₄/K₂HPO₄ pH 8.0, 18 mM L-cysteine, 20 mM ascorbic acid, 0.1 g ml⁻¹ Dowex[®] 1X4-200). The homogenate was centrifuged at 23,000 g for 30 min and the supernatant (enzyme extract) used for the enzyme assay. The reaction mixture (63 μl) contained 50 mM KH₂PO₄/K₂HPO₄ pH 8.0, 20 mM L-cysteine, 2% BSA (w/v), 0.2 mM *p*-coumaroyl-CoA (see below for its synthesis), 0.2 mM [2-¹⁴C]malonyl-CoA and 10 μl enzyme extract (4–6 μg of protein). Incubation was carried out at 35 °C for 1 h. After this time, the reaction was stopped by adding 6 μl 20% HCl. Next, 200 μl ethyl acetate was added and the reaction mixed using a vortex and centrifuged for at least 2 min. The ethyl acetate layer was transferred to a new tube and evaporated to dryness in a SpeedVac concentrator. The sample was redissolved in 20 μl methanol and applied to an aluminum-backed silica gel plate containing a fluorescent indicator (Merck, Cat. No. 5554). The chromatogram was developed for 45 min in chloroform:ethanol (3:1 vol/vol), dried, and the naringenin visualized under a short-wave ultraviolet lamp. The spots of naringenin were cut out and radioactivity determined by scintillation counting.

p-Coumaroyl-CoA was prepared according to Sullivan [32] using recombinant At4CL2 protein. The thioester was synthesized in a 2-ml reaction mixture containing 100 mM Tris-HCl pH 7.8, 0.5 mM CoA, 5 mM ATP, 5 mM MgCl₂, 1 mM *p*-coumaric acid and 4 μg recombinant 4CL2. The reaction mixture was incubated at 37 °C and monitored by measuring the A₃₃₃ up to 2 h. Next, the reaction mixture was applied to a 1-ml ENVI-18 solid-phase extraction column (Supelco) preequilibrated with 3 ml methanol and 3 ml 0.1% acetic acid in water, pH 2.75. The column was washed with 6 ml 0.1% acetic acid in water, pH 2.75, and the coumaroyl-CoA eluted with 1 ml methanol. After methanol evaporation, the thioester was dissolved in 0.5 ml 25 mM MOPS buffer, pH 7.5. The concentration of *p*-coumaroyl-CoA was determined spectrophotometrically (see Section 2.4.2).

2.5. Protein concentration

Total protein concentration was estimated according to Bradford [33], using bovine serum albumin as a standard.

2.6. Statistical analysis

The experiments were carried out in triplicate for the enzyme assays and in duplicate for the mRNA level determination. The results are the mean \pm SD.

3. Results

In initial experiments designed to determine whether exogenously applied Ap₃A or Ap₄A could affect the synthesis and activity of PAL and 4CL in *A. thaliana* seedlings, a concentration of 5 μ M dinucleotide was used in the growth medium. Under these conditions we observed a dramatic time-dependent increase in the expression of the *PAL2* gene with either dinucleotide (Fig. 1A). Ap₄A triggered an increase in *PAL2* expression within the first 5 min of the incubation, reaching a maximum 75-fold increase within 10 min and remaining at this level for at least 3 h. Although we analyzed fewer time points with Ap₃A, this nucleotide appeared to cause a very similar effect. Interestingly, *PAL1* expression remained practically unaffected. Fig. 1B shows that the specific activity of PAL in seedling extracts also increased in response to Ap₃A (up to 8- to 9-fold in 3 h) and Ap₄A, though to a lesser extent and with different kinetics (up to 3-fold by 10 min followed by a decline). The dependence of PAL catalytic activity in the seedling extracts on the concentration of Ap₃A or Ap₄A in the growth medium is shown in Fig. 1C. With Ap₃A, a kind of saturation curve can be seen that reaches a plateau at 0.2 μ M, decreasing slightly thereafter up to 25 μ M. In the case of Ap₄A, the highest increase in PAL activity was observed at 40 nM. The magnitude of the response to 8 nM Ap₃A is quite striking.

Analogous measurements were performed for the *Arabidopsis* 4CL genes (Fig. 2A) and 4CL catalytic activity (Fig. 2B and C). Ap₃A- and Ap₄A-stimulated expression of the 4CL genes was also observed, although it was much less dramatic (less than 3-fold) than that of *PAL2*. In the case of 4CL activity, the effects evoked by Ap₃A and Ap₄A were similar. The plateau of 4CL activity in response to 5 μ M Ap₃A or Ap₄A was reached in 30 min, with that particular concentration of the dinucleotides appearing to exert the strongest effect (Fig. 2C). Finally, we found that the gene and the catalytic activity of chalcone synthase, which catalyzes a more downstream reaction in the phenylpropanoid pathway than does PAL or 4CL, were both poorly stimulated by either Ap₃A or Ap₄A (Fig. 3A and B).

We also found that neither Ap₃A nor Ap₄A affected PAL or 4CL activity directly when added at concentrations up to 50 μ M to the *in vitro* assay mixtures (not shown). Since all organisms [34], including plants [35] possess a number of specific and nonspecific enzymes that can degrade Np_nN's yielding nucleoside mononucleotides, we checked whether these potential degradation products (ATP, ADP, AMP or adenosine), used at the same micromolar concentrations as those of Ap₃A or Ap₄A, could exert the above effects on the genes or activities of PAL and 4CL. When individually tested at a concentration of 5 μ M, none of these four compounds could stimulate the expression or activity of either enzyme in the seedling extracts. Using HPLC, we also monitored changes in the concentration of Ap₃A and Ap₄A in the growth medium during the experiments. In each case, the starting concentration of 5 μ M fell to about 3 μ M after 3 h but no measurable amounts of adenine mononucleotides were detected in the medium. Thus, the apparent "consumption" of these dinucleotides by the seedlings was not

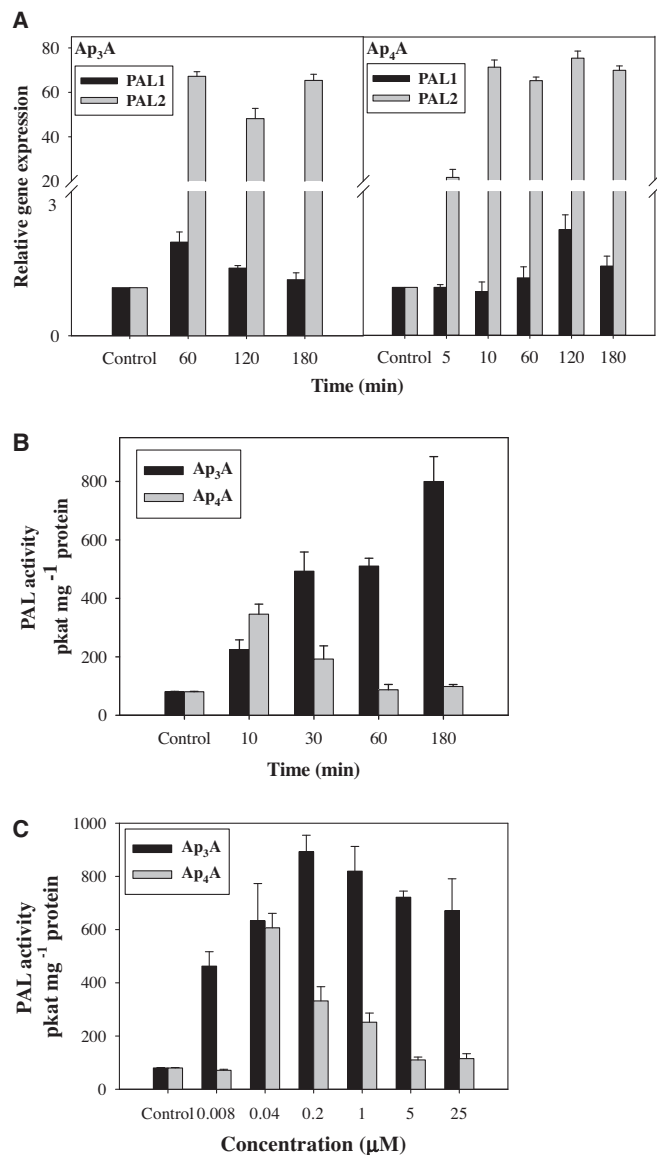


Fig. 1. Phenylalanine ammonia-lyase gene *PAL1* and *PAL2* expression (A) and PAL activity (B and C) in *Arabidopsis thaliana* seedlings treated with Ap₃A or Ap₄A. (A) Treatment with 5 μ M Ap₃A or Ap₄A for 0–180 min; (B) treatment with 5 μ M Ap₃A or Ap₄A for 0–180 min; (C) Treatment with 0–25 μ M Ap₃A or Ap₄A for 180 min. Values are means of three independent experiments \pm SD.

accompanied by the appearance of their potential degradation products in the growth medium.

4. Discussion

For more than three decades, researchers have carried out many different kinds of experiments in different systems to try to answer the question about the biological role of Np_nN's. For example, Ap₄A was found to trigger the initiation of DNA replication *in vitro* [37] and to be a ligand of a 57-kDa protein associated with DNA polymerase α [38]. It also stimulated DNA synthesis when microinjected into *Xenopus laevis* oocytes [39] and induced apoptosis in cultured human cells [40]. Extracellularly, different Ap_nNs can control blood pressure [41,42] and act as neurotransmitters [43]. Recently, Ap₄A has been reported to be a signaling molecule in immunologically activated mast cells [44]. For more examples and a comprehensive discussion on these issues see the review

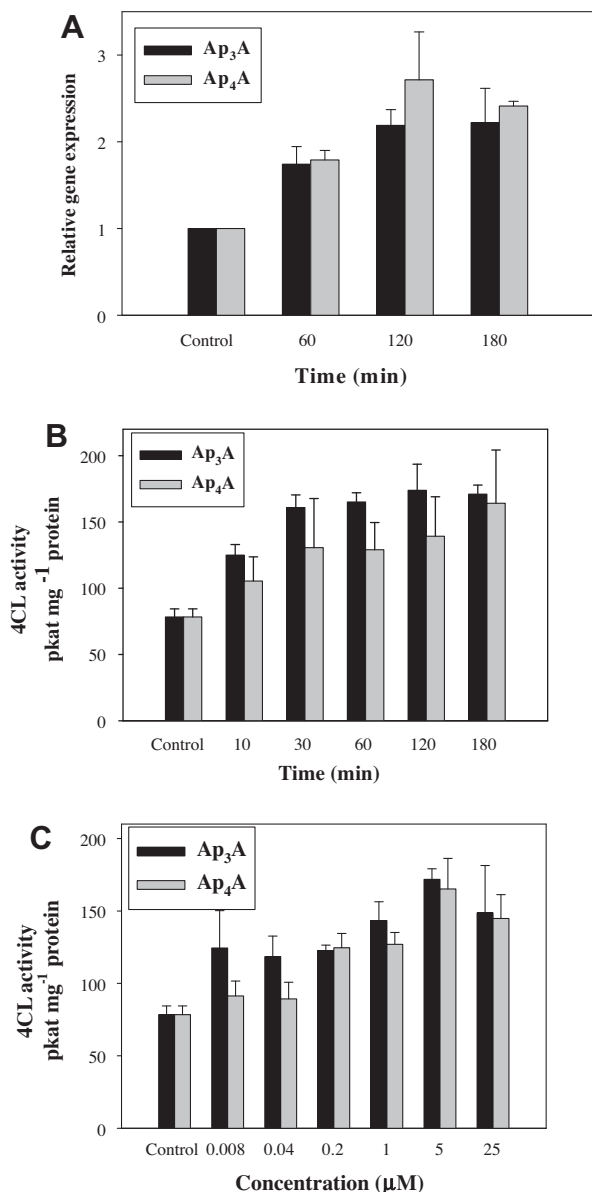


Fig. 2. 4-Coumarate:CoA ligase gene *4CL* expression (A) and *4CL* activity (B and C) in *Arabidopsis thaliana* seedlings treated with Ap₃A or Ap₄A. (A) Treatment with 5 μM Ap₃A or Ap₄A for 0–180 min; (B) treatment with 5 μM Ap₃A or Ap₄A for 0–180 min; (C) treatment with 0–25 μM Ap₃A or Ap₄A for 180 min. Values are means of three independent experiments ±SD.

by McLennan [36]. Until now however, no potential function for Np_nN's has been demonstrated in plants. Our findings described above show that at least Ap₃A and Ap₄A can act as alarmones in plants. They evoked the strongest response in stimulating the expression and activity of PAL, the enzyme that catalyzes the first reaction of the phenylpropanoid pathways. The response of 4CL was much weaker and that of chalcone synthase was insignificant. It is generally believed that the products of the phenylpropanoid pathways protect plants against various stresses caused by wounding, pathogen infection, ultraviolet irradiation and heavy metals, including cadmium [21,45,46]. A large number of studies have shown that *PAL* expression is responsive to these environmental stimuli [45,47,48]. *PAL* activity is a key factor in the increased accumulation of flavonoids and other phenolic compounds under UV-B radiation and water deficit [49,50]. We have checked the expression of *PAL1* and *PAL2* because these genes proved to be important

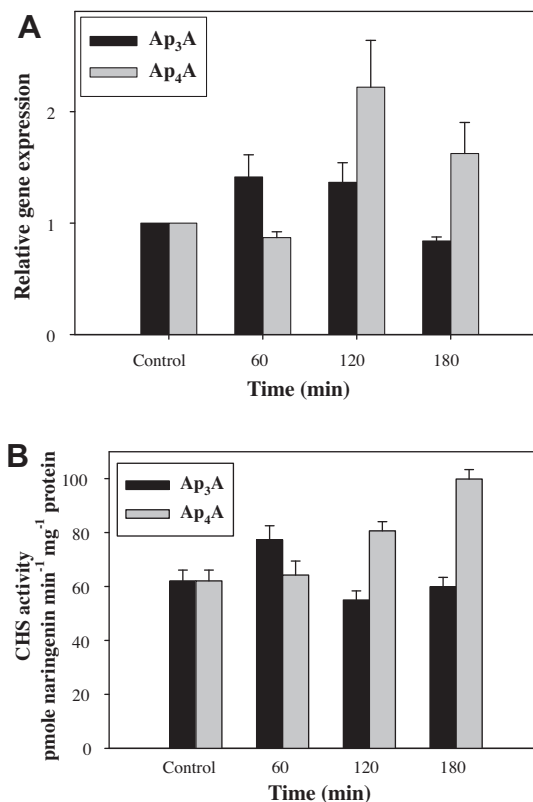


Fig. 3. Expression of chalcone synthase gene *CHS* (A) and *CHS* activity (B) in *Arabidopsis thaliana* seedlings treated with Ap₃A or Ap₄A. (A) Treatment with 5 μM Ap₃A or Ap₄A for 0–180 min; (B) treatment with 5 μM Ap₃A or Ap₄A for 0–180 min. Values are means of three independent experiments ±SD.

for lignin synthesis and also have functional specialization in abiotic environmentally-triggered flavonoid synthesis [51]. Expression of various *4CLs*, including *4CL1*, *4CL2* and *4CL3*, and the *4CL* activity were also shown to respond to different stresses, in particular to pathogen-related elicitor treatment [52,53] and UV-B irradiation [50,54,55]. Our findings suggest that plant tissues possess a specific receptor that recognizes diadenosine tri- and/or tetraphosphates but not adenine mononucleotides and apparently triggers a cascade of events to yield these protective metabolites. Thus our work opens up new avenues for studies on the role of Ap₃A, Ap₄A and of other Np_nN's in plants. In the near future, efforts should be undertaken to at least answer such questions as: (i) do the non-adenylylated Np_nN's evoke the same effects as Ap₃A or Ap₄A; (ii) is this phenomenon common to other plant systems; (iii) can a plant cell receptor be identified with specificity for these dinucleotides; (iv) do the exogenously applied diadenosine polyphosphates affect accumulation of particular phenylpropanoid compound(s) in the plant tissues; and (v) how do other genes and enzymes of the phenylpropanoid pathways respond to those uncommon (di)nucleotides?

Based on existing knowledge of the reactions caused in cells by cadmium [12,13,21–23] and on the observations communicated in this paper, we postulate that in plant cells Cd (II) causes accumulation of Ap₃A and/or Ap₄A and, by analogy with the activation of the MITF transcription factor in mast cells by Ap₄A [44], these compounds interact with transcription factors that control mainly the *PAL2* gene and to a lesser extent the *4CL* genes. Since the metabolites of the phenylpropanoid pathways protect plants against the harmful effects of different types of stress, Ap₃A and Ap₄A behave in our biological system as true alarmones, initiating the rescue action. Finally, as *4CL* is considered to be an enzyme involved in the

response to different harmful factors, one can hypothesize that it plays at least two roles under conditions of stress. First, it is able to synthesize the diadenosine polyphosphate (the putative alarmones) [6] and secondly, as one of the enzymes of the phenylpropanoid pathways, it then contributes to the production of metabolites that minimize the effects of the stress.

Note: Preliminary report of this study was presented as a poster at the 46th Meeting of the Polish Biochemical Society (Cracow, September 5–9, 2011) [56].

Acknowledgements

This study was supported by the Ministry of Science and Higher Education (Grant No. N N303 068634). We thank Professor Alexander McLennan (University of Liverpool, UK) for critical reading of the manuscript and linguistic help in preparation of this paper.

References

- Zamecnik, P.C., Stephenson, M.L., Janeway, C.M. and Randerath, K. (1966) Enzymatic synthesis of diadenosine tetraphosphate and diadenosine triphosphate with a purified lysyl-sRNA synthetase. *Biochem. Biophys. Res. Commun.* 24, 91–97.
- Plateau, P., Mayaux, J.-F. and Blanquet, S. (1981) Zinc (II)-dependent synthesis of diadenosine 5', 5'''-P₁P₄-tetraphosphate by *Escherichia coli* and yeast phenylalanyl transfer ribonucleic acid synthetases. *Biochemistry* 20, 4654–4662.
- Goerlich, O., Foeckler, R. and Holler, E. (1982) Mechanism of synthesis of adenosine(5')tetraphospho(5')adenosine (AppppA) by aminoacyl-tRNA synthetases. *Eur. J. Biochem.* 126, 135–142.
- Jakubowski, H. (1983) Synthesis of diadenosine 5', 5'''-P₁P₄-tetraphosphate and related compounds by plant (*Lupinus luteus*) seryl-tRNA and phenylalanyl-tRNA synthetases. *Acta Biochim. Pol.* 30, 51–69.
- Fontes, R., Günther Sillero, M.A. and Sillero, A. (1998) Acyl-coenzyme A synthetase from *Pseudomonas fragi* catalyzes the synthesis of adenosine 5'-polyphosphates and dinucleoside polyphosphates. *J. Bacteriol.* 180, 3152–3158.
- Pietrowska-Borek, M., Stuiblé, H.P., Kombrink, E. and Guranowski, A. (2003) 4-Coumarate: coenzyme A ligase has the catalytic capacity to synthesize and reuse various (di)adenosine polyphosphates. *Plant Physiol.* 131, 1401–1410.
- Guranowski, A., Günther Sillero, M.A. and Sillero, A. (1990) Firefly luciferase synthesizes P₁P₄-bis(5'-adenosyl) tetraphosphate (Ap₄A) and other dinucleoside polyphosphates. *FEBS Lett.* 271, 215–218.
- Guranowski, A., Just, G., Holler, E. and Jakubowski, H. (1988) Synthesis of diadenosine 5', 5'''-P₁P₄-tetraphosphate (AppppA) from adenosine 5'-phosphosulfate and adenosine 5'-triphosphate catalyzed by yeast AppppA phosphorylase. *Biochemistry* 27, 2959–2964.
- Guranowski, A., de Diego, A., Sillero, A. and Günther Sillero, M.A. (2004) Uridine 5'-polyphosphates (p₄U and p₅U) and uridine(5') polyphospho(5')nucleosides (Up_nNs) can be synthesized by UTP:glucose-1-phosphate uridylyltransferase from *Saccharomyces cerevisiae*. *FEBS Lett.* 561, 83–88.
- Wang, D. and Shatkin, A.J. (1984) Synthesis of Gp₄N and Gp₃N compounds by guanylyltransferase purified from yeast. *Nucleic Acids Res.* 12, 2303–2315.
- Lee, P.C., Barry, R. and Ames, B.N. (1983) Diadenosine 5', 5'''-P₁P₄-tetraphosphate and related adenylated nucleotides in *Salmonella typhimurium*. *J. Biol. Chem.* 258, 6827–6834.
- Coste, H., Brevet, A., Plateau, P. and Blanquet, S. (1987) Non-adenylated bis(5'-nucleosidyl) tetraphosphates occur in *Saccharomyces cerevisiae* and in *Escherichia coli* and accumulate upon temperature shift or exposure to cadmium. *J. Biol. Chem.* 262, 12096–12103.
- Pálfí, Z., Surányi, G. and Borbély, G. (1991) Alterations in the accumulation of adenylated nucleotides in heavy-metal-ion-stressed and heat-stressed *Synechococcus* sp. Strain PCC 6301, cyanobacterium, in light and dark. *Biochem. J.* 276, 487–491.
- Baltzinger, M., Ebel, J.-P. and Remy, P.M. (1986) Accumulation of dinucleoside polyphosphates in *Saccharomyces cerevisiae* under stress conditions: High levels are associated with cell death. *Biochimie* 68, 1231–1236.
- Morioka, M. and Shimada, H. (1984) Synthesis of diadenosine 5', 5'''-P₁P₄-tetraphosphate (AP₄A) in sea urchin embryos. *Cell Differentiation* 14, 53–58.
- Miller, D. and McLennan, A.G. (1986) Changes in intracellular levels of Ap₃A and Ap₄A in cysts and larvae of *Artemia* do not correlate with changes in protein synthesis after heat-shock. *Nucleic Acids Res.* 14, 6031–6040.
- Brevet, A., Plateau, P., Best-Belpomme, M. and Blanquet, S. (1985) Variation of Ap₄A and other dinucleoside polyphosphates in stressed *Drosophila* cells. *J. Biol. Chem.* 260, 15566–15570.
- Flodgaard, H. and Klenow, H. (1982) Abundant amounts of diadenosine 5', 5'''-P₁P₄-tetraphosphate are present and releasable, but metabolically inactive, in human platelets. *Biochem. J.* 208, 737–742.
- Lüthje, J. and Olivie, A. (1983) The presence of diadenosine 5', 5'''-P₁P₃-triphosphate (Ap₃A) in human platelets. *Biochem. Biophys. Res. Commun.* 115, 253–260.
- Garrison, P.N. and Barnes, L.D. (1992) Determination of dinucleoside polyphosphates in: Ap₄A and Other Dinucleoside Polyphosphates (McLennan, A.G., Ed.), pp. 29–61, CRC Press, Boca Raton, FL.
- Schützendübel, A., Schwanz, P., Teichman, T., Gross, K., Langenfeld-Heysler, R., Godbold, D.L. and Polle, A. (2001) Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots pine roots. *Plant Physiol.* 127, 887–898.
- Dai, L.-P., Xiong, Z.-T., Huang, Y. and Li, M.-J. (2006) Cadmium-induced changes in pigments, total phenolics, and phenylalanine ammonia-lyase activity in fronds of *Azolla imbricata*. *Environ. Toxicol.* 21, 505–512.
- Sivaci, A., Sivaci, E.R. and Sökmen, M. (2007) Changes in antioxidant activity, total phenolic and abscisic acid constituents in the aquatic plants *Myriophyllum spicatum* L. and *Myriophyllum triphyllum* Orchard exposed to cadmium. *Ecotoxicology* 16, 423–428.
- Kováčik, J., Klejduš, B., Hedbavny, J. and Zoň, J. (2011) Significance of phenols in cadmium and nickel uptake. *J. Plant Physiol.* 168, 576–584.
- Scheible, W.-R., Morcuende, R., Czechowski, T., Fritz, C., Osuna, D., Palacios-Rojas, N., Schindelasch, D., Thimm, O., Udvardi, M.K. and Stitt, M. (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. *Plant Physiol.* 136, 2483–2499.
- Guranowski, A., Starzyńska, E., Pietrowska-Borek, M., Rejman, D. and Blackburn, G.M. (2009) Novel diadenosine polyphosphate analogs with oxymethylene bridges replacing oxygen in the polyphosphate chain. Potential substrates and/or inhibitors of Ap₄A hydrolases. *FEBS J.* 276, 1546–1553.
- Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by comparative CT method. *Nat. Protoc.* 3, 1101–1107.
- Alokam, S., Li, Y., Li, W., Chinnappa, C.C. and Reid, D.M. (2002) Photoregulation of phenylalanine ammonia-lyase (PAL) in the accumulation of anthocyanin in alpine and prairie ecotypes of *Stellaria longipes* under varied R/FR. *Physiol. Plant.* 116, 531–538.
- Knobloch, K.H. and Hahlbrock, K. (1977) 4-Coumarate:CoA ligase from cell suspension of *Petroselinum hortense* Hoffm. Partial purification, substrate specificity, and further properties. *Arch. Biochem. Biophys.* 184, 237–248.
- Stöckigt, J. and Zenk, M.H. (1975) Chemical syntheses and properties of hydroxycinnamoyl coenzyme A derivatives. *Z. Naturforsch.* 30c, 352–358.
- Fischer, S., Böttcher, U., Reuber, S., Anhalt, S. and Weissenböck, G. (1995) Chalcone synthase in the liverwort *Marchantia polymorpha*. *Phytochemistry* 39, 1007–1012.
- Sullivan, M.L. (2009) A novel red clover hydroxycinnamoyl transferase has enzymatic activities consistent with a role in phaselic acid biosynthesis. *Plant Physiol.* 150, 1866–1879.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 48–54.
- Guranowski, A. (2000) Specific and nonspecific enzymes involved in the catabolism of mononucleoside and dinucleoside polyphosphates. *Pharmacol. Ther.* 87, 117–139.
- Jakubowski, H. and Guranowski, A. (1983) Enzymes hydrolyzing ApppA and/or AppppA in higher plants; purification and some properties of diadenosine triphosphatase, diadenosine tetraphosphatase, and phosphodiesterase from yellow lupin (*Lupinus luteus*) seeds. *J. Biol. Chem.* 258, 9982–9989.
- McLennan, A. (2000) Dinucleoside polyphosphates – friend or foe? *Pharmacol. Ther.* 87, 73–89.
- Grummt, F. (1978) Diadenosine 5', 5'''-P₁P₄-tetraphosphate triggers initiation of in vitro DNA replication in baby hamster kidney cells. *Proc. Natl. Acad. Sci. USA* 75, 371–374.
- Grummt, F., Wälti, G., Jantzen, H.-M., Hamprecht, K., Huebscher, U. and Kuenzle, C.C. (1979) Diadenosine 5', 5'''-P₁P₄-tetraphosphate, a ligand of the 57-kilodalton subunit of DNA polymerase α . *Proc. Natl. Acad. Sci. USA* 76, 6081–6085.
- Zourgui, L., Tharaud, D., Solari, A., Litvak, S. and Tarrago-Litvak, L. (1984) Stimulation of DNA synthesis by microinjection of diadenosine 5', 5'''-P₁P₄-tetraphosphate (Ap₄A) into *Xenopus laevis* oocytes. *Dev. Biol.* 103, 409–413.
- Vartanian, A., Alexandrov, I., Prudovski, I., McLennan, A. and Kisselev, L. (1999) Ap₄A induces apoptosis in cultured human cells. *FEBS Lett.* 456, 175–180.
- Schlüter, H., Offers, E., Bruggemann, G., van der Giet, M., Tepel, M., Nordhoff, E., Karas, M., Spieker, C., Witzel, H. and Zidek, W. (1994) Diadenosine phosphates and the physiological control of blood pressure. *Nature* 367, 186–188.
- Jankowski, V., Tölle, M., Vanholder, R., Schönfelder, G., van der Giet, M., Henning, L., Schlüter, H., Paul, M., Zidek, W. and Jankowski, J. (2005) Uridine adenosine tetraphosphate: a novel endothelium-derived vasoconstrictive factor. *Nat. Med.* 11, 223–227.
- Pintor, J. and Miras-Portugal, M.T. (1993) Diadenosine polyphosphates (Ap_nA) as new neurotransmitters. *Drug Dev. Res.* 28, 259–262.
- Carmi-Levy, I., Motzik, A., Ofir-Birin, Y., Yagil, Z., Yang, Z., Yang, C.M., Kemeny, D.M., Han, J.M., Kim, S., Kay, G., Nechushtan, H., Suzuki, R., Rivera, J. and Razin, E. (2011) Importin beta plays an essential role in the regulation of the LysRS-Ap(4)A pathway in immunologically activated mast cells. *Mol. Cell. Biol.* 2011, 2111–2121.
- Dixon, R.A. and Paiva, N.L. (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell* 7, 1085–1097.

- [46] Ferrer, J.L., Austin, M.B., Stewart Jr, C. and Noel, J.P. (2008) Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiol. Biochem.* 46, 356–370.
- [47] Lawton, M.A., Dixon, R.A., Hahlbrock, K. and Lamb, C. (1983) Rapid induction of the synthesis of phenylalanine ammonia-lyase and of chalcone synthase in elicitor-treated plant cells. *Eur. J. Biochem.* 129, 593–601.
- [48] Olsen, M.K., Lea, U.S., Slimestad, R., Verheul, M. and Lillo, C. (2008) Differential expression of four *Arabidopsis* *PAL* genes; *PAL1* and *PAL2* have functional specialization in abiotic environmental-triggered flavonoid synthesis. *J. Plant Physiol.* 165, 1491–1499.
- [49] Liu, L. and McClure, J.W. (1995) Effects of UV-B on activities of enzymes of secondary phenolic metabolism in barley primary leaves. *Physiol. Plant.* 93, 734–739.
- [50] H. Bandurska, M. Pietrowska-Borek, M. Cieślak, Response of barley seedlings to water deficit and enhanced UV-B irradiation acting alone and in combination. *Acta Physiol. Plant*, 2011. doi:10.1007/s11738-011-0814-9.
- [51] Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y.-H., Yu, J.-Q. and Chen, Z. (2010) Functional analysis of the *Arabidopsis* *PAL* gene family in plant growth, development, and response to environmental stress. *Plant Physiol.* 153, 1526–1538.
- [52] Lindermayr, C., Möllers, B., Fliegmann, J., Uhlmann, A., Lottspeich, F., Meimberg, H. and Ebel, J. (2002) Divergent members of a soybean (*Glycine max* L.) 4-coumarate:coenzyme A ligase gene family. *Eur. J. Biochem.* 269, 1304–1315.
- [53] Gaid, M.M., Scharnhop, H., Ramadan, H., Beuerle, T. and Beerhues, L. (2011) 4-Coumarate:CoA ligase family members from elicitor-treated *Sorbus aucuparia* cell cultures. *J. Plant Physiol.* 168, 944–951.
- [54] Douglas, C., Hoffmann, H., Schulz, W. and Hahlbrock, K. (1987) Structure and elicitor or uv-light stimulated expression of two 4-coumarate:CoA ligase genes in parsley. *EMBO J.* 6, 1189–1195.
- [55] Kimura, M., Yamamoto, Y.Y., Seki, M., Sakuari, T., Sato, M., Abe, T., Yoshida, S., Manabe, K., Shinozaki, K. and Matsui, M. (2003) Identification of *Arabidopsis* genes regulated by high light-stress using cDNA microarray. *Photochem. Photobiol.* 77, 226–233.
- [56] Pietrowska-Borek, M., Nuc, K., Zielezińska, M. and Guranowski, A. (2011) Diadenosine polyphosphates (Ap3A and Ap4A), putative alarmones, trigger synthesis of enzymes of the phenylpropanoid pathway in *Arabidopsis thaliana*. *Acta Biochim. Pol.* 58 (Suppl. 2. P27.4), 264.