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

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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.

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1. Introduction

Dinucleoside 5',5'-P₁,Pᵩ-polyphosphates, Nₚ,N'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 The presence of Nₚ,N's has been demonstrated in yellow lupin seedlings (Guranowski, unpublished observation) but no detailed report of the occurrence of these compounds in plants has appeared yet.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,Pᵩ-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 Nₘ,N's [11–14], these compounds have been termed alarmones. However, no clear metabolic or molecular target (“addressee”) of the postulated “alarm” signalled by the Nₚ,N'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 Nₚ,N'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 Nₚ,N'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...
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 μmol m⁻² 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)₁₈ (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× first strand buffer, DTT and RNase inhibitor (RNase-OUT Invitrogen) were mixed at 4 °C and incubated for 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 PCR 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'-CCGATGTTTGTTATG-C and 55°C, 10 min at 95°C, then 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.

2.4. 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 4X-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 ([ε₂₉₀ = 9.5 mM⁻¹ cm⁻¹]). The PAL assay mixture (0.2 ml) contained 50 mM Tris–HCl, pH 8.8, 5 mM L-phenylalanine and 10 μM 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 4X-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 μM 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 ([ε₃₃₃ = 21 mM⁻¹ 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 4X-200). The homogenate was centrifuged at 23,000g 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 μM 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 aluminium-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-m1 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 4CL. 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×l ENV-18 solid-phase extraction column (Supercyo) prequilled 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 ± 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 Arabidopsis thaliana seedlings, a concentration of 5 μM 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). Ap3A 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 Ap2A, 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 μM, decreasing slightly thereafter up to 25 μM. In the case of Ap4A, the highest increase in PAL activity was observed at 40 mM. The magnitude of the response to 8 mM Ap3A is quite striking.

Analogous measurements were performed for the Arabidopsis thaliana PAL1 and PAL2 expression (A) and PAL 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.

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 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.

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,N’s. For example, Ap2A 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 Ap2Ns can control blood pressure [41,42] and act as neurotransmitters [43]. Recently, Ap2A 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.
by McLennan [36]. Until now however, no potential function for NpN’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 wound- ing, 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 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 tetra- phosphates 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 NpN’s in plants. In the near future, efforts should be undertaken to at least answer such questions as: (i) do the non-adenylylated NpN’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 dinucleoside polyphosphate (the putative alarmones) \[6\] and secondly, as one of the enzymes of the phenylpropionate pathway, it plays at least two roles under conditions of stress. First, it is able to synthesize and release these molecules, and secondly, as one of the enzymes of the phenylpropionate pathway, it is involved in the metabolism of these molecules.

### 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) \[56\].

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