Cloning and Characterization of the Arabidopsis Cyclic Phosphodiesterase Which Hydrolyzes ADP-ribose 1″,2″-Cyclic Phosphate and Nucleoside 2′,3′-Cyclic Phosphates*

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In euakaryotic cells, pre-tRNAs spliced by a pathway that produces a 3′,5′-phosphodiester, 2′-phosphomonoester linkage contain a 2′-phosphate group adjacent to the tRNA anticodon. This 2′-phosphate is transferred to NAD to give adenosine diphosphosphate (ADP)-ribose 1″,2″-cyclic phosphate (Appr p), which is subsequently metabolized in ADP-ribose 1″-phosphate (Appr-1″p). The latter reaction is catalyzed by a cyclic phosphodiesterase (CPDase), previously identified in yeast and wheat. In the work presented here, we describe cloning of the Arabidopsis cDNA encoding the 20-kDa CPDase that hydrolyzes Appr p to Appr-1″p. Properties of the bacterial overexpressed and purified Arabidopsis enzyme are similar to those of wheat CPDase. In addition to their transformation of Appr p, both enzymes hydrolyze nucleoside 2′,3′-cyclic phosphates to nucleoside 2′-phosphates. For the Arabidopsis CPDase, the apparent K_m values for Appr p, A″p, C″p, G″p, and U″p are 1.35, 1.34, 2.38, 16.68, and 17.67 mM, respectively. Southern analysis indicated that CPDase in Arabidopsis is encoded by a single copy gene that is expressed, at different levels, in all Arabidopsis organs that were analyzed. Indirect immunofluorescence, performed with transfected protoplasts, showed that CPDase is localized in the cytoplasm. Based on substrate specificity and products generated, the plant enzyme differs from other known cyclic phosphodiesterases. The Arabidopsis CPDase does not have recognizable structural similarity or motifs in common with proteins deposited in public data bases.

Transcripts of many tRNA genes in eukaryotes contain a single intron, located in a conserved position in the anticodon loop, which is excised by a different mechanism to that utilized during nuclear pre-mRNA processing (Fig. 1; reviewed in Refs. 1 and 2). Splicing of pre-tRNA is initiated by endonucleolytic cleavages that result in removal of the intron and formation of two tRNA half-molecules, a 5′-half terminating in a 2′,3′-cyclic phosphate and a 3′-half bearing a 5′-hydroxyl group (3–7). In yeast and plants, these two tRNA exons are ligated to give an unusual 3′,5′-phosphodiester, 2′-phosphomonoester linkage. This reaction, catalyzed by the RNA ligase (8–11), is a multistep process resulting in formation of the mature length tRNA containing a 2′-phosphate at the splice junction (1, 2) (Fig. 1). The ligation pathway leading to the formation of the 2′-phosphate-bearing tRNA molecules is also conserved in vertebrates (12), despite the fact that in these organisms most of the tRNA splicing appears to involve another RNA ligase, an enzyme which joins two tRNA halves by the regular 3′,5′-phosphodiester (3, 4, 13).

The 2′-phosphate present in the product of the spliced tRNA is removed by a specific phosphotransferase, previously identified in yeast and vertebrates (14, 15). Culver et al. (16) found that this enzyme transfers the 2′-phosphate to an NAD acceptor molecule, to produce ADP-ribose 1″,2″-cyclic phosphate (Appr-1″p). However, Appr p is not the final product of this complex series of reactions. It has been found recently that Appr p is converted into ADP-ribose 1″-phosphate (Appr-1″p) by the action of the cyclic phosphodiesterase (CPDase), identified in yeast and wheat (17). Although all partial reactions leading to the formation of Appr p and Appr-1″p have, to date, only been demonstrated in yeast (16, 17), the available evidence suggests that both compounds are also produced, as a result of the tRNA splicing reaction, in plants and vertebrates (12, 15–17). It has been suggested that Appr p, or its hydrolysis product, may perform some as yet unspecified regulatory function(s) in the cell (16). Conservation of the Appr p-forming pathway in vertebrates (12, 15, 16), despite the fact that most of the cellular tRNA in these organisms seems to be processed by another pathway (see above), offers some support for this hypothesis.

The plant CPDase was originally purified from wheat as an enzyme that hydrolyzes nucleoside 2′,3′-cyclic phosphates to nucleoside 2′-phosphates (18). The biological significance of this reaction is not known, but the ability of the enzyme to convert the 2′,3′-cyclic phosphate to the 2′-phosphate in mono-nucleotides but not in cyclic-phosphate-terminated oligoribonucleotides, together with its ability to hydrolyze Appr p, clearly distinguishes it from other known enzymes having the 2′,3′-phosphate 3′-phosphodiesterase activity (17, 18; see "Discussion"). Although the yeast phosphodiesterase shares many characteristics with the wheat enzyme, it has a different substrate specificity, hydrolyzing Appr p to Appr-1″p, but hav-

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1 The abbreviations used are: Appr p, ADP-ribose 1″,2″-cyclic phosphate; Appr-1″p, ADP-ribose 1″-phosphate; Appr-2″p, ADP-ribose 2″-phosphate; N, any of four (A, G, C, U) nucleosides; pN, N″p, N″p, and N″p, nucleosides 5″, 3″, 2″-, and 2′,3′-cyclic phosphate, respectively; pG″p, guanosine 5″-phosphate, 2′,3′-cyclic phosphate; CIP, calf intestine phosphatase; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; CPDase, cyclic phosphodiesterase; EST, expressed sequence tag; Mes, 2-N-morpholinoethanesulfonic acid; Mops, 3(N-morpholino)propanesulfonic acid; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; TLC, thin layer chromatography.
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**EXPERIMENTAL PROCEDURES**

**Plant Material—**Plantlets of Arabidopsis thaliana, ecotype Columbia C0, were grown in Petri dishes containing 0.8% agar, 1% sucrose, and MS salts (19) in a 22 °C growth chamber under a 12-h light/12-h dark cycle. Three weeks after sowing, leaves, roots, and floral buds were harvested. For leaf strip incubation, the leaves were sliced and incubated in a culture medium described by Nagy and Maliga (20), containing 1 mg/liter of 2,4-dichlorophenoxyacetic acid. Aliquots were harvested in a culture medium described by Nagy and Maliga (20), containing 1 mg/liter of 2,4-dichlorophenoxyacetic acid. Aliquots were harvested.

**Screening of a cDNA Library—**A λZAP cDNA library, prepared with a mixture of the poly(A)* RNA isolated from 24, 48, and 72 h leaf strip cultures (a gift from J. Fleck, Institut de Biologie Moléculaire des Plantes du CNRS, Strasbourg, France), was screened with the partial cDNA clone (the Arabidopsis EST, GenBank®/EBI accession number T12916; kindly provided by the Arabidopsis Biological Resource Center at Ohio State University, Columbus, OH) as a probe. Hybridizations were performed overnight at 42 °C in 5 × SSPE (SSPE: 0.15 M NaCl, 15 mM Na2citrate) and 0.1% SDS. The blots were subsequently washed in 2 × SSC and 0.1% SDS for 30 min at 42 °C and then washed in 0.2 × SSC and 0.1% SDS for 30 min at 42 °C. Twenty-nine clones were isolated after screening 800,000 recombinant phages. After excision of the phagemids, the inserts were analyzed by restriction mapping and sequencing of the ends. The longest clones were subsequently sequenced on both strands.

**Northern, Southern, and Western Blot Analysis—**Total RNA from Arabidopsis organs and leaf strip cultures was isolated as described by Hall et al. (24). RNA (10 μg/lane) was separated on a formaldehyde-agarose gel, blotted onto Hybond-N nylon membrane (Amer sham Corp.) by capillary transfer using 20 × SSPE, and UV-cross-linked to the membrane. The integrity and the amount of RNA applied to each lane were verified by control hybridizations using a tomato 25 S rRNA probe (25). The DNA fragment extending from positions 430–741 (Fig. 2) was used as a CPDase probe. The histone H4 probe corresponds to the 196-base pair restriction fragment AccI/DeeI of the coding region of the gene HA4748 (26). The actin probe corresponds to the 570-base pair PCR-amplified fragment of the Arabidopsis actin gene AAc1 (27). The genomic DNA was isolated from lyophilized Arabidopsis plants using a procedure similar to that of Murray and Thompson (28). The probes were hybridized with [α-32P]dCTP (3000 Ci/mmol, Amersham) by the random priming method (29). RNA as well as DNA gel blots were hybridized overnight at 42 °C in 5 × SSPE, 50% formamide, 10% dextran sulfate, 1% SDS, and 50 μg/ml denatured salmon sperm DNA. The blots were subsequently washed in 2 × SSC and 0.1% SDS for 30 min at 42 °C and in 0.2 × SSC and 0.1% SDS for 30 min at 42 °C and then at 60 °C.

**For immunoblot analysis, proteins were fractionated by SDS-PAGE and electrophoretically transferred to the polyvinylidene difluoride membrane.** The membrane was probed with a 1:1000 dilution of the polyclonal antibody. The immunoreactive proteins were detected using peroxidase-conjugated affinity-purified rabbit anti-chicken IgYs (Dianova) and the ECL Western blotting analysis system from Amersham. **Expression and Purification of the CPDase—**A BanHI site was introduced 3′ to the CPDase coding sequence by site-directed mutagenesis (30). The Ncol/BanHI fragment (the Ncol site is present at the AUG initiation codon of the CPDase cDNA) was cloned into the pQE-60 vector (Qiagen) yielding plasmid pQECPDase. In this construct, 10 additional amino acids (sequence GSRSHHHHHHH) are placed in frame at the C terminus of the recombinant protein. The protein remained soluble during expression in the Escherichia coli strain BL21(DE3) and was purified in the native form, under nondenaturing conditions, using the nickel-nitrilotriacetic acid resin and following the Qiagen protocol. The purified CPDase was applied to a 10-ml Sephadex G-25 column equilibrated and eluted with 20 mM Tris acetate, pH 7.6, 0.5 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5% (v/v) glycerol, 0.01% Triton X-100, and 10 μM phenylmethylsulfonyl fluoride. The protein concentration was measured by the method of Bradford (31) using bovine serum albumin as a standard.

**Preparation of Hen Antibodies—**Two hens were immunized with the purified recombinant CPDase. For primary immunization, 20 μg of the protein, in Freund’s complete adjuvant, was used. After 4 weeks, 20 μg of the protein with Freund’s incomplete adjuvant was injected. Eggs were collected daily starting 2 weeks after the last immunization.
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Antibodies were purified from egg yolk according to the three-step method of Polson and von Wechmar (32). A 2 m (NH₄)₂SO₄ precipitation was used for the complete removal of polyethylene glycol.

Sources of Nucleotides and Oligonucleotides—All nucleoside 2',3'-cyclic and 3',5'-cyclic phosphates, nucleoside 5'-, 3'- and 2'- phosphates, pG>p and inositol 1,2-cyclic phosphate were obtained from Sigma and Pharmacia Biotech Inc. A>p, G>p, and C>p were purified by reverse phase HPLC on Nucleosil C4 RP-300 column using 50 mM triethylammonium acetate-acetonitrile gradient. Products were collected as a single peak. Appr>p was chemically synthesized as described elsewhere (33). The ³²P-labeled oligoribonucleotide AAAAAAAG>³²P was excised and isolated by PAGE, and the 32P-labeled oligoribonucleotide AAAUUAAAAG was folded into the 5'-leader conformation by heating to 90 °C and allowing the 5' leader to anneal to the 3'-terminal phosphate cyclase purified from HeLa cells (34). The oligonucleotide was digested with RNase T1 yielding AAAUUAAAAGp. The latter was quantitatively converted into AAAUUAAAAGp by incubation with the RNA 3'-terminal phosphate cyclase purified from HeLa cells (34). The oligonucleotide was recovered by phenol extraction and ethanol precipitation.

Assays of Cyclic Nucleotide Phosphodiesterase Activity and Thin Layer Chromatography (TLC)—For calculation of specific activities and kinetic analysis, a quantitative assay based on a measurement of the phosphatase-sensitive nucleotide product was used. All incubations (20 µl) contained 50 mM Tris-HCl, pH 7.0, and 0.01% Triton X-100. Concentrations of CPDase and substrates and incubation times at 30 °C were as indicated in the figure legends. Reactions were stopped by boiling for 2 min, and 80 µl of 0.1 M Tris-HCl, pH 8.0, containing 0.2 unit of CIP was added. After incubation for 10 min at 37 °C, liberated phosphate was assayed according to Hess and Derr (35). For determination of the Kₘ and Vₐₘₚ values, the assays contained substrates at concentrations of 1.38–12.5 mM. All velocities were calculated from the initial linear rates. Values were fitted to the Lineweaver-Burk equation by the linear regression method assuming proportional errors.

Products of enzymatic digestion, performed as described previously (8), were analyzed by cellulose TLC in solvent A (saturated (NH₄)₂SO₄/3 M NaCl) and solvent B (0.75 M LiCl). The nucleotide standards and nucleotide sequences are shown in the figure, which has been submitted to the EMBL/GenBank/DDBJ Nucleotide Sequence Libraries under accession number Y11650.
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RESULTS

Cloning of the cDNA Encoding Arabidopsis CPDase—The previously purified wheat germ CPDase (18) was subjected to tryptic digestion, and three peptide sequences were obtained. One of these, the 20-amino acid-long pep3, showed 80% identity and 100% similarity to the 20-amino acid-long pep2. The secondary antibody was diluted 1:100 with buffer B, containing 10 μg/mL Hoechst 33258 dye. After washing four times with 1 mL of solution A and four times with 1 mL of solution C (0.1 M Tris-HCl, pH 7.4). Glass slides were washed four times for 15 min with 1 mL of solution A and overlaid for 30 min with the fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (AffiniPure F(ab')2, fragment; Jackson/Milan Analytica AG). The secondary antibody was diluted 1:100 with buffer B, containing 10 μg/mL Hoechst 33258 dye. After washing four times with 1 mL of solution A and four times with 1 mL of solution C (0.1 M Tris-HCl, pH 7.4), samples were overlaid with a drop of the embedding material and covered with a cover glass. Samples were examined with a Zeiss Axioshot microscope and a Leica TCS 4D confocal scanning laser microscope, using a 63× objective. Images were recorded using the Leica software (SCANware 4.2) provided with the system and analyzed with the Imaris software on a Silicon Graphics work station.

CAUGGA is similar to the consensus (AACCAUGGC) established for plant genes (39). The 5′-terminal leader contains one additional AUG in a much less favorable context, followed by termination codons (Fig. 2). Conceptual translation of the cDNA yields a 20.5-kDa protein of 181 amino acids with a predicted isoelectric point of 4.82. The deduced Arabidopsis protein contains sequences showing significant similarities with all sequenced peptides derived from the wheat protein. The greatest sequence homology is for pep3 (see above). Peptides pep1 and pep2 show 39 and 38% similarity and 39 and 31% identity, respectively.

Enzymatic Properties of the Overexpressed Arabidopsis CPDase and Its Comparison with the Wheat Enzyme—The coding region of the Arabidopsis cDNA was subcloned in the pQE60-inducible expression vector to yield a fusion protein containing six histidine residues at the C terminus. The tagged protein was overproduced in E. coli and purified using the nickel-nitrilotriacetic acid resin (Fig. 3A). The protein was over 95% pure as judged by SDS-PAGE. The polyclonal antibodies, raised in chickens immunized with the overexpressed Arabidopsis CPDase, detected purified Arabidopsis protein on Western blots and also cross-reacted with the purified wheat CPDase (Fig. 3B). The antibodies did not detect the CPDase in crude cellular extracts prepared from the leaves of Arabidopsis, but the protein band likely to correspond to the CPDase could be detected after partial purification of the enzyme (data not shown). Hence, consistent with previous observations (18), the CPDase appears to be a nonabundant protein.

The Arabidopsis CPDase hydrolyzed all four nucleoside 2′,3′-cyclic phosphates to the corresponding 2′-phosphomonoesters as analyzed by cellulose TLC (Fig. 4). No 3′-phosphomonooester formation could be detected. Thus, like the wheat protein, the Arabidopsis enzyme has 2′,3′-cyclic nucleotide 3′-phosphodiesterase activity. Nucleoside 3′,5′-cyclic phosphates (3′,5′-cAMP and 3′,5′-cGMP), inositol 1,2-cyclic phosphate and guanosine 5′-phosphate, 2′,3′-cyclic phosphate (pG-3′-p), and also the cyclic phosphodiesterase in the 2′,3′-cyclic phosphate-terminated oligoribonucleotide (AAAAAAAAG AG-p), the asterisk indicates a position of the 32P label) were not hydrolyzed (data not shown); these compounds are also not substrates for the wheat enzyme (17, 18).

The wheat CPDase was previously shown to cleave the 1′,2′-cyclic phosphate linkage in the enzymatically produced 32P-labeled Appr>3′p to generate Appr-1′p (17). We have used chemically synthesized Appr>3′p (33) to demonstrate that the
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Arabidopsis enzyme has similar activity. The availability of larger amounts of Appr>p also allowed us to determine the kinetic parameters for its hydrolysis by the wheat and Arabidopsis CPDases (see below). Hydrolysis of Appr>p by the Arabidopsis or wheat enzyme yielded products of identical mobility in two different TLC systems (Figs. 5, A and B, lanes 2 and 3). Following additional treatment with CIP, in both cases the compound comigrated with ADP-ribose (Fig. 5B, lane 7). As expected, the RNase T2 hydrolysis product was also sensitive to CIP, yielding ADP-ribose (Fig. 5B, lane 8). Lane 1, untreated Appr>p; lane 6, ADP-ribose marker; A, cellulose TLC in solvent A; B, polyethyleneimine-cellulose in solvent B. Appr>p was not sensitive to CIP treatment as analyzed by TLC in both systems (not shown). Note: use of parentheses indicates that no standard is available for comparison and the identification of the molecule is based on other criteria (see text).

Fig. 5. Hydrolysis of Appr>p by the Arabidopsis and wheat CPDases and characterization of the hydrolysis product. Appr>p was treated with either wheat (lanes 2 and 4) or Arabidopsis (lanes 3 and 5) CPDase or with RNase T2 (lane 7). The samples in lanes 4, 5, and 8 were additionally treated with calf intestinal phosphatase (CIP). Lane 1, untreated Appr>p; lane 6, ADP-ribose marker; A, cellulose TLC in solvent A; B, polyethyleneimine-cellulose in solvent B. Appr>p was not sensitive to CIP treatment as analyzed by TLC in both systems (not shown). Note: use of parentheses indicates that no standard is available for comparison and the identification of the molecule is based on other criteria (see text).

Fig. 6. Hydrolysis of A>p by the Arabidopsis CPDase. A, dependence on enzyme concentration. Assays were incubated for 30 min at 30 °C. B, kinetics of hydrolysis at different temperatures. Assays (20 μl) contained 20 ng of CPDase. C, pH optimum. Assays (20 μl), containing 20 ng of CPDase, were incubated for 30 min at 30 °C. The following buffers were used: Mes-NaOH (■), Mops-NaOH (●), and Tris-HCl (□).

Arabidopsis enzyme has similar activity. The availability of larger amounts of Appr>p also allowed us to determine the kinetic parameters for its hydrolysis by the wheat and Arabidopsis CPDases (see below). Hydrolysis of Appr>p by the Arabidopsis or wheat enzyme yielded products of identical mobility in two different TLC systems (Figs. 5, A and B, lanes 2 and 3). Following additional treatment with CIP, in both cases the compound comigrated with ADP-ribose (Fig. 5, A and B, lanes 4 and 5). Treatment of Appr>p with RNase T2 yielded the Appr-2p isomer, which chromatographs more slowly than Appr-1p on the polyethyleneimine plate (Fig. 5B, lane 7; Ref. 17). As expected, the RNase T2 hydrolysis product was also sensitive to CIP, yielding ADP-ribose (Fig. 5B, lane 8).

Reaction requirements of the Arabidopsis CPDase were determined using A>p as a substrate (Fig. 6). Triton X-100 stimulated the enzyme activity, and 0.01% detergent was included in all reactions. The amount of substrate hydrolyzed was linearly dependent on enzyme concentration up to 20 ng/20 μl. The rates of A>p hydrolysis were similar at 20, 30, and 37 °C. The optimal activity was found at pH 7.0. These reaction requirements are similar to those of the wheat enzyme (18).

The effects of mono- and divalent cations (chloride salts) and EDTA, previously tested with the wheat enzyme, were determined. Addition of NaCl to 0.2 or 0.4 M inhibited A>p hydrolysis by 15 and 31%, respectively. Cu²⁺ and Zn²⁺ at 0.5 mM inhibited A>p hydrolysis by 93 and 87%, respectively. At 0.5 mM, Mn²⁺ slightly stimulated (by 5%) enzyme activity, whereas Ca²⁺, Mg²⁺, Co²⁺, Ni²⁺, and EDTA showed no effect at 0.5 mM and were only weakly inhibitory at 10 mM (data not shown). All these results are consistent with those obtained for the wheat CPDase (18).

The Kₚ and Vₘₐₓ values for the Arabidopsis CPDase were estimated for four nucleoside 2',3'-cyclic phosphates and Appr>p and compared with those obtained with the purified wheat enzyme (Table I). Kₚ values for individual nucleotide substrates were comparable for both enzymes (but see legend to Table I). However, the Vₘₐₓ values obtained with the Arabidopsis CPDase were 10–25 times lower than those measured with the wheat enzyme. It is possible that only a fraction of the Arabidopsis protein overexpressed in E. coli is enzymatically active. Alternatively, activity of the overexpressed enzyme may be lower due to the presence of the histidine tag or the absence of some essential modification of the protein. Based on relative Vₘₐₓ/Kₚ values, the specificity of the Arabidopsis CPDase toward the cyclic nucleotides is C>p:A>p;Appr>p:U>p;G>p=100:54:36:6:6; whereas for the wheat CPDase the specificity is A>p:C>p:Appr>p:U>p:G>p=100:43:39:9:7. Hence, both enzymes have similar substrate specificities with a preference for
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### Table I

| Substrate | Arabidopsis | Wheat |
|-----------|-------------|-------|
|           | $V_{\text{max}}$ | $K_m$ | $V_{\text{max}}/K_m$ | $V_{\text{max}}$ | $K_m$ | $V_{\text{max}}/K_m$ |
| A$\geq$p | 25 ± 2 | 1.34 ± 0.24 | 18.5 ± 3.7 | 597 ± 29 | 1.09 ± 0.13 | 547 ± 72 |
| G$\geq$p | 37 ± 4 | 1.68 ± 2.57 | 22.2 ± 0.4 | 917 ± 121 | 24.44 ± 3.77 | 22 ± 7 |
| C$\geq$p | 79 ± 7 | 2.38 ± 0.39 | 33.3 ± 6.2 | 695 ± 40 | 3.01 ± 0.27 | 231 ± 24 |
| U$\geq$p | 47 ± 3 | 17.67 ± 1.6 | 2.6 ± 0.3 | 762 ± 116 | 15.16 ± 2.65 | 50 ± 12 |
| Appr$\geq$p | 16 ± 2 | 1.35 ± 0.19 | 12.1 ± 2.2 | 261 ± 34 | 1.21 ± 0.23 | 216 ± 49 |

Expression of the CPDase Gene—The CPDase gene copy number was estimated in a Southern blot analysis. Only one hybridizing band was detected in DNA digests carried out with three different restriction enzymes, consistent with the existence of a single-copy gene (Fig. 7).

Expression of the CPDase gene in various tissues of Arabidopsis plants and in germinating seeds and young plantlets was analyzed by Northern blotting (Fig. 8A). The CPDase mRNA is relatively low in abundance, consistent with the results of Western analysis (see above). Roots contained slightly higher levels of mRNA than other tissues analyzed. Still higher levels of mRNA were found in 3-week-old Arabidopsis plantlets.

Expression of the CPDase gene was also investigated during re-initiation of mitotic activity in leaf strip cultures. When Arabidopsis leaf strips are incubated in a culture medium containing 1 mg/liter of the auxin analogue 2,4-dichlorophenoxyacetic acid, the cells start to proliferate very rapidly. $[^{3}H]$Thymidine incorporation and Northern blot hybridization, performed with the histone H4 cDNA as a probe, have shown that 48 h after starting the culture most of the cells are in the S phase (40) (see also Fig. 8B). Using this experimental system, the highest CPDase mRNA level was found at 72 h (Fig. 8B), a time when a high number of cell divisions is observed. 2 RNase A/T1 mapping, performed with the antisense RNA probe covering the coding region of the CPDase cDNA, also indicated that after 72-h incubation, the level of the CPDase mRNA is approximately five times higher than at the start of the culture (data not shown). The significance of this mRNA accumulation is not understood at present.

Cellular Localization of the CPDase Protein Studied by Indirect Immunofluorescence—The intracellular localization of the Arabidopsis CPDase was determined by an epitope tagging approach combined with indirect immunofluorescence. The coding sequence of the CPDase cDNA was cloned in a plant expression vector with an influenza hemagglutinin (flu) epitope fused in frame to the C terminus of the protein. The plasmid expressing the tagged protein was transfected into mesophyll protoplasts of N. plumaginifolia. The protoplasts were processed for immunofluorescence microscopy, using a rabbit anti-flu polyclonal antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. Indirect immunofluorescence showed that the protein is cytoplasmic (Fig. 9B). No staining was seen in untransfected protoplasts visible in the same field (A and B) or in mock-transfected protoplasts (data not shown). Transfected protoplasts (B), in which the nucleus was localized by staining with Hoechst 33258 (A), were also examined by confocal microscopy. The expressed tagged protein was clearly excluded from the nucleus and the chloroplasts (C). In control experiments, in which the flu tag was fused to the protein N-RBP43 known to be targeted to the nucleus, 3 immunofluorescence was predominantly nuclear (D–F). These results indicate that the CPDase is a cytoplasmic protein.

The Arabidopsis CPDase Does Not Share Significant Sequence Similarity with Other Known Phosphodiesterases—A search of current sequence data bases did not reveal any proteins having significant sequence similarity with the Arabidopsis CPDase. The consensus signature motifs of 3',5'-cyclic nucleotide phosphodiesterases (41, 42) are not present in the Arabidopsis enzyme. Cyclic phosphodiesterases from brain (43, 44), and tRNA ligase (45), two proteins having 3'-phosphodiesterase activity, also have no significant similarity with the plant protein.

### DISCUSSION

Removal of the 2'-phosphate group present in the products of tRNA splicing (see Introduction) is catalyzed by a specific phosphotransferase, first identified in yeast and vertebrates by Phizicky and co-workers (14, 15). In this reaction, the 2'-phosphate from tRNA is transferred to give a cyclic phosphate at the 1'-2' positions of NAD, yielding an unusual ADP-ribose derivative, Appr$\geq$p, and nicotinamide (16). Culver et al. (46)

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2 P. Genschik, A. Durr, and J. Fleck, unpublished results.

3 M. Hemmings-Miesenck, U. Klahre, and W. Filipowicz, unpublished results.
have recently cloned the gene encoding this enzyme in yeast and demonstrated that it is essential for viability. We describe here isolation of the Arabidopsis cDNA and properties of the encoded enzyme that metabolizes Appr-p to Appr-1-p. Phosphodiesterases that hydrolyze Appr-p to Appr-1-p have been previously characterized biochemically in both yeast and wheat (17). The purified wheat enzyme also hydrolyzes nucleoside 2',3'-cyclic phosphates in addition to Appr-p. The enzyme partially purified from yeast does not accept nucleoside 2',3'-cyclic phosphates as substrates, but otherwise, its properties are similar to that of the wheat protein (17).

Evidence presented here indicates that the protein encoded by the Arabidopsis cDNA and properties of the encoded enzyme that metabolizes Appr-p to Appr-1-p. Phosphodiesterases that hydrolyze Appr-p to Appr-1-p have been previously characterized biochemically in both yeast and wheat (17). The purified wheat enzyme also hydrolyzes nucleoside 2',3'-cyclic phosphates in addition to Appr-p. The enzyme partially purified from yeast does not accept nucleoside 2',3'-cyclic phosphates as substrates, but otherwise, its properties are similar to that of the wheat protein (17).

Evidence presented here indicates that the protein encoded by the Arabidopsis cDNA and the protein purified previously from wheat (17, 18) are equivalent. Both enzymes have identical pH and temperature dependence and are similarly affected by various inhibitors.

Southern analysis has indicated that CPDase is encoded by a single copy gene (Fig. 7). In previous experiments, only single enzymatic activity for the hydrolysis of Appr-p has been observed upon fractionation of yeast extracts, while chromatography of the wheat germ extract on DEAE-cellulose yielded two pools of CPDase, having similar specificity toward N-p and Appr-p substrates (17, 18). It is not known whether the two active pools represent products of two separate genes or two forms of the same protein.

With respect to substrate specificity, the plant CPDase studied in this work is clearly different from that of other known proteins possessing cyclic 3'-phosphodiesterase activity. The RNA ligase involved in tRNA splicing, extensively characterized for yeast and wheat (see Introduction), efficiently hydrolyzes terminal 2',3'-cyclic phosphates in oligoribonucleotides
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