Lipid Phosphate Phosphatases in Arabidopsis

REGULATION OF THE AtLPP1 GENE IN RESPONSE TO STRESS*

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An Arabidopsis thaliana gene (AtLPP1) was isolated on the basis that it was transiently induced by ionizing radiation. The putative AtLPP1 gene product showed homology to the yeast and mammalian lipid phosphate phosphatase enzymes and possessed a phosphatase signature sequence motif. Heterologous expression and biochemical characterization of the AtLPP1 gene in yeast showed that it encoded an enzyme (AtLpp1p) that exhibited both diacylglycerol pyrophosphate phosphatase and phosphatidate phosphatase activities. Kinetic analysis indicated that diacylglycerol pyrophosphate was the preferred substrate for AtLpp1p in vitro. A second Arabidopsis gene (AtLPP2) was identified based on sequence homology to AtLPP1 that was also heterologously expressed in yeast. The AtLpp2p enzyme also utilized diacylglycerol pyrophosphate and phosphatidate but with no preference for either substrate. The AtLpp1p and AtLpp2p enzymes showed differences in their apparent affinities for diacylglycerol pyrophosphate and phosphatidate as well as other enzymological properties. Northern blot analyses showed that the AtLPP1 gene was preferentially expressed in leaves and roots, whereas the AtLPP2 gene was expressed in all tissues examined. AtLPP1, but not AtLPP2, was regulated in response to various stress conditions. The AtLPP1 gene was transiently induced by genotoxic stress (gamma ray or UV-B) and elicitor treatments with mastoparan and harpin. The regulation of the AtLPP1 gene in response to stress was consistent with the hypothesis that its encoded lipid phosphate phosphatase enzyme may attenuate the signaling functions of phosphatidate and/or diacylglycerol pyrophosphate that form in response to stress in plants.

Phospholipids are major structural components of biological membranes in plants, animals, and yeast. These molecules also serve as a reservoir for several lipid-signaling molecules. PA and DG are intermediates in the biosynthesis of phospholipids and triacylglycerols (1, 2). PA is an intermediate for the synthesis of the major phospholipids, which include phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinerine, and phosphatidylglycerol. The dephosphorylation of PA to DG allows for the subsequent production of phosphatidylcholine and triacylglycerol, the major components of eukaryotic membranes and storage lipid, respectively (1, 2). In addition, PA, DG, and DGPP are products of lipid metabolism that serve as messengers in several cellular signal transduction pathways (3–8). The regulation of these cell-signaling pathways may be achieved, at least in part, by lipid phosphate phosphatase enzymes that catalyze the sequential conversion of DGPP to PA and of PA to DG (5, 7, 9).

Recent studies with a variety of plant systems have shown that PA and DGPP transiently accumulate after elicitor treatment or in response to stress (5, 10–12). These observations have suggested that these phospholipid molecules play a role in plant signal transduction (10, 11). Lipid second messengers in plant cell signaling are produced through the activation of phospholipase C and phospholipase D (5, 13). Enhanced levels of PA, necessary for maintaining housekeeping functions in lipid metabolism and transient induction of PA-related signaling events, are produced by phospholipase D-mediated hydrolysis of structural phospholipids and the combined actions of phospholipase C and DG kinase (5). Stimulation of phospholipase D activity is associated with plant stress responses, particularly in response to pathogens (13), wounding (14, 15), water deficit (11), hyperosmotic stress (12, 16), and the plant stress hormone abscisic acid (17, 18). Some of these effects may be mediated by G-protein-coupled stress receptors, because G-protein activators such as mastoparan activate PLD in the absence of stress (19, 20). Plants appear to have established a mechanism to attenuate PA action through its phosphorylation by PA kinase (21) to yield DGPP (22). DGPP is rapidly produced together with PA during G-protein activation, and high levels of DGPP are rapidly eliminated (20), which raised the question whether signaling functions should also be attributed to DGPP. The plant DGPP phosphatase activity described by Riedel et al. (23) preferentially dephosphorylates DGPP to PA but also dephosphorylates PA to DG, which makes possible the sequential conversion of DGPP to PA and DG. The function

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1 The abbreviations used are: PA, phosphatidate; DG, diacylglycerol; DGPP, diacylglycerol pyrophosphate; PCR, polymerase chain reaction; RT-PCR, reverse-transcribed PCR; DD-RT-PCR, differential display reverse-transcribed polymerase chain reaction; NEM, N-ethylmaleimide; bp, base pair; PLD, phospholipase D.
of DG remains unclear because a protein kinase C, the downstream effector target of DG in mammalian cells, has not been isolated from plants. So far, plant lipid phosphate phosphatase enzymes able to remove elevated levels of PA and DGPP during the early phase of stress response have not yet been described.

During the course of studies to examine global responses of genes to radiation stress in Arabidopsis thaliana, we identified a gene, which we called AtLPP1, whose deduced protein structure resembled lipid phosphate phosphatase enzymes from yeast and mammalian cells. Heterologous expression of the Arabidopsis AtLPP1 cDNA in the yeast Saccharomyces cerevisiae showed that the encoded protein was indeed a lipid phosphate phosphatase that exhibited DGPP phosphatase and PA phosphatase activities. A second gene (AtLPP2) was identified in the Arabidopsis data base that was also shown to encode a lipid phosphate phosphatase enzyme. AtLPP1, but not AtLPP2, was transiently induced by ionizing radiation, UV-B radiation, and elicitor treatments with mastoparan and harpin. The regulation of the AtLPP1 gene in response to stress was consistent with the hypothesis that its encoded protein was indeed a lipid phosphate phosphatase activity that may attenuate the signaling functions of PA and/or DGPP that form in response to plants.

EXPERIMENTAL PROCEDURES

Plant Materials and Treatment Conditions—A. thaliana cell suspensions were grown with constant shaking at 25 °C and continuous white light as described previously (24). A. thaliana (L) Heynh (Columbia ecotype) plants were cultivated in a growth chamber on a 14-h light/10-h dark cycle. Chemical or physical treatments of cell suspensions were applied at the beginning of the exponential phase of growth, which corresponded to 8–12% packed cell volume. Cells in culture medium were treated directly with mastoparan (10 μg/ml) and then collected at the indicated time intervals. Harpin (Eruicia amylovora) elicitation was carried out by mechanic infiltration of the elicitin solution (60 mg/ml) into leaves followed by collection of samples at the indicated time intervals. Gamma irradiation was performed with a 60Co gamma irradiation source at 37 °Ci/mg of tissue (1 Gy/min) at 1.5% NaCl (1/3 normal saline) (pH 7.4). Media was added to the culture medium of DH5α cells that carried plasmids. Media were supplemented with 2 (yeast) or 1.5% (bacteria) agar for growth on plates. Cell numbers were determined spectrophotometrically at an absorbance of 600 nm.

The diploid S. cerevisiae strain YPH500×YPH499 (33) was used as a genetic background for the disruption of the DPP1 and LPP1 genes and the heterologous expression of the Arabidopsis-soluble (32) and AtLPP2 cDNAs. To obtain the dpp1Δ lpp1Δ double mutant, the resident genes were successively disrupted by the insertion of selection marker genes allowing growth on appropriate selective media. Gene disruption was carried out according to the method of Baudin et al. (34). The DPP1 gene was disrupted by insertion of the S. cerevisiae HIS3 gene, and the LPP1 gene was disrupted by insertion of the Kluyveromyces lactis URA3 gene. Homologous dpp1Δ lpp1Δ mutant strains were selected for the disruption analysis. A dpp1Δ mutant was crossed with an lpp1Δ strain to form a diploid strain that was heterozygous for the DPP1 and LPP1 alleles. Putative dpp1Δ lpp1Δ double mutants were selected for their ability to grow on complete synthetic SD media lacking both histidine and uracil. The disruption of the chromosomal copies of the DPP1 and LPP1 genes was confirmed by PCR and Northern blot analysis. Strain 259 was one of the haploid dpp1Δ lpp1Δ mutants that was isolated and used for the experiments of the Arabidopsis cDNAs.

Preparation of Yeast Membranes, Protein Determination, Enzyme Assays, and Analysis of Kinetic Data—The total membrane fraction was isolated from exponential phase yeast cells as described by Toke et al. (35). Protein concentration was determined with the BCA microassay reagent (Pierce) using bovine serum albumin as the standard. MγCD- independent PA phosphatase activity was measured by following the formation of water-soluble [γ-32P]DGPP (5000–10,000 cpm/nmol). The reaction mixture contained 50 mM Tris maleate buffer (pH 6.5), 0.1 mM PA, 1 mM Triton X-100, 2 mM Na2EDTA, 10 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. DGPP-phosphatase activity was measured by the release of water-soluble [32P] from chloroform-soluble [β-32P]DGPP (5000–10,000 cpm/nmol) as described by Wu et al. (36). The reaction mixture contained 50 mM Tris maleate buffer (pH 5.0), 1 mM DGPP, 1 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. [32P]PA was synthesized from DG using E. coli DG kinase (37), and DGPP was synthesized from PA using purified Catharanthus roseus PA kinase as described previously (16). All enzyme assays were conducted at 30 °C in triplicate. The enzyme reactions were linear with time and protein concentration. Activities were expressed as pkatal (pmol of product/s) per mg of protein. Kinetic data were analyzed according to the Michaelis-Menten equation using SigmaPlot software with nonlinear regression algorithms.

RESULTS

Identification of Arabidopsis AtLPP1 as an Ionizing Radiation-induced Gene—By using differential display methodology (26), we identified an mRNA species that rapidly increased in response to severe radiation stress. The display gel shown in Fig. 1A revealed a 400-bp RT-PCR DNA fragment that accumulated in mRNA preparations from irradiated cells with respect to RNA preparations from unirradiated control cells. In Northern blot hybridization experiments, the purified DNA fragment hybridized to a single RNA species of about 1.3 kilobase pairs that rapidly and transiently accumulated in mRNA populations extracted from irradiated cell suspensions (Fig. 1B). The 8-fold elevation of hybridizing mRNA levels in response to radiation stress was restricted in time to 30 min.
 immediately following the radiation injury. After this initial induction, the mRNA levels rapidly returned to near basal levels. We isolated a 1350-bp cDNA from an Arabidopsis cDNA library (31), which shared complete sequence identity with the DD-RTPCR fragment at its 3' end. We also isolated the genomic DNA for this gene from an A. thaliana genomic DNA library. Comparison of the genomic DNA and cDNA revealed the existence of two putative initiator ATG codons, separated by the insertion of an intron (Fig. 2A). This gene is located on chromosome II (Arabidopsis Genome Sequencing Program). The open reading frame of 984 nucleotides on the cDNA codes for a putative 335-kDa integral membrane protein, containing six highly hydrophobic regions of sufficient length to be membrane spanning that are designated TM1–6 (Fig. 2D). The putative protein contains a 3-domain phosphatase sequence motif (38) that is conserved in several lipid phosphate phosphatase enzymes in S. cerevisiae and in mammalian cells (7, 38) (Fig. 2D). Because of this structural conservation, we named this gene AtLPP1 (A. thaliana lipid phosphate phosphatase) and its product AtLpp1p.

Identification of the Arabidopsis AtLPP2 and AtLPP3 Genes—A BLAST search in Arabidopsis data bases with the deduced AtLpp1p protein sequence revealed the existence of two additional proteins of similar size that possessed six putative transmembrane-spanning domains and the phosphatase signature motif (Fig. 2, E and F). We named the proteins AtLpp2p and AtLpp3p and their genes AtLPP2 and AtLPP3, respectively. The size of AtLPP2 cDNA (Arabidopsis EST clone 158j20-XP3’) is 1131 bp with an open reading frame of 873 nucleotides coding for a putative protein of 291 amino acids (Fig. 2B). The corresponding gene was identified by systematic sequencing of chromosome I (GenBank™ accession number AC007591). The gene encoding the putative AtLpp3p protein of 35 kDa (Fig. 2C) was found by sequencing of chromosome III BAC F16B3 genomic DNA (GenBank™ accession number AC021640). In striking difference to AtLPP1, the AtLPP2 and AtLPP3 genes are organized in similar exon/intron structures (Fig. 2, B and C). The AtLPP2 gene, deduced from sequence comparison of its cDNA with the genomic DNA, is interrupted by seven introns in the genome. The start of the open reading frame is near the 5' end of the second intron. The genomic AtLPP3 DNA downstream of the putative ATG initiator codon is organized into seven exons of similar size and separated by six introns of similar size, which may indicate that the two genes have evolved by a recent duplication from a common ancestor gene. This assumption is further strengthened by the extent of sequence conservation at the level of nucleic acids (AtLPP2/AtLPP3 47.8%, AtLPP1/AtLPP2 30.2%, and AtLPP1/AtLPP3 18.3%). The data shown in Fig. 3 indicate that the deduced amino acid sequence of the three putative lipid phosphate phosphatase proteins are highly conserved (AtLpp1p/AtLpp2p 54.1%, AtLpp1p/AtLpp3p 54.9%, and AtLpp2p/AtLpp3p 59.7%). This sequence conservation does not take into account the hypervariable C-terminal extensions and the AtLpp1p-specific extension at the N terminus, which brings about the question whether two AtLpp1p splice variants exist, and whether this extension has the function of a leader peptide triggering subcellular localization. However, we have not found a short version of the AtLPP1 mRNA. The lipid phosphatase motif (denoted by asterisks) is located in virtually invariant domains of the three Arabidopsis lipid phosphate phosphatase proteins. These domains are juxtaposed to the proposed membrane-spanning regions in a way that the conserved domains probably form an important three-dimensional component for the proteins (7, 38). This model has been confirmed by the mutational analysis of the S. cerevisiae DPP1 and mouse LPP1 genes, demonstrating that single amino acid changes in each phosphatase consensus domain (large asterisks in Fig. 3) are sufficient to produce severe losses of lipid phosphate phosphatase activity (39, 40). These amino acid residues are conserved in the Arabidopsis proteins.

We constructed a phylogenetic tree of members of the lipid phosphate phosphatase protein family including the Arabidopsis AtLpp1p, AtLpp2p, and AtLpp3p proteins (Fig. 4). Although it was not possible to determine the exact location of the tree root, nevertheless the phylogenetic tree was clearly split into two major branches whose separation is a remote event. The Arabidopsis proteins were grouped with the S. cerevisiae Dpp1p and Lpp1p proteins in one main branch, whereas all animal lipid phosphate phosphatase proteins were grouped in the other main branch, with the exception of the putative Drosophila melanogaster Lpp-like protein Q9VND6 (Fig. 4). The recent multiplication of the A. thaliana Lpp-like proteins leads to the formation of two distinct subgroups, one specified by the AtLpp1p protein and the other composed of the AtLpp2p and AtLpp3p proteins. Collectively, these analyses indicated that the Arabidopsis proteins were lipid phosphate phosphatase enzymes. The analysis of one member of each subgroup, namely the AtLPP1 and AtLPP2 genes and their encoded proteins, is described below. The analysis of AtLPP3 gene and its product will be described elsewhere.

Functional Analysis of the Arabidopsis AtLPP1- and AtLPP2-encoded Proteins Expressed in S. cerevisiae—The lipid
phosphate phosphatase enzymes from *S. cerevisiae* (35, 41) and mammalian cells (7) exhibit activity toward a variety of substrates. Since AtLPP1 gene was identified on the basis of its induction by radiation stress, and in light of the well-documented transient accumulation of PA and DGPP in plants in response to stress, it was of particular interest to compare these two substrates. We examined the hypothesis that the AtLpp1p and AtLpp2p enzymes from *Arabidopsis* exhibited both PA phosphatase and DGPP phosphatase activities. The AtLPP1 and AtLPP2 cDNAs were expressed in a *S. cerevisiae* lpp1Ddpp1D mutant that we have constructed (see “Experimental Procedures”) as a genetic background to examine their gene-enzyme relationships. As described previously (35), the lpp1Ddpp1D mutant that we have obtained did not possess DGPP phosphatase activity, and the PA phosphatase activity was reduced to about 1% of the activity measured in the control wild-type strain. Thus, this yeast mutant was an appropriate system to examine the functional expression of the plant enzymes. The Arabidopsis cDNAs were expressed at similar levels in exponential phase cells (Fig. 5A). Moreover, these cDNAs directed the expression of both PA phosphatase and DGPP phosphatase activities in the membrane fraction of exponential phase cells. Under standard assay conditions, with saturating concentrations of substrates, the DGPP phosphatase activity of the AtLpp1p enzyme was 3.4-fold greater when compared with its PA phosphatase activity (Table I). On the other hand, the PA phosphatase and DGPP phosphatase activities of the AtLpp2p enzyme were not significantly different (Table I). The lipid phosphate phosphatase activities of *S. cerevisiae* (9) and mammalian cells (7) are generally described as being NEM-insensitive and Mg2+-independent. We examined the effects of these molecules on the PA phosphatase activity of the Arabidopsis AtLpp1p and AtLpp2p enzymes. The effects of NEM on the PA phosphatase activities of the AtLpp1p and AtLpp2p enzymes are shown in Fig. 5B. The addition of NEM to the assay system for the AtLpp1p enzyme resulted in a dose-dependent inhibition of PA phosphatase activity with an IC50 value of 10 μM, whereas NEM did not affect the PA phosphatase activity of the AtLpp2p enzyme. The PA phosphatase activity of each enzyme was indeed independent of a Mg2+ ion requirement (Fig. 5C). However, the addition of Mg2+ ions to the assay system for the AtLpp2p enzyme, but not the AtLpp1p enzyme, resulted in a transient increase (2.5-fold) in PA phosphatase activity (Fig. 5C). Relatively high concentrations of...
Asterisks of different sizes indicate the amino acid residues that have been used for site-directed mutagenesis of the three domains of the phosphatase sequence motif. mouse Arabidopsis thaliana phosphate phosphatase-like proteins identified in the Drosophila melanogaster, C. elegans, and Schizosaccharomyces pombe genomes were used for comparison. The tree was built applying the Neighbor Joining Method to PAM distances computed on 88 reliably aligned sites (49). SwissProt accession numbers (in brackets) designate all protein sequences. The length of horizontal branches is such that the evolutionary distance between two proteins is proportional to the total length of the horizontal branches that connect them. Bootstrap values are shown at the nodes.

Mg$^{2+}$ ions (e.g. 20 mM) resulted in a small decrease in the PA phosphatase activities of both the AtLpp1p and AtLpp2p enzymes (Fig. 5C).

The dependence of the AtLpp1p and AtLpp2p enzymes on PA and DGPP was examined using Triton X-100/phospholipid mixed micelles according to the surface dilution kinetic model (42). Accordingly, the dependence of the enzymes on their substrates was measured as a function of surface concentration (in mol %) as opposed to a molar concentration (42). Under the conditions used here, the activities of the AtLpp1p and AtLpp2p enzymes were essentially independent of the molar concentration of substrates. The AtLpp1p (Fig. 6A) and AtLpp2p (Fig. 6B) enzymes exhibited saturation kinetics with respect to PA. The $K_{m}$ value for PA of the AtLpp2p enzyme (0.04 mol %) was 30-fold lower than that of the AtLpp1p enzyme (1.26 mol %). This suggested that the AtLpp2p enzyme had a greater affinity for PA when compared with the AtLpp1p enzyme. Owing to the fact that these enzyme preparations were not homogeneous, we could not make comparisons of their relative turnover numbers using PA as a substrate. The dependence of the AtLpp1p enzyme activity on the surface concentration of DGPP is shown in Fig. 6C. The enzyme displayed saturation kinetics toward DGPP with a $K_{m}$ value of 0.25 mol %. For the AtLpp1p enzyme, the $K_{m}$ value for DGPP was 5-fold lower than the $K_{m}$ value for PA. Thus, based on relative $K_{m}$ values, DGPP was a better substrate for the enzyme when compared with PA. We were unable to obtain kinetic data for the AtLpp2p enzyme using DGPP as a substrate. The dose-response curve for DGPP was already saturated at the lowest surface concentration that was possible to use in these experiments. Collectively, these data showed that the Arabidopsis AtLPP1 and AtLPP2 genes encoded lipid phosphate phosphatase enzymes with distinct enzymological properties.

Expression of the AtLPP1 and AtLPP2 mRNAs in Arabidopsis Tissues—To examine the expression profile of the Arabidopsis AtLPP1 and AtLPP2 genes, we compared the distribution of their transcripts in the major plant organs. Northern blot analyses of total RNA preparations revealed that the AtLPP1 mRNA was strongly expressed in leaves, moderately expressed in roots, and weakly expressed in floral hamps and flower buds (Fig. 7A). The AtLPP1 mRNA was not detected in adult flowers and seedpods (Fig. 7A). To analyze simultaneously the levels of AtLPP1 and AtLPP2 mRNAs in the different RNA preparations, we employed the quantitative RT-PCR method (28). We confirmed the preferential expression of AtLPP1 in leaves, whereas the AtLPP2 gene was expressed at detectable levels in all plant organs analyzed (Fig. 7B).

Differential Regulation of the Arabidopsis AtLPP1 and AtLPP2 Genes in Response to Stress—We examined the steady state levels of AtLPP1 and AtLPP2 mRNAs in the response to different extracellular stimuli. Because we identified the AtLPP1 gene based on its induction in response to radiation injury, we analyzed the levels of the AtLPP1 and AtLPP2...
mRNAs after exposure to two different genotoxic stresses, namely gamma rays and UV-B radiation. Both cause DNA damage but by different mechanisms (reviewed in Ref. 43). Ionizing radiation preferentially induces double strand breaks on genomic DNA, whereas UV-B radiation induces thymidine dimers. Exposure of cell suspensions to ionizing radiation resulted in a rapid and transient accumulation (8-fold) of AtLPP1 mRNA (Fig. 8A). Maximum induction occurred at the 15-min time point following treatment (Fig. 8A). These data were consistent with the Northern blot data shown in Fig. 1B. There was a linear correlation between the level of induction and the applied dose up to 100 Gy (data not shown). The application of UV-B radiation caused a transient induction of AtLPP1 mRNA, with maximum accumulation (7−fold) at 5 J/cm² of UV-B (Fig. 8B). The lower levels of AtLPP1 mRNA expression with higher doses of UV-B radiation correlated with the appearance of increasing amounts of cellular degradation that was observable on whole leaves (data not shown). In contrast to the AtLPP1 gene, the expression levels of the AtLPP2 mRNA were not significantly affected by gamma rays (Fig. 8A) or by UV-B radiation (Fig. 8B).

Munnik and co-workers (16, 19, 20, 22) have shown that the levels of PA and DGPP transiently accumulate in the green alga *Chlamydomonas moewusii* in response to mastoparan, a G-protein elicitor. In the light of these observations, we examined the effects of mastoparan on the expression of the AtLPP1 and AtLPP2 genes using *Arabidopsis* cells. In control experiments, mastoparan elicited changes in the lipid content of *Arabidopsis* cells similar to that shown by Munnik et al. (19). Mastoparan elicited a rapid and transient accumulation (3-fold) of AtLPP1 mRNA within 30 min after treatment (Fig. 8C).

The levels of AtLPP1 mRNA were not significantly affected by mastoparan over the same period (Fig. 8C).

As a second elicitor, we have used harpin, a 44-kDa bacterial protein that elicits hypersensitive response in plant cells (44). In contrast to mastoparan, the hypersensitive response is induced by oxidative stress but is independent of PLD activity (45). We examined the effects of harpin on the expression of AtLPP1 and AtLPP2. Infiltration of *Arabidopsis* leaves with harpin resulted in a transient accumulation (8-fold) of AtLPP1 mRNA in leaves, whereas AtLPP2 mRNA levels remain unchanged (Fig. 8D). Interestingly, AtLPP1 mRNA induction was also observed in the leaves adjacent to those that were infiltrated with harpin (data not shown). Collectively, these experiments showed that the *Arabidopsis AtLPP1* gene was induced in response to a variety of signaling events including DNA damage, G-protein activation, and oxidative stress.

**DISCUSSION**

Several lipid phosphate phosphatase enzymes have been described from *S. cerevisiae* and mammalian cells (7, 9). These enzymes utilize a variety of lipid phosphate substrates in vitro including PA, DGPP, lyso-PA, ceramide 1-phosphate, and sphingosine 1-phosphate (7, 9). It has been proposed that the function of these enzymes is to attenuate the signaling functions that are associated with their substrates (3, 4, 7, 9). A number of studies have shown that PA and DGPP accumulate in plants in a transient manner in response to various forms of stress (5, 10−12). In this work we identified a new *Arabidopsis* lipid phosphate phosphatase gene family. The AtLPP1 gene was identified on the basis that it was induced in response to ionizing radiation. The other members of the family (AtLPP2 and AtLPP3) were identified based on their homology with AtLPP1. The deduced proteins encoded by these genes showed structural similarities to the lipid phosphate phosphatase enzymes of yeast and mammalian cells, including the presence of a 3-domain phosphatase sequence motif (38). Heterologous expression of the *Arabidopsis AtLPP1* and AtLPP2 cDNAs in a *S. cerevisiae* dpp1Δ lpp1Δ mutant showed that the plant genes did indeed encode lipid phosphate phosphatase enzymes.

The *Arabidopsis* AtLpp1p and AtLpp2p enzymes utilized PA and DGPP as substrates, similar to the lipid phosphate phosphatase enzymes from yeast (9) and mammalian cells (7). AtLpp1p and AtLpp2p showed differences in their enzymological properties with respect to effects by Mg²⁺ ions and NEM. Kinetic data showed that the AtLpp1p enzyme preferred DGPP to PA as a substrate, whereas the AtLpp2p enzyme did not show a preference for either substrate. Based on relative $K_m$ values, the AtLpp2p enzyme exhibited a greater affinity for its.

**TABLE I**

| Enzyme      | Specific activity | PA phosphatase | DGPP phosphatase |
|-------------|-------------------|----------------|------------------|
|             | Activity (pkatal/mg) |                |                  |
| AtLpp1p     | 82.6 ± 2.6        | 282 ± 16       |
| AtLpp2p     | 25 ± 2.0          | 20 ± 4         |

*Fig. 5. Expression of the *Arabidopsis* AtLPP1 and AtLPP2 genes in a *S. cerevisiae* dpp1Δ lpp1Δ mutant. A, Northern blot analysis was performed with RNA samples (5 μg) extracted from the *S. cerevisiae* dpp1Δ lpp1Δ mutant bearing plasmids pCM185 (lanes 1 and 3), pCM185AtLPP1 (lane 2), and pCM185AtLPP2 (lane 4). *S. cerevisiae* dpp1Δ lpp1Δ mutant cells bearing plasmids pCM185AtLPP1 and pCM185AtLPP2 were harvested in the exponential phase of growth, and the membrane fraction was isolated. PA phosphatase activity in the membrane fractions from these cells was measured in the absence and presence of the indicated concentrations of Mg²⁺ ions (B) and the indicated concentrations of NEM (C).*
lipid phosphate substrates when compared with the AtLpp1p enzyme. The substrate preference and apparent affinities of the Arabidopsis AtLpp1p and AtLpp2p enzymes for PA and DGPP were generally similar to the DPP1-encoded (36, 41) and LPP1-encoded (35, 45) lipid phosphate phosphatase enzymes of S. cerevisiae, respectively. Indeed, the phylogenetic tree of the Arabidopsis and yeast lipid phosphate phosphatase enzymes support these relationships.

The Arabidopsis AtLpp1p and AtLpp2p enzymes appear to have different functions in cell physiology. Expression of the AtLPP1 gene was mainly found in leaves and roots, whereas the AtLPP2 gene was expressed in all plant organs examined. The expression of AtLPP1 gene was regulated in response to stress, whereas the AtLPP2 gene was constitutively expressed. Treatment of cells with both ionizing radiation and UV-B radiation, both of which induce DNA damage, resulted in a transient increase in AtLPP1 gene expression. Elicitation with mastoparan, which induces G-protein-mediated activation of phospholipase D and subsequently accumulation of PA and DGPP (19, 22), caused the transient induction of the AtLPP1 gene. Elicitation with harpin, which induces oxidative stress but not PLD activity, also caused the transient induction of AtLPP1. Taken together, these observations suggest that the AtLpp1p enzyme plays a general role in cellular responses to stress. The AtLpp2p enzyme may play a general "housekeeping role" in lipid metabolism; however, additional studies will be needed to establish this. The S. cerevisiae DPP1-encoded lipid phosphate phosphatase also plays a role in cellular responses to stress. The DPP1 gene is induced by inositol supplementation (47), the stress condition of stationary growth phase (47), and by zinc deprivation (48).

The rapid synthesis of PA and its subsequent conversion to DGPP by PA kinase are newly discovered common signaling events that take place in plants after elicitor treatment (10), wounding (14), dehydration (11), and hyperosmotic stress (12, 16). The increases in PA and DGPP levels measured in the resurrection plant Craterostigma plantagineum early after onset of dehydration correlates with the induction of PLD2 gene expression, a global increase in phospholipase D activity, and the combined actions of phospholipase C and DG kinase (11). These observations demonstrate a link between stress, phospholipase activities, and the transient accumulation of PA and DGPP, which may activate pathways leading to stress adaptation. Interestingly, dehydration causes an increase in expression of the PLD genes in leaves and roots, the organs where the AtLPP1 gene was expressed the most. This further suggests a link between the phospholipases and lipid phosphate phosphatases, one creating the signal and the other attenuating the signal. From the responsiveness of the AtLPP1 gene to a variety of stress

![Fig. 6. Kinetic analysis of the PA phosphatase and DGPP phosphatase activities of the Arabidopsis AtLpp1p and AtLpp2p proteins expressed in S. cerevisiae membranes. The PA phosphatase activity of the AtLpp1p (A) and AtLpp2p (B) proteins expressed in the membranes of the S. cerevisiae dpp1Δ lpp1Δ mutant was measured as a function of the surface concentration of PA. The molar concentration of PA was held constant at 0.1 mM. The DGPP phosphatase activity of the AtLpp1p protein (C) was measured as a function of the surface concentration of DGPP. The molar concentration of DGPP was held constant at 0.1 mM. The insets shown in each of the panels are double-reciprocal plots of the data.](image)

![Fig. 7. Expression of the Arabidopsis AtLPP1 and AtLPP2 mRNAs in various plant organs. A, samples (20 μg) of total RNA prepared from roots, leaves, stems, flower buds, mature flowers, and siliques were transferred to nylon filter and hybridized with a mixture of radiolabeled AtLPP1 cDNA and 25 S cDNA. The 25 S RNA was visualized after a 6-h exposure, whereas the AtLPP1 mRNA was visualized after a 10-day exposure. B, the same RNA preparations were used for the analysis of AtLPP1, AtLPP2, and Act8 mRNA by quantitative RT-PCR with gene-specific primers.](image)
conditions, we cannot rule out that other bioactive plant phospholipids may be substrates for the AtLPP1p enzyme.

Before PA and DGPP can be generally accepted as second messengers in plants, specific downstream targets and responses must be identified, and a rapid and direct down-regulation mechanism should exist to remove signaling when appropriate. PA has been shown to activate deflagellation in Chlamydomonas spp. (19), inhibit α-amylase synthesis in barley aleurone cells (17), and activate stomatal closure via inhibition of the inward K+ channel in fava bean leaves (18). The formation of DGPP, barely detectable in non-stimulated cells and strictly coupled to increases in PA, has been initially interpreted as inactivation of the PA signal (5, 22). Recent observations, demonstrating that DGPP is able to activate a mitogen-activated protein kinase pathway in macrophages (8), and the fact that DGPP is synthesized when PA levels decline (20), suggest a signaling function for DGPP.

In summary, these studies advance the understanding of the lipid phosphate phosphatase protein family in higher plants and the regulation of the AtLPP1 gene in response to stress. To understand better the individual roles of the Arabidopsis lipid phosphate phosphatase protein family, ongoing research in our laboratory focuses on the isolation and characterization of AtLPP1 and AtLPP2 knockout mutant plants to determine their roles in lipid metabolism and cellular responses to stress.

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