Intracortical projections of the RSPagl may be divided into three streams. One major stream extends rostrally to end in the anterior cingulate, caudal prelimbic, and ventrolateral orbital areas, and our double-injection results suggest that the first two areas project back to the RSPagl. This is of interest here because, in the rat, anterior cingulate and to a lesser extent caudal prelimbic areas are thought to be associated with the frontal eye fields, along with the adjacent secondary motor areas, mainly because they project to several brainstem regions involved in oculomotor control, including the superior colliculus (2, 26). Anterior cingulate as well as secondary motor areas again receive inputs from the LP (this work) and mediodorsal nucleus (17), and the anterior cingulate area receives inputs from the LD (20) and REr. Interestingly, both cortical areas receive dense inputs from the same site in AM that projects to the RSPagl (16) (that is, perhaps AMv; see also ref. 17).

Other major cortical projections of the RSPagl extend caudally and medially to pre- and parasubicular areas of the hippocampal formation and to other parts of the RSP that also project to the hippocampal formation (27). Note that in Fig. 3 information from the rostromedial hypothalamus can potentially reach the intrahippocampal circuit (28) in a number of ways. The most direct way involves projections to the REr/AMv, which in turn project directly to the intrahippocampal circuit as well as to the RSPagl, which then projects to the intrahippocampal circuit. Furthermore, the intrahippocampal circuit projects back to the REr/AMv, as well as to the medial zone of the hypothalamus, particularly by way of the septal region (see ref. 7).

Thus, it would appear that the flow of information descending from the RSPagl to midbrain motor regions could be modified continuously by feedforward and feedback information from the intrahippocampal circuit. In this context, it is important to recall that the intrahippocampal circuit plays a critical role in short-term episodic or declarative memory. The physiological characterization of place and navigation neurons in the intrahippocampal circuit (29) serves to emphasize a potential role of the circuitry outlined in Fig. 3 in the foraging behavior associated with certain classes of goal-oriented or motivated behavior.

Anterior and posterior cingulate areas have been implicated in several aspects of spatial memory (30). In particular, it has been postulated that posterior cingulate areas, along with posterior parietal association areas, are involved in evaluating the significance of identified and spatially located stimuli, as well as in the elaboration of spatially accurate movements in relation to topographic cues (30, 31). We did not observe any significant connections of the RSPagl with what is usually referred to as posterior parietal cortex in the rat. However, the third RSPagl cortical projection ends in caudoventral parts of the temporal association area that project back to RSPagl. More medial parts of the RSP in the rat have been reported to share bidirectional connections with established visual areas (in what are referred to as area 17 and subdivisions of area 18) (32, 33).

The model presented in Fig. 3 suggests that information arriving at the rostral medial hypothalamus from pheromonal cortex (in the corticomedial amygdala) is then sent to midbrain motor regions by descending pathways as well as to parts of the cerebral cortex involved in regulating eye and head movements by ascending pathways through the REr/AMv. Interestingly, the hippocampal formation participates in conceptually similar circuitry involving the caudal medial hypothalamus (mammillary body), which gives rise to the mammillothalamic and mammillotegmental tracts, and isocortical regions project to the basal ganglia, which in turn generate descending projections to midbrain motor regions and ascending projections to secondary motor cortical regions by way of the ventral anterior thalamic nucleus. Significantly, the REr, anterior group, ventral anterior nucleus together occupy the rostral end of thalamus, while secondary motor areas and the cingulate g also lie adjacent to one another in the cerebral cortex. summary, this model predicts that the REr/AMv to RSP pathway conducts pheromonal information to a polymo cortical–midbrain pathway eliciting attentional sponses involved in the procurement phase of ap motivated or goal-oriented behavior (34).

This work was supported by National Institutes of Hea n Gra RO1 NS16686.

1. Barnes, G. R. (1993) *Prog. Neurobiol.* **41,** 435–472.
2. Cooper, J. D. & Phillipson, O. T. (1993) *Prog. Neurobiol.* 4 209–279.
3. Segovia, S. & Guillamón, A. (1993) *Brain Res. Rev.* **18,** 51–74.
4. Swanson, L. W. (1987) in *Handbook of Chemical Neuroanatomy* eds. Björklund, A., Hökfelt, T. & Swanson, L. W. (Elsevier Amsterdam), Vol. 5, pp. 1–124.
5. Canteras, N. S. & Swanson, L. W. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10089–10093.
6. Canteras, N. S., Simerly, R. & Swanson, L. W. (1994) *J. Comp. Neurol.* **348,** 41–79.
7. Risold, P. Y., Canteras, N. S. & Swanson, L. W. (1994) *J. Comp. Neurol.* **348,** 1–40.
8. Krieg, W. J. S. (1946) *J. Comp. Neurol.* **84,** 221–324.
9. Rose, M. (1928) *J. Psychol. Neurol.* **40,** 1–51.
10. Swanson, L. W. (1992) *Brain Maps: Structure of the Rat Brain* (Elsevier, Amsterdam).
11. Herkenham, M. (1978) *J. Comp. Neurol.* **177,** 589–610.
12. Gerfen, C. R. & Sawchenko, P. E. (1984) *Brain Res.* **290,** 219–238.
13. Schmued, L. C. & Fallon, J. H. (1986) *Brain Res.* **377,** 147–154.
14. Wouterlood, F. G., Saldana, E. & Witter, M. P. (1990) *J. Comp. Neurol.* **296,** 179–203.
15. Halpern, M. (1987) *Annu. Rev. Neurosci.* **10,** 325–362.
16. Shibata, H. (1993) *J. Comp. Neurol.* **330,** 533–542.
17. Thompson, S. M. & Robertson, R. T. (1987) *J. Comp. Neurol.* **265,** 175–188.
18. Thompson, S. M. & Robertson, R. T. (1987) *J. Comp. Neurol.* **265,** 189–202.
19. Vogt, B. A., Pandya, D. N. & Rosene, D. L. (1987) *J. Comp. Neurol.* **262,** 256–270.
20. Van Groen, T. & Wyss, J. M. (1992) *J. Comp. Neurol.* **324,** 427–448.
21. Van Groen, T. & Wyss, J. M. (1992) *J. Comp. Neurol.* **315,** 200–216.
22. Sparks, D. L. (1988) *Brain Behav. Evol.* **31,** 49–56.
23. Jones, E. G. (1985) *The Thalamus* (Plenum, New York).
24. May, J. G., Keller, E. L. & Suzuki, D. A. (1988) *J. Neurophysiol.* **59,** 952–977.
25. Bandler, R., Carrive, P. & Depaulis, A. (1991) in *The Midbrain Periaqueductal Gray Matter: Functional, Anatomical, and Neurochemical Organization*, eds. Depaulis, A. & Bandler, R. (Plenum, New York), pp. 1–8.
26. Leichnetz, G. R. (1987) *Exp. Brain Res.* **68,** 255–264.
27. Van Groen, T. & Wyss, J. M. (1990) *J. Comp. Neurol.* **302,** 593–606.
28. Swanson, L. W., Köhler, C. & Björklund, A. (1987) in *Handbook of Chemical Neuroanatomy*, eds. Björklund, A., Hökfelt, T. & Swanson, L. W. (Elsevier, Amsterdam), Vol. 5, pp. 125–277.
29. Muller, R. U., Kubic, J. L., Bostock, E. M., Taube, J. S. & Quirk, G. J. (1991) in *Brain and Space*, ed. Paillard, G. (Oxford Univ. Press, Oxford), pp. 296–333.
30. Sutherland, R. J., Whitshaw, I. Q. & Kolb, B. (1988) *J. Neurosci.* **8,** 1863–1872.
31. Pandya, D. N. & Yeterian, E. H. (1984) *Neuropsychologia* **22,** 109–122.
32. Paperna, T. & Malach, R. (1991) *J. Comp. Neurol.* **308,** 432–456.
33. Vogt, B. A. & Miller, M. W. (1983) *J. Comp. Neurol.* **216,** 192–210.
34. Swanson, L. W. & Mogenson, G. J. (1981) *Brain Res. Rev.* **3,** 1–34.

92, pp. 3903–3907, April 1995
Biology

A gene encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*

TAKA HI HIRAYAMA*, CHIKARA OHTO†, TSUYOSHI MIZOGUCHI*, AND KAZUO SHINOZAKI*‡

*Laboratory of Plant Molecular Biology, Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), 3-1-1 Koyadai, Tsukuba, Ibaraki, 305, Japan; and †Bio Research Laboratory, Research Division, Toyota Motor Corporation, 1 Toyota-cho, Toyota, Aichi, 471, Japan

Communicated by Clarence A. Ryan, Washington State University, Pullman, WA, January 13, 1995

ABSTRACT **A cDNA corresponding to a putative phosphatidylinositol-specific phospholipase C (PI-PLC) in the higher plant *Arabidopsis thaliana* was cloned by use of the polymerase chain reaction. The cDNA, designated cAtPLC1, encodes a putative polypeptide of 561 aa with a calculated molecular mass of 64 kDa. The putative product includes so-called X and Y domains found in all PI-PLCs identified to date. In mammalian cells, there are three types of PI-PLC, PLC-β, -γ, and -δ. The overall structure of the putative AtPLC1 protein is most similar to that of PLC-δ, although the AtPLC1 protein is much smaller than PLCs from other organisms. The recombinant AtPLC1 protein synthesized in *Escherichia coli* was able to hydrolyze phosphatidylinositol 4,5-bisphosphate and this activity was completely dependent on Ca^{2+}, as observed also for mammalian PI-PLCs. These results suggest that the AtPLC1 gene encodes a genuine PI-PLC of a higher plant. Northern blot analysis showed that the AtPLC1 gene is expressed at very low levels in the plant under normal conditions but is induced to a significant extent under various environmental stresses, such as dehydration, salinity, and low temperature. These observations suggest that AtPLC1 might be involved in the signal-transduction pathways of environmental stresses and that an increase in the level of AtPLC1 might amplify the signal, in a manner that contributes to the adaptation of the plant to these stresses.**

In animal cells, phosphatidylinositol-specific phospholipase C (PI-PLC) plays a key role in various signal-transduction pathways. Extracellular stimuli such as hormones and growth factors activate PI-PLCs. PI-PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) and generates two second messengers, inositol 1,4,5-trisphosphate (IP_3), which induces the release of Ca^{2+} from intracellular stores, and 1,2-diacylglycerol (DG), which activates protein kinase C (1–3). These initial events in the transmembrane signal-transduction process are referred to as the PI turnover system. PI turnover in higher organisms is involved in a variety of cellular processes, including fertilization, development, the growth and differentiation of cells, and phototransduction, as well as in neural behavior, such as memory (1). In addition, PI, PIP_2, and DG seem to be involved in the regulation of the organization of the cytoskeleton (4–6). In animal cells, PI-PLCs have been classified into three subgroups, β, γ, and δ. Cloning and structural analysis of cDNAs that correspond to the three types of PI-PLC have shown that only two domains, X and Y, are conserved among the three isoforms. The X and Y domains are both essential and sufficient for PI-PLC activity (7). The nonconserved regions of each particular isoform are thought to be involved in the regulation of its respective activity. The PLC-β1 isoform is regulated by $G_{\alpha q}$ protein (8). By contrast, PLC-γ isoforms interact with certain receptor tyrosine kinases through Src homology 2 (SH2) domains located between the X and Y domains and are activated by tyrosine phosphorylation (9, 10). The mechanism(s) of the activation of PLC-δ is not well characterized.

Recently, genes or cDNAs related to PI-PLC have been cloned from *Saccharomyces cerevisiae* (PLC1; refs. 11–13) and *Dictyostelium discoideum* (DdPLC; ref. 14). The overall structures of PLC1 and DdPLC are similar to that of the PLC-δ isoforms of animals. In yeast, PLC1 is required for cell growth and functions in nutritional and stress-related responses. By contrast, mutants with disruption of the DdPLC gene grow and undergo development normally even though no PI-PLC activity is detectable (15). The function of PLC of *Dictyostelium* is unknown.

In higher plants, Ca^{2+} ions play important roles as second messengers in various signal-transduction pathways. Marked increases in intracellular Ca^{2+} concentration have been observed upon stimulation by wind, touch, cold shock, or fungal elicitors (16). Many genes for calmodulins and Ca^{2+}-dependent protein kinases, which appear to act as signal transducers, have been cloned from plants (17). In addition, considerable research on the role of PI turnover in plant signal-transduction pathways has been reported (reviewed in ref. 18). For example, the rapid breakdown of inositol phospholipid in plant cells was observed upon treatment of *Catharanthus roseus* with an auxin (19). IP_3 stimulates the release of Ca^{2+} from the vacuoles of plant cells (20). In spite of remarkable progress, there is still no direct evidence for a role of PI-PLC in the PI turnover system that has been observed to be operative during plant responses to various environmental stimuli, because the gene for PI-PLC has not been isolated and its role has not been identified. Here we describe the cloning and the characterization of a cDNA, cAtPLC1, that corresponds to a putative PI-PLC of the higher plant *Arabidopsis thaliana*. We report that the AtPLC1 gene is induced by environmental stress.§

MATERIALS AND METHODS

Plant Materials and Treatments. *A. thaliana* (Columbia ecotype) was used in this study. Growth and stress conditions were reported previously (21).

Primers and Conditions for PCR. Primer 1 was 5′-CTGCAGTYNSCNAYNTCYYKYTGNGT-3′, and primer 2 was 5′-GGATCCWCNTWCNTWYGCNRYNCAYTG-GATHAA-3′. The PCR mixture contained template cDNA (2.5 ng/μl) synthesized from poly(A)$^+$ RNAs from normally grown plants, 0.2 mM each dNTP, 10 pmol of both primers, and 4 units of AmpliTaq DNA polymerase (Takara Shuzo,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DG, 1,2-diacylglycerol; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; SH2 and SH3, Src homology 2 and 3.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. D38544).

Kyoto). The mixture was subjected to 15 cycles of 94°C for 90 sec, 48°C for 2 min, and 72°C for 3 min and then to 15 cycles of 94°C for 30 sec, 55°C for 3 min, and 70°C for 1 min. Amplified DNA fragments were subcloned into pBluescript II SK(+) (Toyobo, Osaka) and sequenced. The nucleotide sequence of both ends of the PI-PLC-like PCR fragment corresponded to primer 1.

Cloning and Sequencing of cDNA. A cDNA library constructed with mRNA from 1-hr-dehydrated *Arabidopsis* plants by using λZAPII (22) was screened with the PLC-like PCR fragment as probe. The plasmids were excised from λ clones by induction with helper phage (23). DNA sequences were determined by a dye-primer sequencing method on a DNA sequencer (model 373A; Perkin–Elmer). GENETYX software (Software Development, Tokyo) was used for analysis of DNA and amino acid sequences.

Northern Blot Hybridization. Samples (30 μg) of total RNA were denatured and fractionated by electrophoresis in a 1 M formaldehyde/1.25% agarose gel and then transferred to a Hybond-N membrane (Amersham). Hybridization conditions were described previously (24). The signals were visualized by autoradiography or with a Fujix BAS-2000 bioimage analyzer (Fuji). rRNAs blotted on the membrane were visualized by staining with methylene blue.

Expression of a Glutathione *S*-Transferase (GST)–AtPLC1 Fusion Protein. The truncated cDNA (clone 1; see *Results*) was inserted in the *Bam*HI site of pGEX-2T (Pharmacia), to yield pNH496. Cells of *Escherichia coli* strain JM109 (25) transformed with pNH496 or pGEX-2T were grown in L broth (26) at 37°C. When OD_{600} reached about 0.4, isopropyl β-D-thiogalactopyranoside (IPTG, 0.5 mM) was added and incubation was continued for 12 hr at 15°C. The cells were harvested, washed, and suspended in extraction buffer [50 mM Tris · HCl, pH 7.2/150 mM NaCl/10% (vol/vol) glycerol/1.4 mM 2-mercaptoethanol/1 mM dithiothreitol]. The procedures for the purification of the fusion protein and digestion with thrombin have been described (27). Protein concentration was determined with a Coomassie dye-based protein assay kit (Bio-Rad).

Assay of PI-PLC Activity. Two assays for PI-PLC activity, to monitor the hydrolysis of PIP_2 and of PI, respectively, were carried out. To assay PIP_2 hydrolysis, the reaction mixture (40 μl) contained 50 mM Mes buffer (pH 6.8), 250 μM KCl, 0.05 bovine serum albumin, 125 μM PIP_2, 2 μCi of [^{3}H]PIP_2 (5. Ci/mmol; DuPont/New England Nuclear; 1 Ci = 37 GBq), μM phosphatidylethanolamine (Sigma), and the protein sam ple. Ca^{2+} concentration in the reaction mixture was kept belo 0.1 mM with a Ca^{2+}/EGTA buffer system (various amounts Ca^{2+} plus 1 mM EGTA). PI hydrolysis was assayed in the sam way as PIP_2 hydrolysis activity except that 125 μM PI and 2 μC of [^{3}H]PI (13.0 Ci/mmol; DuPont/New England Nuclear were used instead of PIP_2. The mixtures were subjected t sonication before use. The reaction was carried out at 37°C fo 10 min and was stopped by the addition of 2 ml of chloroform/ methanol (2:1, vol/vol). Inositol phosphates were extracted with 0.5 ml of 1 M HCl. The upper, aqueous phase (0.8 ml) wa removed and its radioactivity was determined.

RESULTS

Cloning and Structural Analysis of cAtPLC1 cDNA. We performed several series of PCR experiments using *Arabidopsis* cDNAs as templates and a variety of primers in an effort to clone plant homologs for signal transducers, such as protein kinases and transcription factors. We found a PCR fragment whose deduced amino acid sequence resembled that of the Y domain of PI-PLCs (Fig. 1). Using this PI-PLC-like PCR fragment as probe, we screened a cDNA library constructed from mRNAs isolated from normally grown plants, but we failed to obtain any positive clones. Then we screened a cDNA library that had been prepared from *Arabidopsis* plants after dehydration stress for 1 hr (22), and we obtained numerous positive clones (1 positive clone per ≈20,000 plaques). Fifteen positive clones were selected and analyzed further. Physical maps revealed that all of these cDNA clones were derived from the same gene. We chose the longest cDNA clone, no. 17 (2.0 kb), and the second longest, no. 1 (1.9 kb), for further analysis. The nucleotide sequences of these clones were identical except that clone 1 was about 80 bp shorter in the 5′ region than clone 17. We found the nucleotide sequence identical to that of the PI-PLC-like PCR fragment in both these clones (nt 1195–1427 in Fig. 1). Clone 17 encodes a putative polypeptide of 561 aa (Fig. 1). There is an in-frame stop codon 24 bp upstream of the putative initiation codon. In addition, a single band of 2.2-kb

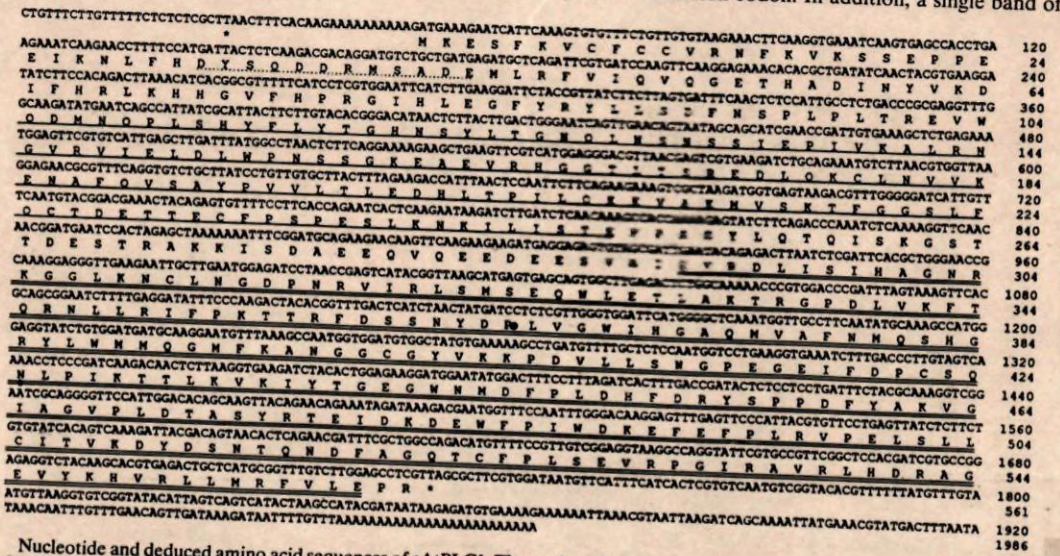

FIG. 1. Nucleotide and deduced amino acid sequences of cAtPLC1. The nucleotide sequence of cDNA clone 17 includes a putative coding region, and 5′ and 3′ noncoding regions. The amino acid sequence of the putative coding region is shown beneath the nucleotide sequence. The putative E–F hand motif, X domain, and Y domain are underlined with a dotted line, a single line, and a double line, respectively. Stars indicate the in-frame stop codons. The nucleotide sequence from 1195 to 1427 corresponds to the PI-PLC-like PCR fragment (see text).

mRNA was detected by Northern blotting with clone 17 or the PI-PLC-like PCR fragment as probe (see below). We concluded that clone 17 encodes a full-length polypeptide.

The amino acid sequence of the predicted protein contains the X and Y domains that have been reported to be conserved in various PI-PLCs (Figs. 1 and 2). We concluded that clone 17 encodes a PI-PLC homolog in *Arabidopsis*, and we designated the cDNA clone cAtPLC1. The extent of amino acid sequence identity between the deduced AtPLC1 protein and PI-PLCs of other species is 34–42% for the X domain and 19–33% for the Y domain. The overall structure of AtPLC1 appeared to be most closely related to that of δ-type PI-PLCs in several respects. For example, in AtPLC1, the Y domain is located in the C-terminal region as it is in δ-type PI-PLCs. AtPLC1 lacks the SH2 and SH3 motifs that are found in γ-type PI-PLCs. In addition, AtPLC1 has an E–F hand motif in the N-terminal region as do δ-type PI-PLCs. However, the predicted molecular mass of AtPLC1 (64.3 kDa) is lower than that of δ-type PI-PLCs (≈85 kDa) because AtPLC1 does not have the N-terminal nonconserved region that is found in δ-type PI-PLCs, as well as in β- and γ-PI-PLCs. AtPLC1 appears to be the smallest PI-PLC identified so far.

To examine the existence of additional AtPLC1-like genes in the genome of *Arabidopsis*, we performed genomic Southern blot analysis using the full-length cAtPLC1 cDNA as probe. Under high-stringency conditions, hybridized bands were detected in each restriction digest. However, additional bands were detected under low-stringency conditions (data not shown). These results indicate that additional PI-PLC-like genes might exist in the genome of *Arabidopsis*.

A Recombinant AtPLC1 Fusion Protein Possesses PI-PLC Activity. To confirm that the AtPLC1 gene encodes a genuine PI-PLC, we analyzed the biochemical properties of a recombinant AtPLC1 protein expressed in *E. coli*. The cDNA from clone 1 was introduced in pGEX-2T, an expression vector for GST fusion protein, to construct the chimeric plasmid pNH496. In pNH496 DNA, the gene for GST was fused in frame to cAtPLC1 at nt 76 (see Fig. 1) via an adapter sequence (5′-GATCCGGGTACCATGG-3′). A GST–AtPLC1 fusion protein of 88 kDa accumulated in *E. coli* cells carrying pNH496 DNA when IPTG was added to the medium. Although almost all of the fusion protein was found in the insoluble fraction, a small amount was detected in the soluble fraction and was purified on a column of glutathione-Sepharose 4B (Fig. 3, lanes 4 and 5). The AtPLC1 moiety of the fusion protein could be separated from GST by digestion with thrombin (Fig. 3, lane 6).

The crude extract from *E. coli* cells that harbored the pGEX-2T vector only and that had been grown under the inducing conditions (plus IPTG) had no PIP_2-hydrolyzing activity (Fig. 4, bar 1), whereas the crude extract from *E. coli* cells that harbored pNH496 grown under the same conditions had weak PIP_2-hydrolyzing activity (bar 3). Further, both the GST–AtPLC1 fusion protein and the product of digestion with thrombin (rAtPLC1) exhibited high PIP_2-hydrolyzing activity (bars 4 and 5), whereas the GST protein purified on the column of glutathione-Sepharose 4B had no such activity (bar 2). These results suggest that the PI-PLC activity that we detected was due to the AtPLC1 moiety of the fusion protein.

Since an important feature of PI-PLC is the Ca^{2+} dependence of its reaction, we examined the Ca^{2+} dependence of the PIP_2-hydrolyzing activity of AtPLC1, using the thrombin-digested recombinant protein, rAtPLC1. The PIP_2 hydrolysis of rAtPLC1 was completely Ca^{2+}-dependent (Fig. 5). Another important feature of PI-PLCs is that the optimum concentration of Ca^{2+} for PI hydrolysis is higher than that for PIP_2 hydrolysis. rAtPLC1 hydrolyzed PIP_2 more rapidly at a Ca^{2+} concentration of 1 μM, which has been reported to be the concentration in native plant cells, than at higher concentrations (>0.1 mM). By contrast, the PI-hydrolyzing activity was

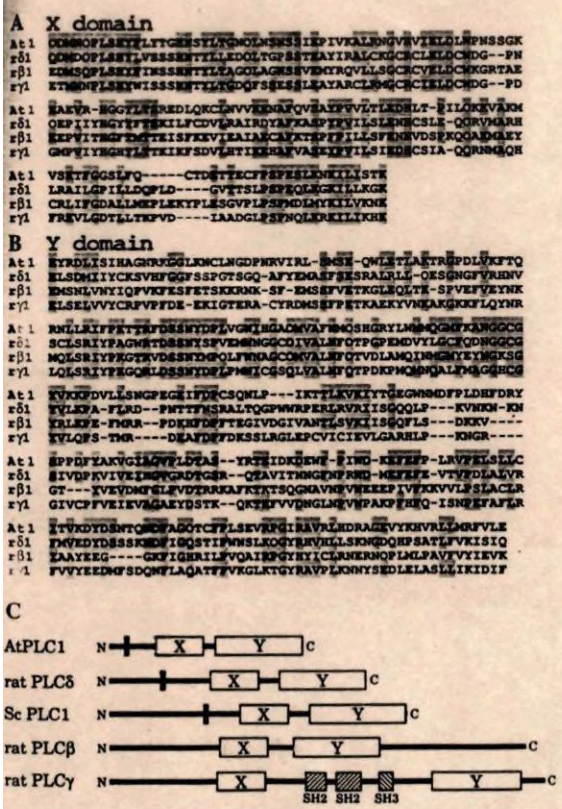

FIG. 2. (*A* and *B*) Alignment of the putative AtPLC1 protein with known PI-PLCs. The putative X domain (*A*) and Y domain (*B*) of AtPLC1 (At1) are aligned with those of rat PLC-δ1 (rδ1) (28), PLC-β1 (rβ1) (28), and PLC-γ1 (rγ1) (29). The amino acid residues that are identical in AtPLC1 and other PI-PLCs are shaded. (*C*) Comparison of a schematic model of the structure of AtPLC1 with those of various PI-PLCs. Sc PLC1, *S. cerevisiae* PLC1 (11). Two conserved regions, the X and Y domains, are marked by open boxes. The putative E-F hand motifs (narrow striped boxes) and Src homology 2 and 3 (SH2 and SH3) domains (hatched boxes) are emphasized.

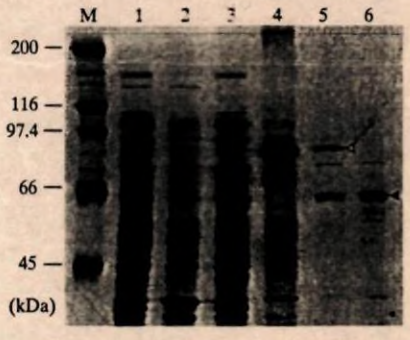

FIG. 3. Expression and partial purification of the recombinant GST–AtPLC1 protein from *E. coli* cells. The soluble fraction (lanes 1 and 3) and the insoluble fraction (lanes 2 and 4) of cellular extracts prepared from IPTG-treated (lanes 3 and 4) and untreated (lanes 1 and 2) *E. coli* cells that harbored pNH496 were analyzed by SDS/PAGE. The GST-AtPLC1 fusion protein was partially purified on a column of glutathione-Sepharose 4B column from the soluble fraction (lane 5, open triangle). The purified fusion protein was digested with thrombin (lane 6; solid triangle). Lane M, marker proteins with molecular masses in kilodaltons.

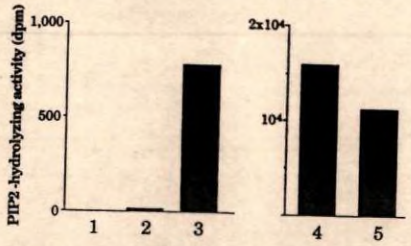

FIG. 4. PIP_2-hydrolyzing activity of the recombinant AtPLC1 protein assayed in a crude extract (10 μg) from IPTG-treated *E. coli* cells that harbored pGEX-2T (bar 1), purified GST protein (2 μg) after chromatography on a column of glutathione-Sepharose 4B (bar 2), a crude extract (10 μg) from IPTG-treated *E. coli* cells that harbored pNH496 (bar 3), the purified GST–AtPLC1 fusion protein (1.4 μg) after chromatography on a column of glutathione-Sepharose 4B (bar 4), and the thrombin-generated rAtPLC1 protein (1.4 μg) (bar 5).

low at lower concentrations of Ca^{2+} and increased gradually as the concentration of Ca^{2+} was increased. These results indicate that AtPLC1 has characteristics similar to those reported for mammalian PI-PLCs with respect not only to structure but also to enzymatic properties. The rate of hydrolysis of PIP_2 by rAtPLC1 was 0.2 μM/min per mg of protein at 33 μM Ca^{2+}. Taking the purity of rAtPLC1 into consideration, we find that the PIP_2-hydrolyzing activity of this protein is similar to that of purified mammalian PI-PLCs. The PI-hydrolyzing activity was about 2.2 nM/min per mg of protein at 5 mM Ca^{2+}.

Expression of the AtPLC1 Gene Is Induced by Dehydration, High Salt, and Low Temperature. The cAtPLC1 cDNA clone was obtained from a cDNA library that had been constructed with mRNA from 1-hr-dehydrated plants, whereas no positive clones were obtained from the cDNA library prepared from normally grown plants. This observation suggests that the AtPLC1 gene is expressed at a very low level under normal growth conditions and is induced under environmental stress, such as dehydration. To examine this hypothesis, we performed Northern blot analysis of total RNA prepared from plants that had been subjected to high-salt stress, dehydration, low temperature, heat, or low osmotic stress for 10 hr. A very small amount of the 2.2-kb AtPLC1 mRNA was detected under normal growth conditions (Fig. 6*A*). By contrast, AtPLC1 mRNA accumulated to a significant level under high-salt, dehydration, and cold stress conditions. Heat stress had no effect. Quantitative analysis revealed that the level of AtPLC1 mRNA under high-salt stress was >10 times that under normal growth conditions. Treatment with water slightly induced the expression of the AtPLC1 gene, suggesting that low osmotic stress might also function as a trigger. The plant hormone

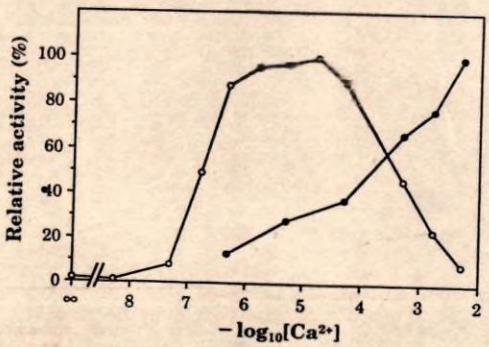

FIG. 5. Ca^{2+} dependence of PIP_2- and PI-hydrolyzing activities (○ and ●, respectively) of the thrombin-generated recombinant rAtPLC1 protein (1.4 μg).

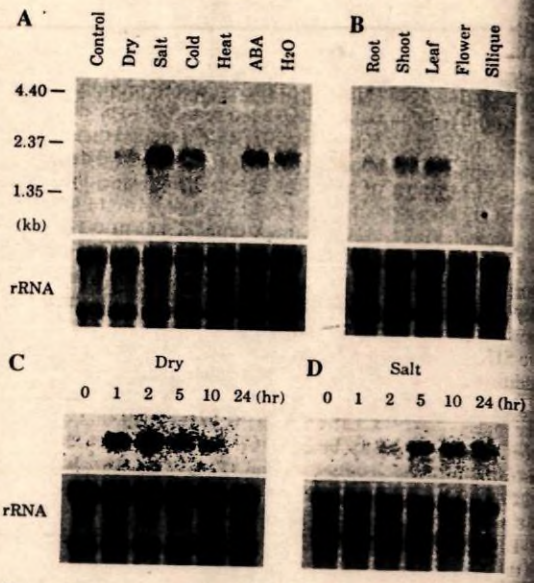

FIG. 6. Expression of the AtPLC1 gene in response to various stresses and in various organs. (*A*) Induction of AtPLC1 by dehydration (Dry), high salinity (Salt), low temperature (Cold), heat stress (Heat), abscisic acid treatment (ABA), and low osmotic stress (H_2O) (see *Materials and Methods*). (*B*) Organ-specific expression of the AtPLC1 gene. (*C* and *D*) Time course of accumulation of AtPLC1 mRNA during dehydration (*C*) and high-salinity stress (*D*).

abscisic acid, which functions in the induction of many genes under dehydration and high-salt conditions (30), also induced the accumulation of AtPLC1 mRNA. Under dehydration conditions, AtPLC1 mRNA increased rapidly within 1 hr, reached a maximum at 2 hr, and then decreased gradually (Fig. 6*C*). By contrast, the accumulation of AtPLC1 mRNA began at about 2 hr after the start of salt treatment and reached a maximum at 5–10 hr (Fig. 6*D*). The discrepancy between the patterns of induction under dehydration stress and under salt stress might be due to the differences between the effects of these stresses upon the plants. Organ-specific expression of the AtPLC1 gene was examined under normal growth conditions. AtPLC1 mRNA was detected in vegetative organs, such as stems, leaves, and roots, whereas it was hardly detected at all in reproductive organs, such as siliques (Fig. 6*B*). These results indicate that AtPLC1 functions only in vegetative organs, in particular under stressful conditions.

DISCUSSION

In plants, as in animals, Ca^{2+} ions play crucial roles in a variety of signal-transduction pathways in response to extracellular stimuli. In mammalian cells, the release of Ca^{2+} ions is controlled mainly by the PI turnover system, in which PI-PLC plays a key role. Therefore, it has been proposed that the PI turnover system and homologs of PI-PLC also function in plant cells. Many reports suggestive of PI turnover systems in plants and their involvement in signal-transduction pathways of extracellular stimuli have been published. However, no direct evidence was presented for the existence of PI-PLCs in plant cells. In this study, we demonstrate that the higher plant *Arabidopsis* has a gene, which we designated AtPLC1, that is significantly similar to genes for PI-PLCs. The recombinant AtPLC1 protein expressed in *E. coli* had considerable PI-PLC activity and its catalytic properties were similar to those of PI-PLCs from other organisms (Figs. 4 and 5). These results strongly suggest that AtPLC1 represents a PI-PLC in *Arabi-*

opsis. The overall structure of AtPLC1 is most closely related to that of the δ isotype among the three isotypes of PI-PLC. However, AtPLC1 has a unique structure with a quite short, nonconserved N-terminal region, whereas PI-PLC-β, -γ, and -δ have long N-terminal regions. AtPLC1 is the smallest protein among PI-PLCs identified to date and it is composed, for the most part, of just the E–F hand and the X and Y domains. This structural difference might reflect differences in regulatory properties or subcellular localization.

PI-PLCs hydrolyze PIP_2 to produce IP_3 and DG. In animal cells, IP_3 binds to an IP_3 receptor and opens a Ca^{2+} channel, localized on the membrane of the endoplasmic reticulum, to release Ca^{2+} into the cytoplasm. The other product, DG, activates protein kinase C. Alexandre *et al.* (20) proposed that a Ca^{2+} channel coupled with an IP_3 receptor might exist on the vacuolar membrane of plant cells. However, no evidence has been presented for the presence of protein kinase C, the target protein kinase of DG. None the less, our finding of a homolog of PI-PLC, AtPLC1, in *Arabidopsis* supports the possibility of the existence of protein kinase C homologs in higher plants.

The expression of the AtPLC1 gene was induced under environmental stresses such as dehydration, high salinity, and low temperature, results that suggest that AtPLC1 might function in signal-transduction pathways under these stress conditions. It has been reported that these stresses transiently increase free cytosolic Ca^{2+} in plant cells (31). The release of free Ca^{2+} under these stresses is thought to be induced via the PI turnover system in plants. Dehydration and abscisic acid induce changes in turgor of stomatal guard cells with resultant closure of stomata. IP_3 functions in the regulation of cytosolic levels of Ca^{2+} in guard cells. Ca^{2+} released by IP_3 into the cytoplasm is implicated in the regulation of guard-cell turgor via modulation of K^+ channels (32, 33). In the unicellular green alga *Dunaliella salina*, the levels of PI metabolites change rapidly in response to osmotic stress (34). In addition, a yeast PI-PLC, PLC1, is presumed to be involved in responses to environmental changes, such as changes in nutrient conditions (11, 13). Therefore, the PI-PLC homolog AtPLC1 can be presumed to function in signal transduction in higher plants under environmental stress. The AtPLC1 gene is induced by dehydration and salinity stress, a result that suggests the accumulation of the AtPLC protein in plant cells that have been exposed to these stresses. The accumulation of AtPLC1 probably contributes to the enhancement of the efficiency of signal transduction under stress conditions and increases the ability of plant cells to adapt to these conditions. Analysis of transgenic plants in which the AtPLC1 gene is overexpressed or downregulated should give us some ideas as to the function of AtPLC1.

This work was supported in part by the Special Coordination Fund of the Science and Technology Agency of the Japanese Government and by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan to K.S. Further support was provided by a Grant for Biodesign Research Programs from The Institute of Physical and Chemical Research to T.H.

1. Berridge, M. J. (1993) *Nature (London)* **361,** 315–325.
2. Nishizuka, Y. (1992) *Science* **258,** 607–614.
3. Majerus, P. W., Ross, T. S., Cunningham, T. W., Caldwell, K. K., Jefferson, A. B. & Bansal, V. S. (1990) *Cell* **63,** 459–465.
4. Goldschmidt-Clermont, P. J., Machesky, L. M., Baldassare, J. J. & Pollard, T. D. (1990) *Science* **247,** 1575–1578.
5. Payrastre, B., Van Bergen en Henegouwen, P. M. P., Breton, M., den Hartigh, J. C., Plantavid, M., Verkleij, A. J. & Boonstra, J. (1991) *J. Cell Biol.* **115,** 121–128.
6. Shariff, A. & Luna, E. J. (1992) *Science* **256,** 245–247.
7. Emori, Y., Homma, Y., Sorimachi, H., Kawasaki, H., Nakanishi, O., Suzuki, K. & Takenawa, T. (1989) *J. Biol. Chem.* **264,** 21885–21890.
8. Wu, D., Jiang, H., Katz, A. & Simon, M. I. (1993) *J. Biol. Chem.* **268,** 3704–3709.
9. Nishibe, S., Wahl, M. I., Hernández-Sotomayor, S. M. T., Tonks, N. K., Rhee, S. G. & Carpenter, G. (1990) *Science* **250,** 1253–1256.
10. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C. & Pawson, T. (1991) *Science* **252,** 668–674.
11. Yoko-o, T., Matsui, Y., Yagisawa, H., Nojima, H., Uno, I. & Toh-e, A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 1804–1808.
12. Payne, W. E. & Fitzgerald-Hayes, M. (1993) *Mol. Cell. Biol.* **13,** 4351–4364.
13. Flick, J. S. & Thorner, J. (1993) *Mol. Cell. Biol.* **13,** 5861–5876.
14. Drayer, A. L. & Van Haastert, P. J. M. (1992) *J. Biol. Chem.* **267,** 18387–18392.
15. Drayer, A. L., Van der Kaay, J., Mayr, G. W. & Van Haastert, P. J. M. (1994) *EMBO J.* **13,** 1601–1609.
16. Knight, M. R., Campbell, A. K., Smith, S. M. & Trewavas, A. J. (1991) *Nature (London)* **352,** 524–526.
17. Trewavas, A. & Gilroy, S. (1991) *Trends Genet.* **7,** 356–361.
18. Coté, G. G. & Crain, R. C. (1993) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44,** 333–356.
19. Ettlinger, C. & Lehle, L. (1988) *Nature (London)* **331,** 176–178.
20. Alexandre, J., Lassalles, J. P. & Kado, R. T. (1990) *Nature (London)* **343,** 567–572.
21. Yamaguchi-Shinozaki, K. & Shinozaki, K. (1994) *Plant Cell* **6,** 251–264.
22. Kiyosue, T., Yamaguchi-Shinozaki, K. & Shinozaki, K. (1994) *Plant Mol. Biol.* **25,** 791–798.
23. Short, J. M., Fernandez, J. M., Sorge, J. A. & Huse, W. D. (1988) *Nucleic Acids Res.* **16,** 7583–7600.
24. Hirayama, T. & Oka, A. (1992) *Plant Mol. Biol.* **20,** 653–662.
25. Yanisch-Perron, C., Vieira, J. & Messing, J. (1985) *Gene* **33,** 103–109.
26. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
27. Smith, D. B. & Johnson, K. S. (1988) *Gene* **67,** 31–40.
28. Suh, P.-G., Ryu, S. H., Moon, K. H., Suh, H. W. & Rhee, S. G. (1988) *Cell* **54,** 161–169.
29. Suh, P.-G., Ryu, S. H., Moon, K. H., Suh, H. W. & Rhee, S. G. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5419–5423.
30. Bray, E. A. (1993) *Plant Physiol.* **103,** 1035–1040.
31. Läuchli, A. (1990) in *Calcium in Plant Growth and Development*, eds. Leonard, R. T. & Hepler, P. K. (Am. Soc. Plant Physiol., Rockville, MD), pp. 26–35.
32. Blatt, M. R., Thiel, G. & Trentham, D. R. (1990) *Nature (London)* **346,** 766–769.
33. Gilroy, S., Read, N. D. & Trewavas, A. J. (1990) *Nature (London)* **346,** 769–771.
34. Einspahr, K. J., Peeler, T. C. & Thompson, G. A., Jr. (1988) *J. Biol. Chem.* **263,** 5775–5779.