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Molecular and Enzymatic Characterization of Three Phosphoinositide-Specific Phospholipase C Isoforms from Potato[1]

Joachim Kopka, Christophe Pical, Julie E. Gray, and Bernd Müller-Röber*

Max-Planck-Institut für Molekulare Pflanzenphysiologie, Karl-Liebknecht-Strasse 25, Haus 20, D-14476 Golm/Potsdam, Germany (J.K., B.M.-R.); and Department of Molecular Biology and Biotechnology, University of Sheffield, P.O. Box 594, Sheffield, S10 2UH, United Kingdom (C.P., J.E.G.)

Many cellular responses to stimulation of cell-surface receptors by extracellular signals are transmitted across the plasma membrane by hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), which is cleaved into diacylglycerol and inositol-1,4,5-trisphosphate by phosphoinositide-specific phospholipase C (PI-PLC). We present structural, biochemical, and RNA expression data for three distinct PI-PLC isoforms, StPLC1, StPLC2, and StPLC3, which were cloned from a guard cell-enriched tissue preparation of potato (*Solanum tuberosum*) leaves. All three enzymes contain the catalytic X and Y domains, as well as C_2-like domains also present in all PI-PLCs. Analysis of the reaction products obtained from PIP_2 hydrolysis unequivocally identified these enzymes as genuine PI-PLC isoforms. Recombinant StPLCs showed an optimal PIP_2-hydrolyzing activity at 10 μM Ca^{2+} and were inhibited by Al^{3+} in equimolar amounts. In contrast to PI-PLC activity in plant plasma membranes, however, recombinant enzymes could not be activated by Mg^{2+}. All three *stplc* genes are expressed in various tissues of potato, including leaves, flowers, tubers, and roots, and are affected by drought stress in a gene-specific manner.

In animal cells many cellular responses to stimulation of cell-surface receptors by extracellular signals are transmitted across the plasma membrane by hydrolysis of PIP_2, which is catalyzed by PI-PLC. This reaction generates the two secondary messengers: IP_3, a compound soluble in the cytosol that triggers transient increases of the cytosolic Ca^{2+} level, and DG, a lipid that stays within the plasma membrane and activates PKC (Berridge, 1993). In plants an enzyme with PKC activity has been partially purified from *Brassica campestris* (Nanmori et al., 1994), and evidence has been obtained for the participation of PKC in the elicitor-induced defense response in potato (*Solanum tuberosum*; Subramaniam et al., 1997). DG has been shown to induce both ion pumping in patch-clamped guard cell protoplasts and the opening of intact stomata (Lee and Assmann, 1991). Furthermore, it is now well established that IP_3-mediated Ca^{2+} release occurs in plant cells (Drøbak, 1992, 1993; Coté and Crain, 1993, 1994).

Increasing evidence strongly suggests that IP_3-mediated signal transduction is functional in guard cells. The phytohormone ABA, which is involved in multiple stress responses, also triggers stomatal closure and inhibits stomatal opening. Extracellular application of ABA or injection of this phytohormone into guard cells of *Commelina communis* and *Vicia faba* results in transient increases in cytosolic Ca^{2+} (McAinsh et al., 1990; Schroeder and Hagiwara, 1990). In addition, Lee et al. (1996) demonstrated that ABA induces an increase in cytosolic IP_3 concentration within seconds after the extracellular application of ABA to *V. faba* guard cell protoplasts, which is accompanied by a rapid turnover of inositol phospholipids. Photolysis of "caged" IP_3 within guard cells of *C. communis* (Gilroy et al., 1990) and *V. faba* (Blatt et al., 1992) causes an increase of cytosolic Ca^{2+} and a subsequent stomata-closing reaction. Finally, increasing concentrations of both Ca^{2+} (Schroeder and Hagiwara, 1989) and IP_3 (Blatt, 1992) reduce inward-rectifying K^+ currents in plasma membranes of *V. faba* guard cells. Inward-rectifying K^+ channels are believed to be involved in driving the opening of guard cells.

Current evidence supports a model in which the transduction of the ABA signal in guard cells utilizes changes in cytosolic concentrations of IP_3 and Ca^{2+} and ultimately leads to the inactivation of inward-rectifying K^+ channels and stomatal closure. However, in *C. communis* ABA can induce stomatal closure by a second, Ca^{2+}-independent pathway (Allan et al., 1994). In addition, both cyclic ADP-Rib and IP_3 independently are able to trigger Ca^{2+} release from plant vacuoles (Allen et al., 1995). Therefore, it appears that guard cells operate different, and in part complementary, signal transduction pathways that all lead to stomatal closure.

To investigate the role of the IP_3-mediated pathway in guard cells from a new angle we decided to clone cDNAs of PI-PLCs and other enzymes of the phosphoinositide-signaling pathway expressed in epidermal fragments and

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* Corresponding author; e-mail mueller@mpimp-golm.mpg.de; fax 49–331–977–2301.

Abbreviations: CDS, CDP-DG synthase; DG, diacylglycerol; GST, glutathione *S*-transferase; IP_3, inositol-1,4,5-trisphosphate; PA, phosphatidic acid; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate; PI-PLC, phosphoinositide-specific phospholipase C; PKC, protein kinase C.

possibly guard cells of potato leaves. Epidermal fragments were used as the source for the isolation of guard cell mRNA, even though it is possible to isolate mRNA from guard cell protoplasts (Nakajima et al., 1995) or from single plant cells (Karrer et al., 1995). These latter methods, however, exhibit inherent analytical problems, as discussed previously (Kopka et al., 1997b).

Phosphoinositide metabolism in higher plants has recently been approached by molecular cloning of cDNAs encoding multiple PI-PLC isoforms from *Arabidopsis thaliana* (Hirayama et al., 1995, 1997; Yamamoto et al., 1995) and *Glycine max* (Shi et al., 1995; GenBank accession nos. U41473, U41474, and U41475). Our laboratory has recently cloned CDS from potato and Arabidopsis (Kopka et al., 1997a). An eye-specific CDS in *Drosophila melanogaster* has been shown to be essential for IP_3-mediated signal transduction during light perception (Wu et al., 1995), where the enzyme is required for the regeneration of PIP_2 from DG (one of the reaction products of PI-PLC activity) via PA, and thus participates in PI-PLC-signaling cascades. Another enzyme involved in the resynthesis of PIs, DG kinase, has also been cloned recently from higher plants (*A. thaliana*; Katagiri et al., 1996).

To obtain a probe for heterologous screening of potato PI-PLC cDNAs we initially cloned a novel PI-PLC homolog from *A. thaliana* ecotype C24, which was identified by a search of the plant database of expressed sequence tags (J. Kopka and B. Müller-Röber, unpublished data). This clone was successfully used to isolate cDNAs coding for PI-PLCs from *Nicotiana rustica* (Pical et al., 1997). Here we report the isolation of cDNAs representing three PI-PLC isoforms from epidermal fragments of fully expanded potato leaves. We demonstrate gene expression of the three isoforms in a variety of tissues and differential gene regulation under different stress regimes. Using purified recombinant proteins we identified the reaction products produced by the potato PI-PLCs and characterized the cation requirements of the three isoforms.

MATERIALS AND METHODS

Cloning and Sequencing of cDNAs

A novel PI-PLC homolog was cloned from *Arabidopsis thaliana* (J. Kopka and B. Müller-Röber, GenBank accession no. X85973). Two *Hin*dIII fragments (0.7 and 0.8 kb) containing most of the coding region of the novel cDNA were used for heterologous screening of a λ-ZAP II cDNA library (Stratagene), which was prepared from poly(A^+) RNA that was isolated from epidermal fragments of potato (*Solanum tuberosum* L. cv Désiree) leaves (Müller-Röber et al., 1995; Kopka et al., 1997b). DNA fragments were labeled with [α^{32}P]dCTP using a random-primed DNA-labeling kit (Boehringer Mannheim). Hybridization of plaque lifts overnight at 42°C in PEG buffer (Amasino, 1986) was followed by washes at 45°C in 6× SSC and 0.5% SDS for 15 min and in 5× SSC and 0.5% SDS for 15 min. pBluescript II SK plasmids containing target inserts were obtained from hybridizing phages by in vivo excision according to the manufacturer's (Stratagene) protocol. The plasmids containing the longest inserts were manually sequenced (T7 sequencing kit, Pharmacia). Complete nucleotide sequences of full-length cDNAs representing the genes *stplc1, stplc2,* and *stplc3* were submitted to the EMBL nucleotide sequence database. Standard molecular biology methods were performed as described previously (Sambrook et al., 1989).

Computational Analysis of Predicted Amino Acid Sequences

The computational services and options of the Wisconsin package (version 8.1, Genetics Computer Group, Madison, WI) were used with default parameters. Analysis of amino acid sequence homology was performed with the BLAST program (Altschul et al., 1990). Multisequence alignments were created with the PILEUP option. The percentage of sequence identity and similarity was determined by pairwise alignment using the GAP program. Hydropathy plots were generated according to the algorithm of Kyte and Doolittle (1982). Relative molecular masses and pIs were calculated from the deduced amino acid compositions with the Compute pI/Mw tool available at the ExPASy Molecular Biology Web server (Geneva, Switzerland). The secondary structure of plant PI-PLCs was predicted by submitting a multisequence alignment of all known plant PI-PLCs to the SSPRED Web server at the European Molecular Biology Laboratory (Heidelberg, Germany). Subcellular sorting was predicted at the PSORT Web server for analyzing and predicting protein-sorting signals at the Institute for Molecular and Cellular Biology (Osaka, Japan). Analysis of StPLC primary structures for conserved protein domains was performed with the PROFILESCAN program at the Web server of the ISREC Bioinformatics Group (Lausanne, Switzerland).

Plant Material

Potato plants were obtained through Saatzucht Fritz Lange KG (Bad Schwartau, Germany). Plants were grown in soil in individual 3-L pots in a greenhouse under periods of 16 h of light (with additional illumination giving a total light intensity of approximately 100–200 μmol photons s^{-1} m^{-2}; 22°C) and 8 h of darkness (15°C).

Material for RNA analysis of steady-state mRNA levels in different tissues (Fig. 3) was harvested from well-watered, flowering potato plants at approximately 3 PM, 8 h after the beginning of the light period. Epidermal fragments for this experiment were prepared from source leaves, i.e. fully expanded, nonsenescent leaves from the fifth node downward (Müller-Röber et al., 1995; Kopka et al., 1997b). Sink leaves were immature leaves (<1 cm in length) harvested from the first visible node of nonflowering shoots.

Leaflets of fully expanded leaves used for analysis of the short-term effect of local wounding on transcript levels (Fig. 4) were crushed once in vertical orientation to the major vein of the leaflet with a 5-cm clamp used to seal dialysis tubes. Total RNA was prepared from these leaves 6 h after application of wound stress. Fast wilting of leaves for RNA analysis of short-term effects on gene expression

(Fig. 4) was achieved by air-drying the root system after gentle removal of the pot and adhering soil. Wilted leaves were harvested after 6 h. In this experiment leaf fresh weight was reduced by approximately 20% compared with untreated plants.

The effect of slowly developing, long-term drought stress was studied on leaves of the upper third to fifth node because most leaves of the lower nodes were subject to fast senescence under the experimental conditions applied. Plants were not watered for 4 d before the leaves were collected, whereas control plants were subjected to a normal watering regime and were kept under otherwise identical growth conditions. Leaves and epidermal fragments were prepared as described by Kopka et al. (1997b).

RNA Analysis

RNA from different tissues of potato plants was prepared according to the method of Logemann et al. (1987), except RNA of epidermal fragments, which was prepared as described by Kopka et al. (1997b). Total RNA was quantitated spectrophotometrically at 260 nm. Total RNA from each sample (25 or 50 μg) was electrophoretically separated in a denaturing 15% (v/v) formaldehyde-1.5% (w/v) agarose gel and blotted onto Hybond-N^+ membranes (Amersham). Fixation of RNA to the membrane was achieved by incubation in 50 mM NaOH for 5 min at room temperature and a subsequent wash in 2× SSC (Noonberg et al., 1994). Labeling of cDNA fragments and hybridizations were performed as described for plaque lifts. Washes were performed at 65°C in 3× SSC and 0.5% SDS for 15 min and in 0.2× SSC and 0.5% SDS for 20 min. Blots were exposed to Kodak X-OMAT AR film.

The following DNA probes were used for hybridizations: the *Spe*I/*Xho*I fragments containing the complete cDNA inserts of *stplc1*, *stplc2*, and *stplc3*; the 3.5-kb *Asp*718/*Bam*HI fragment of plasmid efEST G56 coding for 25S rRNA of potato (GenBank accession no. R28706); the 2.7-kb *Asp*718/*Bam*HI fragment of POTSSYN coding for potato Suc synthase (Salanoubat and Belliard, 1987); the 0.6-kb *Pst*I fragment of PINR5 coding for the wound-inducible potato proteinase inhibitor II (*pin2* gene; Peña-Cortés et al., 1996); and the full-length fragment of a tomato cDNA coding for the drought-stress-inducible *tas-14* gene (Godoy et al., 1990). Transcript sizes of *stplc1*, *stplc2*, and *stplc3* were determined in relation to the electrophoretic mobility of rRNA species with a single RNA blot, which was successively probed.

Analysis of Recombinant StPLC Expressed in *Escherichia coli*

Recombinant StPLC1, StPLC2, and StPLC3 proteins were expressed in *E. coli* BL21 cells as GST-StPLC fusion proteins using the pGEX-4T-2 vector (Pharmacia), which provides a thrombin cleavage site for removal of the GST part of the fusion proteins. The complete coding regions of *stplc1*, *stplc2*, and *stplc3* were amplified by PCR using *Taq*-DNA polymerase. The forward primers were specific for each isoform and introduced *Bam*HI restriction sites, which were used for in-frame cloning of the PCR fragments into the pGEX-4T-2 vector. The reverse primer was the T7 primer, which anneals to the T7 promoter region of pBluescript II SK plasmids. The second restriction site for cloning of PCR fragments was *Xho*I, which was the cloning site that was used for construction of the epidermal fragment λ-ZAP II cDNA library (Müller-Röber et al., 1995) and therefore immediately follows the poly(A^+) tail of the cDNA inserts. The resulting expression vectors were named pGEX-StPLC1, pGEX-StPLC2, and pGEX-StPLC3.

The cloning strategy preserved the native stop codons of StPLCs but introduced an N-terminal Gly-Ser dipeptide to thrombin-cleaved, recombinant StPLCs in place of the native Met residue. *E. coli* BL21 was transformed with pGEX-StPLC1, pGEX-StPLC2, or pGEX-StPLC3. Five to 10 transformants per isoform, each containing a vector derived from an independent PCR fragment, were screened for protein expression using a small-scale purification protocol. *E. coli* BL21 transformants (3-mL cultures) were grown at 28°C to A_{600} = 0.6 and were subsequently induced for 2 h with 2 mM isopropyl-β-D-galactopyranoside. Fusion protein was purified using a glutathione-Sepharose resin according to the manufacturer's (Pharmacia) instructions, and GST- and PIP_2-hydrolyzing activities were determined in the affinity-purified fractions. Fusion protein isolated from most transformants had similar, high ratios of PI-PLC activity compared with GST activity; only a few did not exhibit PI-PLC activity, which may have been due to errors induced during DNA amplification or cloning.

A single *E. coli* BL21 transformant carrying either pGEX-StPLC1, pGEX-StPLC2, or pGEX-StPLC3 and showing high PI-PLC activity compared with GST activity was selected for a large-scale production of recombinant StPLC1, StPLC2, and StPLC3. Thrombin-cleaved StPLCs and uncleaved fusion proteins were purified from 1-L cultures according to the manufacturer's recommendations using PBS buffer (10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.14 M NaCl, and 2.7 mM KCl, pH 7.3). Thrombin cleavage was performed overnight at room temperature with fusion protein that was bound to a glutathione-Sepharose column.

Purification was monitored by protein determination (Bradford, 1976), by determination of GST activity with 1-chloro-2,4-dinitrobenzene as substrate (Habig et al., 1974), by determination of PI-PLC activity with PIP_2 as substrate (see below), and by SDS-PAGE (Laemmli, 1970). Protein purity and molecular masses were estimated by densitometry of Coomassie brilliant blue G-250-stained SDS-PAGE gels. Yield of a representative purification from a 1-L *E. coli* culture was 0.38 mg of GST-StPLC fusion protein or 0.15 mg of recombinant StPLC. Purity of recombinant StPLCs was greater than 90% and GST activity was not detectable.

Biochemical Analysis of Recombinant StPLCs

The PI-PLC assay was performed according to the method of Melin et al. (1992) and Drøbak et al. (1994). The reaction mixture contained 50 mM Tris/maleate, pH 6.25, 0.2 mM of ^{3}H-head-group-labeled PI or PIP_2 at approxi-

mately 5000 dpm $nmol^{-1}$ (Amersham) and varying amounts of Ca^{2+} in a volume of 50 μL. A 0.6 mM micellar lipid stock solution was prepared by mixing unlabeled (PI, PIP_2; Sigma) and ^3H-labeled lipid in chloroform:methanol (2:1, v/v), evaporation of solvent under a stream of nitrogen, addition of 166 mM Tris/maleate, pH 6.25, and sonication for 10 min. The reaction was performed at 25°C for 15 min. Substrate consumption did not exceed 15%. The reaction was stopped by the addition of 1 mL of chloroform:methanol (2:1, v/v). Phases were separated by adding 0.25 mL of 1 N HCl, and liquid scintillation counting of water-soluble reaction products was carried out as described previously (Melin et al., 1992).

Cations were added to the reaction mixtures as chloride salts. The standard reaction mixture contained 10 μM Ca^{2+}. Ca^{2+} concentrations $\leq$10 μM were buffered with 1 mM EGTA (Owen, 1976). Studies of the effect of Al^{3+} and Mg^{2+} on plant PI-PLC activity were performed in the presence of 10 μM Ca^{2+}. Recovery of IP_3 from standard reaction mixtures in the presence of Al^{3+} and Mg^{2+} was determined with [^3H]IP_3 (5000 dpm $nmol^{-1}$; Amersham).

Analysis of Plant PI-PLC Reaction Products

Standard reactions were performed in 50 μL with 0.1 μg μL^{-1} recombinant StPLC, 10 μM Ca^{2+}, and 0.2 nmol μL^{-1} phosphatidyl[2-^3H]inositol-4,5-bisphosphate (5000 dpm $nmol^{-1}$). Reactions were stopped by chloroform:methanol (2:1, v/v), and lipophilic and water-soluble reaction products were separated as described above.

The chloroform phase was concentrated under a stream of nitrogen and applied quantitatively onto silica gel plates with a concentration zone (type Si 250 PA, J.T. Baker, Deventer, The Netherlands). TLC was performed with hexane:diethylether:acetic acid (9:1:0.5, v/v) to analyze neutral lipids or with chloroform:methanol:acetic acid:water (25:15:4:2, v/v) for separation of PA. Lipids were visualized by iodine staining. The standard lipid mixtures contained 10 μg of DG and 10 μg of PA (both from Sigma). PIP_2 was immobile in both solvent systems.

The aqueous phase of the chloroform:methanol extraction was neutralized with 2 N NaOH and concentrated by freeze-drying. The reaction products were analyzed on a 25-cm Partisil SAX HPLC column (Whatman, Maidstone, UK). The chromatographic conditions of Brearley and Hanke (1996) were applied. The columns were eluted at 0.5 mL min^{-1} applying a linear gradient of 0 to 2.5 M NaH_2PO_4 with a slope of 0.416 M min^{-1}. ^3H was monitored with a flow-through counter. Recovery and retention time of IP_3 was determined with authentic [^3H]IP_3 (50,000 dpm per reaction mixture). [^3H]Inositol-bisphosphate was prepared by limited alkaline phosphatase treatment of [^3H]IP_3 (Brearley and Hanke, 1996). Separation of inositol phosphates on the Partisil SAX column was possible but limited by severe peak tailing and did not meet the superior quality of Partisphere SAX columns (Brearley and Hanke, 1996).

RESULTS

Cloning and Sequence Analysis

Using the coding region of an *A. thaliana* cDNA encoding a novel plant PI-PLC homolog as a molecular probe, we cloned three PI-PLC isoforms from a λ-ZAP II cDNA library, which was prepared from poly(A^+) RNA isolated from epidermal fragments of potato leaves (Müller-Röber et al., 1995). The 1981-bp cDNA of *stplc1* codes for a 596-amino acid protein; the cDNA of *stplc2* has 1940 bp with an open reading frame representing 565 amino acids, and *stplc3* encodes a 585-amino acid polypeptide within a cDNA of 2009 bp. All clones were full length, as was shown by determining transcript sizes corresponding to these *stplc* genes (compare with Fig. 3). The primary structures of StPLC1, StPLC2, and StPLC3 (Fig. 1) were similar to those of currently known plant PI-PLC homologs. Relative molecular weights of the potato proteins were between 64,294 and 67,766 and the pIs ranged from 5.27 to 6.19. The homologies between the coding regions of *stplc1*, *stplc2*, and *stplc3* were 68 to 81%, and the deduced proteins showed 63 to 77% identity at the amino acid level.

Analysis of homology between the potato PI-PLC proteins (not shown) indicated that StPLC2 and StPLC3 are more closely related to each other than to any other plant PI-PLC. StPLC1 appeared to be as distantly related to StPLC2 and StPLC3 as to PI-PLCs from other plant species. In general, homology among plant PI-PLCs was higher than 52.0% (identical amino acids). In addition to high amino acid homology, plant PI-PLCs also appeared to share common features on the secondary structure level. Using the SSPRED program for the analysis of conserved structural elements (see "Materials and Methods"), we identified 14 α-helix-forming and 19 β-sheet-forming domains in a multisequence alignment based on all presently known plant PI-PLCs. The positions of these putative structural elements are shown in Figure 1. StPLC isoforms do not contain obvious subcellular sorting signals or transmembrane-spanning domains and therefore these proteins are most likely located in the cytosol and, considering their presumed role, may be associated with the plasma membrane.

Compared with other PI-PLCs, plant PI-PLCs seem to form a distinct subgroup. All PI-PLCs identified so far exhibit high homology within three domains: X and Y, which together constitute the catalytic domain of these enzymes, and a C_2-like domain (Fig. 1). The C_2-like domain of StPLCs was identified by the C_2 domain profile (PS50004) of the PROSITE database. C_2 domains are Ca^{2+}-dependent protein-phospholipid interaction domains, which could mediate membrane attachment of the amphipathic plant PI-PLCs. C_2 domains were first identified in Ca^{2+}-regulated PKC isoforms (Hug and Sarre, 1993) and are present in a variety of mammalian enzymes involved in transmembrane signaling, such as phospholipase A_2 (Clark et al., 1991), PI-PLC (Essen et al., 1996), synaptotagmin (Perin et al., 1991), and rabphilin-3A (Shirataki et al., 1993).

The C_2-like domains of StPLCs align to the eight-stranded β-sandwich structure of the C_2 domain in rat PLCδ1 (Essen et al., 1996). The predominant predicted

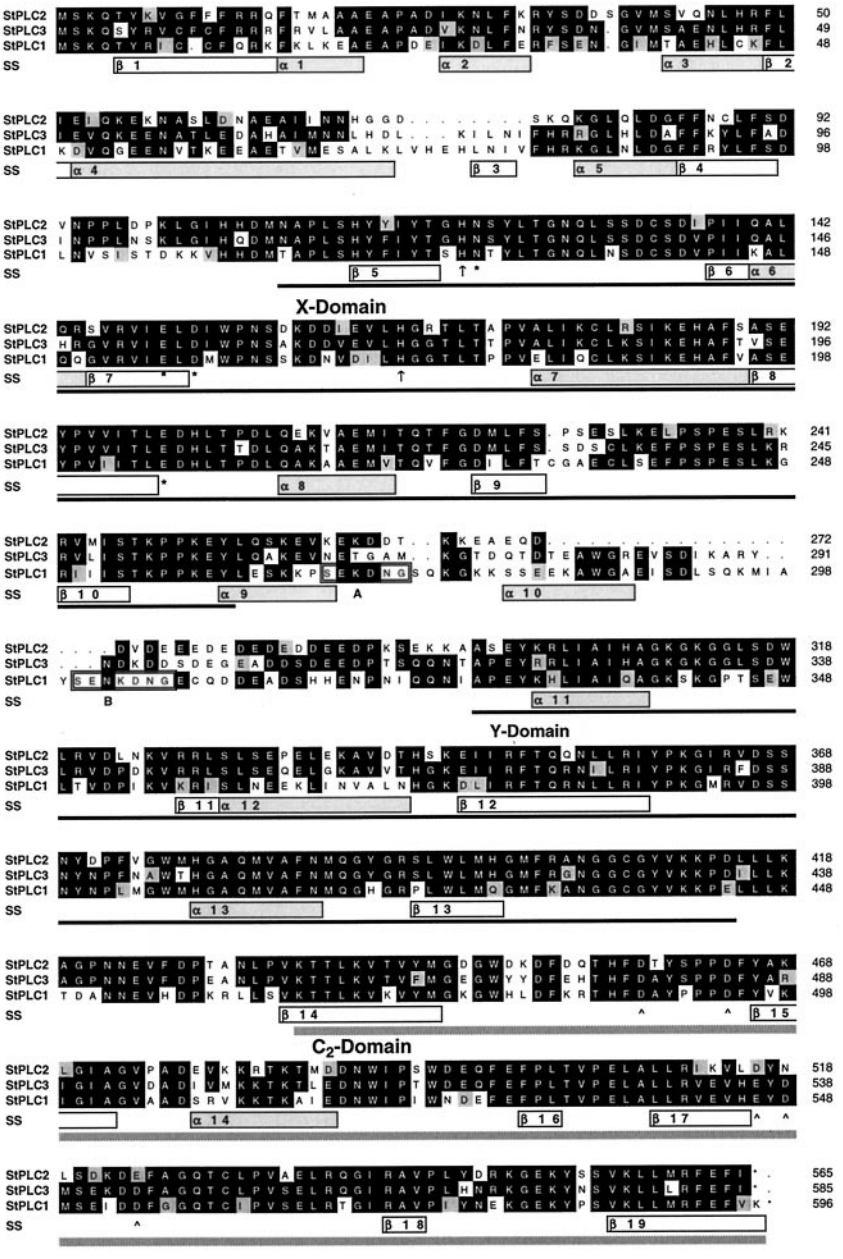

Figure 1. Multisequence alignment of the amino acid sequences of three PI-PLC isoforms, StPLC1, StPLC2, and StPLC3, as deduced from corresponding cDNA clones isolated from a potato epidermal fragment cDNA library. Amino acid residues conserved in at least two of the three sequences are shaded in black; conservative substitutions are marked in gray. Predicted domains of conserved secondary structure (SS) are indicated by bars (α, α-helix; β, β-sheet). Conserved functional domains are underlined in black (X and Y domain) or gray (C_2-domain; PROSITE database at the ExPASy Molecular Biology Web server; see Essen et al., 1996). His residues of the reaction center, which are invariable within the X domains of all known enzymatically active PI-PLCs, are marked by arrows. Ca^{2+}-binding amino acids within the active site are marked by asterisks (*). Putative Ca^{2+}-binding amino acids within the C_2-like domain are indicated by arrowheads. Note that the linking region between X and Y domains of StPLC1 contains a short sequence repeat (A and B), which is highlighted by frames.

secondary structure of StPLCs within this region is β-sheet conformation (Fig. 1, β14–β19). The location of most of these β-sheet domains is in agreement with the two known crystal structures of C_2 domains within rat PLCδ1 (Essen et al., 1996) and rat synaptotagmin I (Sutton et al., 1995). Moreover, StPLCs contain the polybasic core region K-(K,R)-T-K typical for C_2 domains (Fig. 1, α-14) and five putative Ca^{2+}-binding residues (Fig. 1, arrowheads), which correspond to the Ca^{2+}-interacting amino acids identified in crystallized C_2 domains (Sutton et al., 1995; Essen et al., 1996; Shao et al., 1996).

Hydropathy plots of StPLC isoforms illustrate the pattern of sequence conservation and diversity. Within both amphipathic X and Y domains, a large number of conserved hydrophilic and hydrophobic subdomains can be identified in all three StPLC isoforms (Fig. 2). N termini of StPLC isoforms are also amphipathic. In this region, StPLC2 and StPLC3 exhibit almost identical hydropathy patterns, whereas StPLC1 is more variable. The linking region between the X and Y domains is highly hydrophilic and appears to differ between isoforms in length and hydrophilicity. A bipartite hydrophilic linking region, which contains a short sequence repeat that is not present in the single hydrophilic domains of StPLC2 and StPLC3, is characteristic of StPLC1 (Figs. 1 and 2).

RNA Expression

Analysis of RNA expression of the three potato PI-PLC isoforms was performed on various tissues from potato

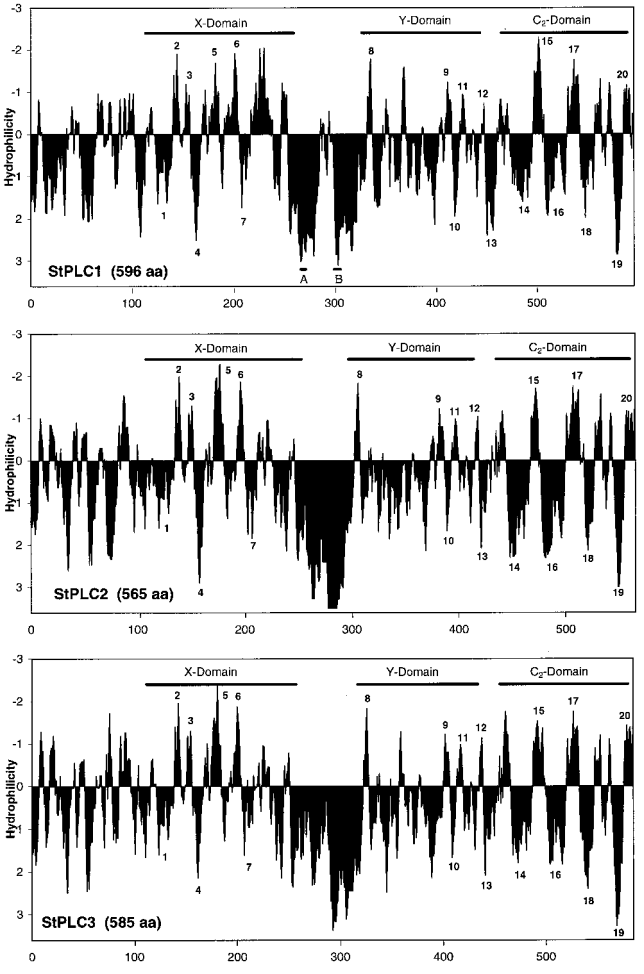

Figure 2. Hydropathy plots of the deduced amino acid sequences of StPLC1, StPLC2, and StPLC3. The total number of amino acids (aa) constituting each isoform is listed. The relative positions of the X and Y domains, the C_2 domain, and the sequence repeat in StPLC1 (A and B) are indicated by bars. The conserved hydropathy pattern of StPLC isoforms is marked by 20 individually numbered hydrophilic and hydrophobic domains. Note that significant differences between isoforms are present at the N terminus and within the highly hydrophilic linker region between the X and Y domains.

plants (Fig. 3). Steady-state mRNA levels of all isoforms were high in flower, petiole, and stem. In contrast, fully expanded source leaves and a preparation of epidermal fragments contained low amounts of *stplc* transcripts. Epidermal fragment RNA is 90% pure guard cell RNA and may be contaminated only by RNA from trichomes (Kopka et al., 1997b). As judged by phosphorimaging analysis, intensities of hybridization signals observed with RNA isolated from epidermal fragments and source leaves were approximately equal (data not shown). Expression in sink leaves and in tubers differed between isoforms and, in the case of *Stplc1*, was approximately 10-fold higher than in fully expanded source leaves. *Stplc2* and *stplc3* transcript levels were also elevated in sink leaves but showed only an approximately 2- to 3-fold difference compared with fully expanded leaves (for definitions of leaf stages, see "Materials and Methods").

The *Stplc1* transcript was hardly detectable in preparations of tuber RNA. In contrast, transcript levels of *stplc2* and *stplc3* in tubers were equal or slightly higher than in stems. Cross-hybridization between the three *stplc* probes and their corresponding mRNAs in this and the following experiments can largely be excluded because successive hybridizations of the probes to a single membrane revealed slightly different transcript sizes (compare with Fig. 3) or exhibited differential response of *stplc* transcript levels to environmental signals (see below; compare with Fig. 4). Furthermore, no cross-hybridization between *stplc* cDNAs was observed in DNA-blot experiments performed under the same hybridization conditions as used here for the RNA analysis (not shown).

Gene expression of *stplc* isoforms in source leaves showed different short-term responses to local wound stress and air drying. Whereas *stplc1* exhibited strong reduction of its transcript level in response to both stresses, *stplc2* mRNA was equally strongly induced by both treatments (Fig. 4). Local wounding induced only a minor, short-term alteration in the expression of *stplc3*. Analysis of transcript levels in this experiment was performed 6 h after application of wound or drought stress, respectively (see "Materials and Methods"). The effect of the applied stresses on mRNA composition was verified by monitoring wound-induced *pin2* gene expression (Peña-Cortés et al., 1996) and the drought-induced increase in *tas-14* mRNA levels (Godoy et al., 1990; Harms et al., 1995). In addition, application of both wound and drought stress could also be monitored by an increase in gene expression of Suc synthase (Fig. 4; Kopka et al., 1997b).

We also compared long-term and short-term drought effects (see "Materials and Methods") on *stplc* transcript levels in RNA isolated from whole leaves. Under both conditions, *stplc1* and *stplc2* showed the same behavior as described above, whereas *stplc3* transcript levels increased only after long-term drought stress. The same observations were made with RNA extracted from epidermal fragments (not shown). Previously, Hirayama et al. (1997) reported transient induction by drought of the *AtPLC1S* gene in

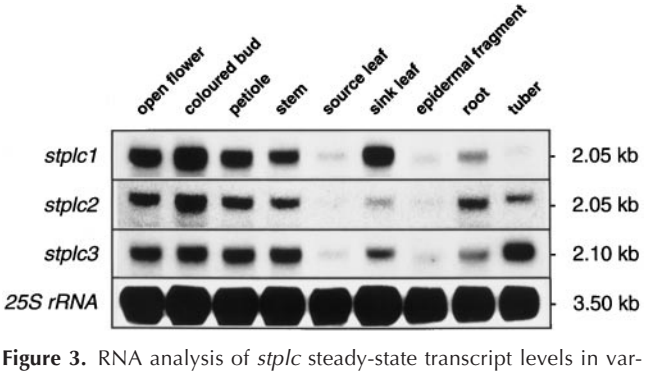

Figure 3. RNA analysis of *stplc* steady-state transcript levels in various tissues of potato plants. Epidermal fragments were prepared from source leaves. Total RNA (50 μg per lane) was probed with the complete cDNAs of *stplc1*, *stplc2*, and *stplc3* and with a cDNA encoding potato 25S rRNA (see "Materials and Methods"). Transcript sizes are indicated.

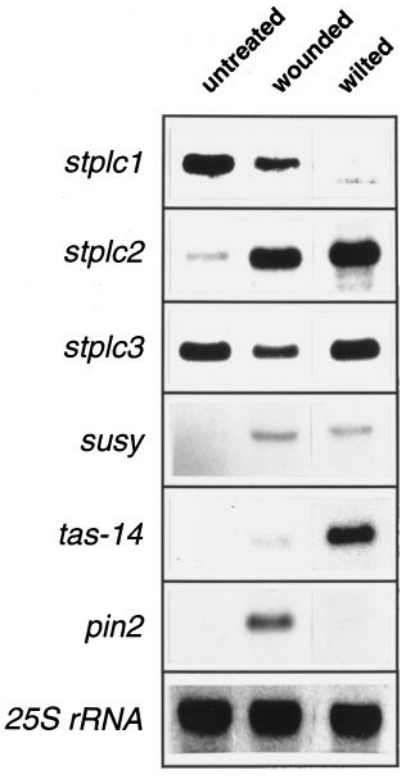

Figure 4. RNA analysis of *stplc* steady-state transcript levels in fully expanded leaves of potato plants that were untreated, locally wounded, or wilted by severe short-term drought. Leaves for RNA preparation were detached 6 h after application of stresses at approximately 4:30 PM. RNA preparations (25 μg per lane) were also tested for wound-induced expression of the proteinase inhibitor II gene (*pin2*), for drought-induced expression of the *tas-14* gene, and for induction of Suc synthase (*susy*) gene expression.

Arabidopsis rosette plants but constitutive expression of the *AtPLC2* gene.

Analysis of Recombinant StPLC Isoenzymes

Recombinant StPLC isoenzymes were expressed in *E. coli* BL21 cells as GST-StPLC containing a thrombin recognition site between the N-terminal GST-tag and StPLC. Recombinant fusion proteins were affinity purified on glutathione-Sepharose 4B and either eluted with reduced glutathione to yield GST-StPLC fusion proteins or cleaved while bound to the column with thrombin to prepare recombinant StPLC proteins. These preparations contained proteins that showed the expected electrophoretic mobilities for the relative molecular weights predicted from the cDNA sequences. Recombinant StPLCs were at least 90% pure and, as judged by SDS-PAGE and enzyme assays, contained no residual GST (not shown). Preparations of StPLC3 contained approximately equal amounts of two polypeptides, one polypeptide being smaller than expected. Because of differences in protein degradation, the specific enzyme activities of recombinant StPLC isoenzymes could not be compared.

We characterized the nature of the PIP_2 hydrolysis products in reaction mixtures incubated under standard conditions with StPLC1, StPLC2, and StPLC3. All StPLC isoenzymes produced the same reaction products from PIP_2. As shown for StPLC1, both an unlabeled lipid that co-migrated on TLC plates with authentic DG (Fig. 5) and a ^3H-labeled, water-soluble product that co-eluted in HPLC chromatograms with authentic IP_3 (Fig. 6) accumulated with increasing reaction time. Under the chosen conditions hydrolysis was complete in 15 min. The amount of product at this time was approximately 10 μg of lipid (Fig. 5), and recovery of ^3H into the aqueous phase was approximately 90%. Because of severe peak tailing (Fig. 6), recovery of IP_3 from HPLC columns was not determined. Our data indicate complete conversion of the initial amount of educt PIP_2 (M_r = approximately 1020 g mol^{-1}) into DG and IP_3.

All reaction products analyzed after 0 to 30 min of incubations contained a minor, nonaccumulating substance that co-migrated with authentic PA when analyzed by TLC with a solvent system for the separation of polar lipids, i.e. chloroform:methanol:acetic acid:water (25:15:4:2, v/v; not shown). The origin and chemical nature of this lipid was not further investigated. The water-soluble reaction product contained a minor but accumulating compound that co-eluted with inositol-1,4,5-bisphosphate in HPLC chromatograms. Given the fact that PA did not accumulate, inositol-1,4,5-bisphosphate can only result from the cleavage of IP_3. The origin of the underlying phosphatase activity in our preparations of recombinant StPLC was not investigated.

A comparative study of the cation requirements for activation of phosphoinositide hydrolysis by StPLC isoenzymes was performed. All recombinant StPLCs showed a maximum activity of PIP_2 hydrolysis at 10 μM Ca^{2+} and a lower activity at higher Ca^{2+} concentrations (Fig. 7A). A detailed investigation of the activation of StPLC by low concentrations of Ca^{2+} normalized relative to 10 μM Ca^{2+}

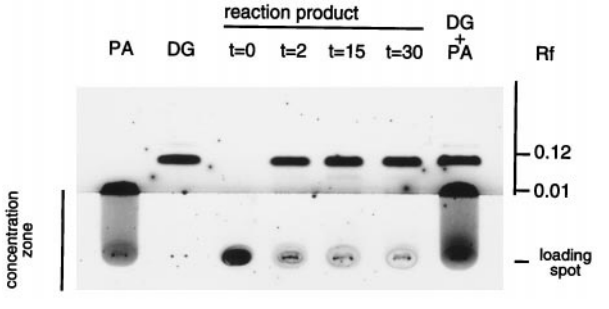

Figure 5. Analysis of the lipid products of PIP_2 hydrolysis catalyzed by recombinant StPLC1. Reactions were performed under standard reaction conditions (see "Materials and Methods") in 50 μL with 0.1 μg μL^{-1} StPLC1, 10 μM Ca^{2+}, and 0.2 nmol μL^{-1} PIP_2 (5000 dpm $nmol^{-1}$). Reactions were started with PIP_2, and lipophilic reaction products were extracted with chloroform:methanol (2:1, v/v) at 0, 2, 15, and 30 min. Lipids were concentrated under a stream of nitrogen, applied quantitatively onto TLC plates, separated by hexane:diethylether:acetic acid (9:1:0.5, v/v), and visualized by iodine staining. The standard lipid mixtures contained approximately 10 μg of DG and 10 μg of PA. PIP_2 was immobile in the chosen TLC solvent system (autoradiogram not shown).

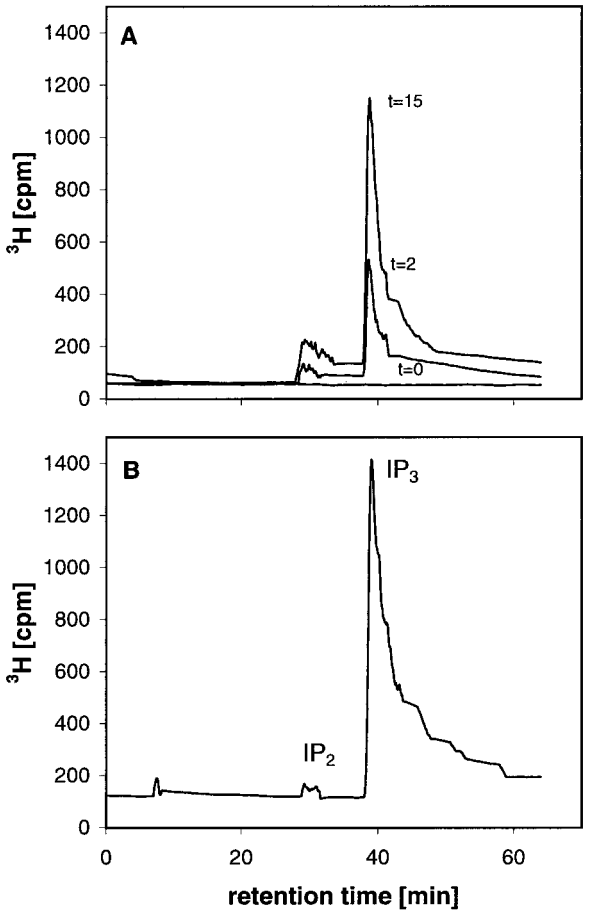

Figure 6. Analysis of the water-soluble products of PIP_2 hydrolysis, which was catalyzed by recombinant StPLC1. Reactions were performed in 50 μL, as described in "Materials and Methods." Reactions were started with PIP_2 and stopped at 0, 2, and 15 min by extraction with chloroform:methanol (2:1, v/v). Reactions were complete at 15 min (see Fig. 5). The aqueous phase of the chloroform:methanol extractions was neutralized and concentrated by freeze drying. A, A volume representing one-half of the reaction products was applied onto a 25-cm HPLC column. Chromatography was performed as described in "Materials and Methods." ^3H was monitored. B, To demonstrate product recovery, authentic [^3H]IP_3 (50,000 dpm) was added to a reaction mixture, extracted at 0 min, and analyzed by applying the same conditions. IP_2, Inositol bisphosphate.

showed no significant difference in the activation pattern between StPLC isoenzymes. Only at 0.5 μM Ca^{2+} did StPLC2 show a tendency for lower activity compared with the other isoforms (Fig. 7C).

We also investigated the hydrolysis of PI, a metabolic lipid precursor of PIP_2, under standard PI-PLC reaction conditions in the presence of recombinant StPLCs (Fig. 7B). All StPLC isoenzymes showed a minor PI-hydrolyzing activity at 10 μM Ca^{2+}, which was substantially increased at 10 mM Ca^{2+}. At this Ca^{2+} concentration preferential cleavage of PIP_2 was lost and the specific activities of all StPLC isoenzymes were approximately the same with PI or PIP_2 (Fig. 7, A and B). In contrast to StPLC2 and StPLC3, PI hydrolysis by StPLC1 was already significantly increased at 100 μM Ca^{2+}.

Competition experiments with increasing amounts of Mg^{2+} and Al^{3+} were performed in the presence of 10 μM Ca^{2+}. Significant inhibition of PIP_2 hydrolysis starting at equimolar concentrations of Al^{3+} was observed for all StPLC isoenzymes. In contrast, within the range of applied concentrations, Mg^{2+} did not affect the activity of recombinant StPLC (Fig. 8). The recovery of [^3H]IP_3 from standard reaction mixtures after extraction with chloroform: methanol (2:1, v/v) was determined in the presence of 0.01 $\mu g\ \mu L^{-1}$ recombinant StPLC1 and 0.5, 1.0, 5.0, 10, 50, 100, and 200 μM Mg^{2+} or Al^{3+}. Recovery of [^3H]IP_3 was approximately 95 ± 2.5% (mean ± SD; n = 3) and, within the

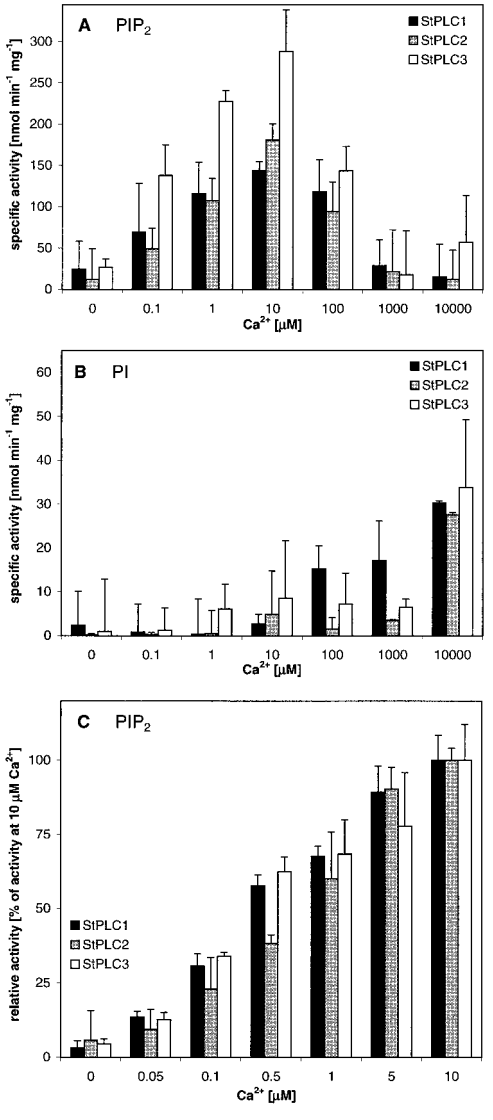

Figure 7. PI-PLC activity in preparations of recombinant StPLC1, StPLC2, and StPLC3 in the presence of varying amounts of free Ca^{2+}. In all experiments, 2-^3H-labeled, water-soluble product was determined after lipids were removed with chloroform:methanol (2:1, v/v). A, PI-PLC substrate was PIP_2 (n = 3). B, PI-PLC substrate was PI (n = 3). C, Activation of PIP_2 hydrolysis at low concentrations of Ca^{2+} (n = 8). Data presented in A and B are specific enzyme activities, whereas data in C are expressed as percentages of maximum activity at 10 μM free Ca^{2+}.

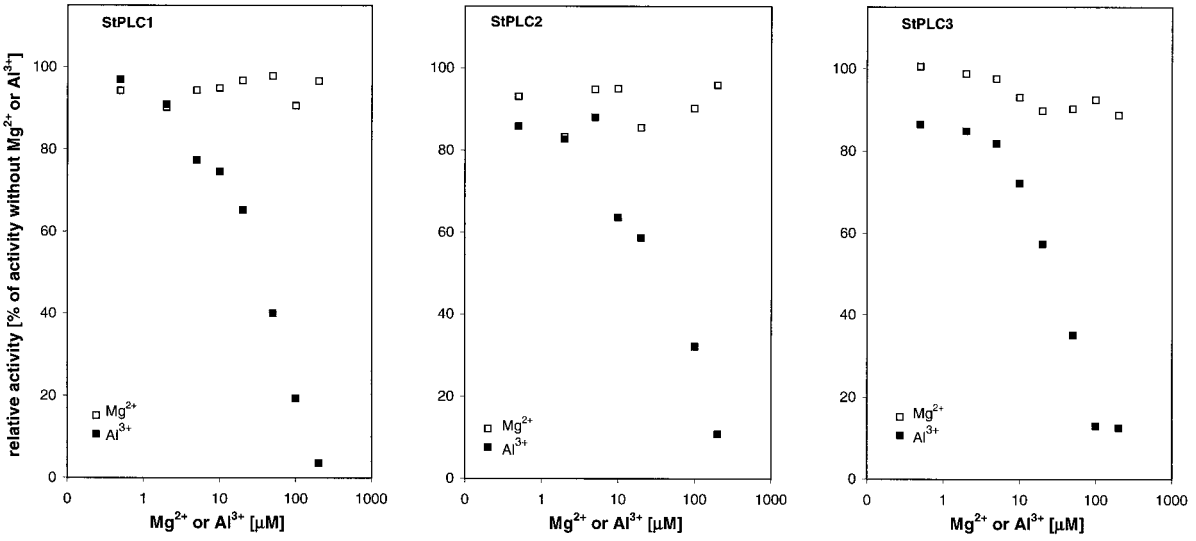

Figure 8. Influence of Mg^{2+} and Al^{3+} on PIP_2 hydrolysis in preparations of StPLC1, StPLC2, and StPLC3 in the presence of 10 μM free Ca^{2+} ($n = 2$). Mg^{2+} and Al^{3+} cations were added as chloride salts. Recovery of authentic [^{3}H]IP_3 from reaction mixtures after extraction with chloroform:methanol (2:1, v/v) was approximately 95% and independent of the presence of 0.5 to 200 μM Mg^{2+} or Al^{3+} (data not shown).

range of applied concentrations, was independent of both the concentration and chemical nature of the cation.

DISCUSSION

The primary structures of all cloned StPLC isoforms show the typical features of PI-PLC enzymes. Based on amino acid sequence homology, plant PI-PLCs, including StPLCs, form a distinct group within the family of eukaryotic PI-PLCs (not shown). This group is closely related to the mammalian PLCδ subfamily (Irvine, 1996). The X domains of StPLCs appear to contain the putative active site, including two conserved His residues. These His residues not only were shown to be located within the active site of crystallized rat PLCδ1 (Essen et al., 1996), but were also essential for enzyme activity, as was demonstrated by site-directed mutagenesis of PLCδ1 (Cheng et al., 1995; Ellis et al., 1995). A similar reaction center with two His residues was also found in the prokaryotic PI-PLC from *Bacillus cereus* (Heinz et al., 1995).

These observations suggest that the StPLCs encoded by the *stplc* cDNAs represent enzymatically active PI-PLCs. Previous investigations of cloned plant PI-PLCs demonstrated specificity for phosphoinositides (Hirayama et al., 1995; Shi et al., 1995). However, the fate of the phosphodiester group that links the inositol phosphate head group to the DG part of the glycerolipid was not analyzed; therefore, it could not be ruled out that the cloned enzymes might catalyze a phospholipase D reaction and form PA instead of DG. The latter would be the expected product of an authentic PI-PLC. Partial complementation of a PI-PLC-deficient yeast mutant by a plant PI-PLC cDNA as performed by Shi et al. (1995) is important evidence but could also occur if the PI-PLC activity were only a side reaction catalyzed by the cloned plant enzyme. To confirm the activity of StPLC, we demonstrated that recombinant StPLC almost exclusively forms DG and IP_3 from PIP_2 (Figs. 5 and 6).

StPLCs, like other plant PI-PLCs (Hirayama et al., 1995; Shi et al., 1995), are activated by Ca^{2+} concentrations in the nanomolar range (Fig. 7). PI-PLCs from soybean (*G. max*; Shi et al., 1995) and *A. thaliana* (Hirayama et al., 1995) have previously been reported to contain EF-hand motifs, i.e. putative Ca^{2+}-binding domains, which, however, are absent from the StPLCs described here. Regulation of plant PI-PLCs by Ca^{2+}, therefore, most likely involves domains other than EF-hands. In accordance with this assumption is the observation that mutating an EF-hand motif in PI-PLC from *Dictyostelium discoideum* did not affect the Ca^{2+}dependence of this enzyme (Drayer et al., 1995). We suggest that the Ca^{2+} sensitivity of StPLCs might be brought about by one of two alternative mechanisms. A Ca^{2+} ion could bind to the charged residues in the reaction center, which are conserved in all PI-PLCs (i.e. N_{127}, E_{156}, D_{158}, and E_{206} of StPLC1; Fig. 1) and might aid to position the inositol phosphate head group within the active site. The presence of Ca^{2+} within the active site of rat PLCδ1 and interaction with the conserved charged residues (i.e. N_{312}, E_{341}, D_{343}, and E_{390} of PLCδ1) was shown by a high-resolution crystal structure of a Ca^{2+}- and IP_3-containing complex (Essen et al., 1996). A second possibility is that the Ca^{2+}-regulatory domain in PI-PLC could be the C_2-like domain that is present C-terminal to the Y domains of all plant PI-PLCs (Fig. 1). As suggested by Essen et al. (1996), the C_2 domain of PI-PLC could regulate enzyme activity by mediating membrane interaction of the mainly amphipathic PI-PLC enzymes (Fig. 2) and thus give access to the phosphoinositide substrate.

Mg^{2+} ions are known to activate PI-PLC activity in the presence of 10 μM Ca^{2+} in wheat plasma membranes (Pical et al., 1992; Jones and Kochian, 1995). In contrast, Al^{3+} is a strong inhibitor of PI-PLC activity in preparations of

plasma membranes from wheat roots (Jones and Kochian, 1995). We demonstrated that Al^{3+} inhibited the activity of all recombinant StPLCs and that Mg^{2+} was not able to stimulate the recombinant enzyme preparations (Fig. 8). Therefore, we propose that Mg^{2+} does not directly interact with plant PI-PLC but might stimulate the enzyme via an as-yet-unidentified PI-PLC-binding component that could be present in preparations of plant plasma membranes. In contrast, Al^{3+} appears to act directly on PIP_2 hydrolysis. Two explanations might apply to explain the inhibition of PIP_2 hydrolysis: (a) inhibition could be caused by inactivation of the enzyme because of displacement of an essential Ca^{2+} ion from the putative binding sites at the reaction center or the C_2-like domain of the PI-PLC protein (see above), or (b) an Al^{3+}/PIP_2 complex could be formed that might not be cleavable by PI-PLC. It has been reported that Al^{3+} can substitute for Ca^{2+} in a liposome complex with a different phospholipid, i.e. phosphatidylcholine, due to 560-fold higher affinity of Al^{3+} for the glycerolipid (Akeson et al., 1989), and it was proposed that Al^{3+} binds to the phosphodiester group of phosphatidylcholine (Hunter and Etherton, 1989).

We found that, as in other plant species, multiple PI-PLC isoforms exist in potato plants and do not appear to be expressed in a strict tissue-specific manner (Fig. 3). Although *stplc* cDNA clones were isolated from an epidermal fragment cDNA library, we found high expression for all three genes in several other tissues (cf. Fig. 3), indicating that phosphoinositide metabolism in guard cells most likely does not require cell-specific isoforms of PI-PLC or high expression levels of the corresponding genes. Moreover, expression of three *stplc* genes in epidermal fragments under both normal and environmental stress conditions strongly suggests that multiple PI-PLC isoforms might be utilized in a single cell type, i.e. guard cells (not shown). The attempt to prove simultaneous expression of multiple PI-PLC isoforms in guard cells by promoter studies is part of ongoing investigations in our group. Whereas we have not yet proven that multiple isoforms are expressed in guard cells, we have identified one PI-PLC isoform in potato (J. Kopka and B. Müller-Röber, unpublished results) and one isoform in *N. rustica* (Pical et al., 1997), for which no RNA is detectable in epidermal fragments and therefore do not appear to be expressed in guard cells.

We performed a comparative analysis at the levels of protein structure, gene expression, and biochemical properties of the StPLC isoforms isolated from epidermal fragments. StPLC isoforms show high sequence homology and conservation of hydropathy patterns within the X and Y domains (Figs. 1 and 2), which are involved in enzymatic function. In agreement with this observation we were not able to demonstrate significant catalytic differences between the recombinant enzymes at physiological Ca^{2+} concentrations ($\leq$10 μM; Figs. 7 and 8). However, specificity toward PIP_2 started to decline at 100 μM Ca^{2+} in the case of StPLC1, whereas the other isoforms lost specificity only at higher Ca^{2+} concentrations (compare A and B in Fig. 7, 100–10,000 μM Ca^{2+}). Whether these differences are of physiological relevance remains an open question.

In contrast to the catalytic domains, StPLC isoforms show rather high sequence diversity in regions that in mammalian PI-PLC subfamilies are known to contain regulatory domains, i.e. the linking region between X and Y domains and the N terminus. We were not able to identify pleckstrin-homology domains or the *src*-homology domains SH2 and SH3 in plant PI-PLCs. However, the linking regions of StPLCs contain a high number of charged amino acids (Fig. 2). The pattern of hydrophilicity in this region appears to be characteristic for each StPLC isoform. StPLC1, for example, contains a unique bipartite hydrophilic domain, each part of which contains a short sequence repeat (Fig. 2). The structural and biochemical analyses strongly indicate that StPLC2 and StPLC3 belong to one group, and StPLC1 represents a less closely related form of plant PI-PLC isoform. This observation was also substantiated by RNA-expression analysis. Even though there appears to be no distinct tissue specificity in the expression of these genes (Fig. 3), the regulation of transcript levels in response to short-term (Fig. 4) and long-term drought stress (not shown) is markedly different. After long-term stress, *stplc2* and *stplc3* showed an increase in mRNA levels, but, in contrast, *stplc1* mRNA level decreased under identical conditions. Taken together, these results indicate that adaptation to drought might involve changes in the relative levels of PI-PLC isoforms.

In conclusion, in this paper we present the cloning and comparative analysis of three phosphoinositide-specific StPLC isoforms, which are expressed in epidermal fragments and at the mRNA level are regulated in a differential manner. Previously, we reported the isolation of the first cDNA coding for a plant CDS, which among other tissues appears to be also expressed in potato epidermal fragments (Kopka et al., 1997a). The product of the CDS reaction is the initial substrate for the resynthesis of PI from DG produced in plant plasma membranes (Wissing et al., 1992). Thus, we are now able to modulate in planta biosynthesis as well as cleavage of PIP_2, which is the lipid precursor of the second messenger IP_3. Transgenic approaches should allow us to modulate expression of PI-PLC by attempting guard cell-targeted antisense inhibition studies under the control of cell-specific promoters (Müller-Röber et al., 1994).

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The nucleotide sequence data reported will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession numbers X93564 (*stplc1*), X94183 (*stplc2*), and X94289 (*stplc3*).

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