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Characterization of a novel phosphoinositide-specific phospholipase C from *Zea mays* and its expression in *Escherichia coli*

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Abstract

A cDNA encoding a phosphoinositide-specific phospholipase C (PI-PLC) has been isolated from *Zea mays* by screening a cDNA library. The cDNA, designated *ZmPLC*, encodes a polypeptide of 586 amino acids, containing the catalytic X, Y and C2 domains found in all PI-PLCs from plants. Northern blot analysis showed that the expression of the *ZmPLC* gene in roots is up-regulated under conditions of high salt, dehydration, cold or low osmotic stress conditions. Recombinant ZmPLC protein was expressed in *Escherichia coli*, purified and used to produce polyclonal antibody, this polyclonal antibody is important for further studies to assess the ultimate function of the *ZmPLC* gene in plants.

Introduction

Phosphoinositide-specific phospholipase C (PI-PLC) is a key enzyme in the phospholipid signaling pathway. Genes encoding biochemically active PI-PLCs and PI-PLC-like proteins have been identified in a number of different plants such as *Arabidopsis thaliana* (Hirayama *et al.* 1995, 1997), soybean (Shi *et al.* 1995), *Nicotiana rustica* (Pical *et al.* 1997), potato (Kopka *et al.* 1998), and rice (Song & Goodman 2002). Recently, a cDNA encoding an inactive isoform of PI-PLC was isolated from pea (Venkataraman *et al.* 2003). Sequence analysis indicates that all PI-PLCs from plants are similar in size and the overall structure is most closely related to the PLCδs in animals except that they lack the pleckstrin homology (PH) domain. Notably, Otterhag *et al.* (2001) demonstrated that the *N*-terminal EF-hand-like domain is required for PI-PLC activity in *Arabidopsis thaliana*, suggesting that PI-PLCs in plants probably contain an EF-hand domain corresponding to the second loop of the EF-hand domain of PI-PLCs in animals.

Previous studies have shown that the PI-PLCs from plants play an important role in environmental stress signaling and the disease resistance signaling pathway (Hirayama *et al.* 1995, Kopka *et al.* 1998, Song & Goodman 2002, Kim *et al.* 2004). In addition, using sense- and antisense-transgenic *Arabidopsis thaliana*, Sanchez & Chua (2001) demonstrated that *AtPLC1* might be involved in secondary abscisic acid (ABA) responses.

In this report, we focus on the cloning and molecular characterization of a novel PLC gene from maize and the expression of ZmPLC as a fusion protein in *E. coli*.

Materials and methods

Growth of maize seedlings and stress treatment

Sterilized germinated seeds from maize (inbred 90110) were transferred to a 25 °C growth chamber and incubated with 70% relative humidity and no light for up to 10 days. Then seedlings used for cDNA library construction were treated

with 137 mM NaCl for 24 h. In the treatment of high salt or dehydration stress, the seedlings of maize were grown in a solution containing 200 mM NaCl or 30 mM polyethylene glycol (PEG) (MW 6000) under normal conditions (16-h light/28 °C, 8-h dark/19 °C). For cold treatment the plants grown at 28 °C were shifted to 4 °C. For low osmotic treatment the liquid medium A (full-strength Murashige and Skoog salts, pH 5.7, and 1% (m/v) sucrose) was substituted with water. These methods of plant stress treatment were based on a previous study (Yamaguchi-Shinozaki & Shinozaki 1994). Leaf samples were collected at the indicated times and frozen in liquid N_2 until required.

Cloning and sequencing of ZmPLC *cDNA*

A cDNA library was constructed with the SMART Kit from Clontech following the manufacturer's instructions with mRNA from maize seedlings that were treated with 137 mM NaCl for 24 h. According to the conserved domains of PI-PLCs from plants, two primers (5′-GTT TCC TTC TCC AGC ATC TC-3′) and (5′-CAT ACC GGC AAG ACT GTC TG-3′) were designed, and a PI-PLC-like cDNA fragment about 1 kb was obtained. This cDNA fragment was used as a probe to screen the maize cDNA library, using the protocol from the Roche Dig System. DNA sequencing was performed by the BioAsia Company. Protein sequences of PI-PLCs from plants were obtained from the GenBank database and alignments were performed using the ClustalW program.

Northern blot analysis

Total RNA (30 μg) was separated in a 1.2% agarose/formaldehyde gel and transferred to a nylon membrane. The full length *ZmPLC* cDNA sequence was labelled with [α-^{32}P]-dCTP by the random primer labeling system. Prehybridization and hybridization were performed following the standard method. The hybridization signals were detected by autoradiography.

Expression of the recombinant ZmPLC proteins in E. coli

The complete coding sequence for *ZmPLC* was amplified by PCR. The amplified product was initially inserted into the T-easy-Vector (Promega) and then sub-cloned into the expression vector pET-42c(+) (Novegen). All constructs were confirmed by restriction enzyme analysis and DNA sequencing. Successful constructs were transformed into *E. coli* BL21(DE3) and expected to express a ZmPLC fusion protein with a hexahistidine tag and a glutathione *S*-transferase (GST) tag at the *N*-terminus, which contained a thrombin cleavage site for removal of the tag. Recombinant ZmPLC protein was purified by His·Bind Resin Chromatography (Novagen) according to the manufacturer's protocol.

Polyclonal antibody preparation and immunoblotting

The preparation of polyclonal antibodies against the ZmPLC fusion protein was performed by following the standard method. For the immunoblotting analysis, a small amount of recombinant protein was purified from the soluble fraction of BL21(DE3) cells and digested with thrombin. The thrombin-digested recombinant protein and the *ZmPLC* fusion protein were separated on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated in anti-ZmPLC polyclonal antibody solution. The ZmPLC-antibody complex was visualized by staining with alkaline phosphatase conjugated to Goat anti-Rabbit IgG.

Results

Cloning and sequence analysis of a cDNA encoding a phosphoinositide-specific phospholipase C from maize

Using the coding region of a rice PI-PLC cDNA as bait, we conducted a BLAST search of the GenBank database and found several maize ESTs that may encode potential PI-PLC. Based on the sequences of these ESTs and the conserved domains of PI-PLCs from plants, two specific primers were designed. PCR amplification of cDNA with these primers yielded a fragment of about 1 kb. Sequence analysis showed that the fragment contained the conserved domain of the PI-PLCs and was very similar to a maize mRNA

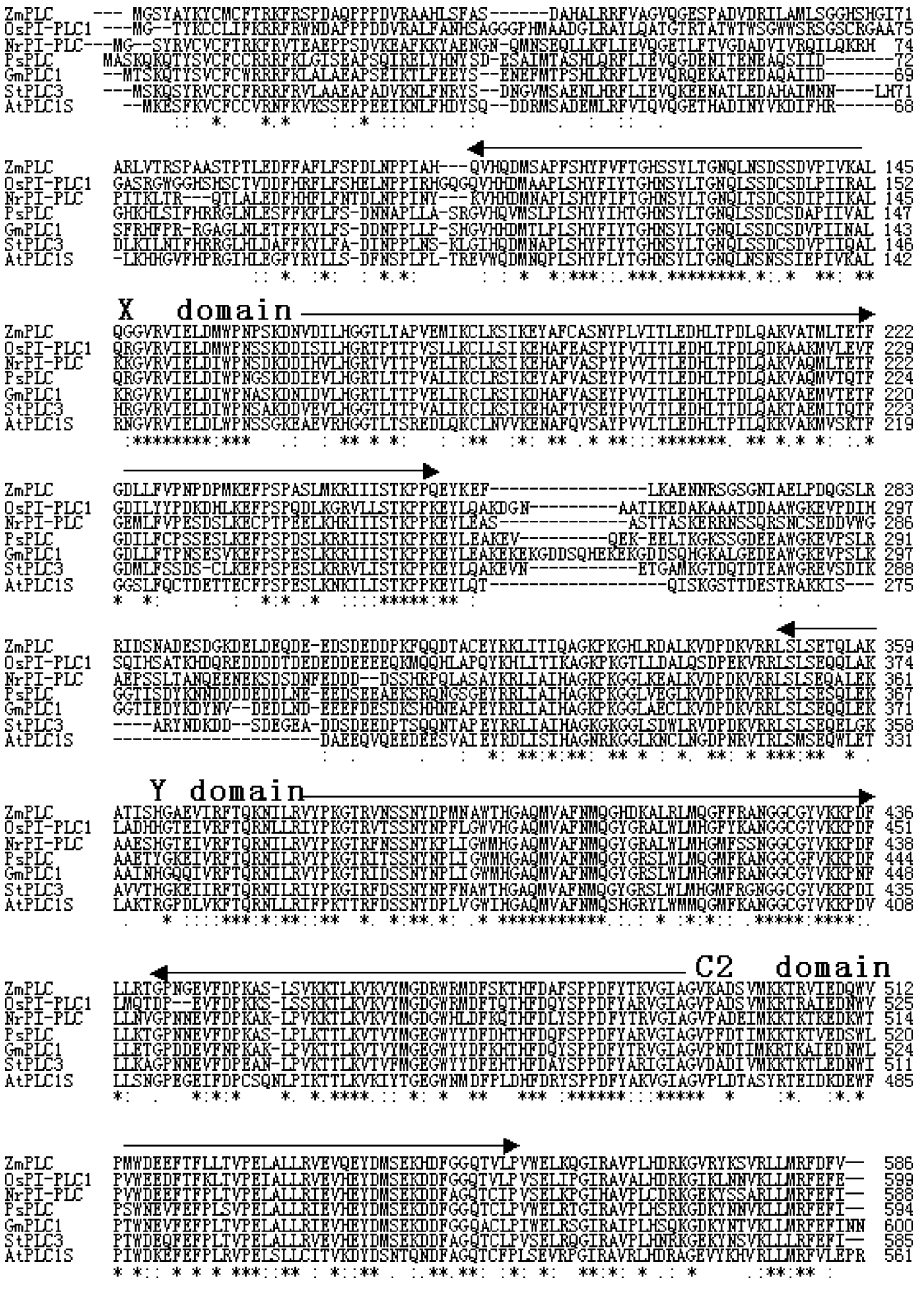

Fig. 1. Multiple alignment of deduced amino acid sequences of phospholipase C from seven plants species. The PLCs used for alignment are: *Zea may* (ZmPLC, AY536525), *Oryza sativa* (OsPI-PLC, AF332874), *Nicotiana rustica* (NrPI-PLC, X95877), *Pisum sativum* (PsPLC, Y15253), *Glycine max* (GmPLC1, U25027), *Solanum tuberosum* (StPLC3, X94289), *Arabidopsis thaliana* (AtPLC1S, D38544). The positions of the X, Y and C2 domains are indicated by arrows.

sequence (Accession No. AY108097) in the GenBank Database. Screening of the maize cDNA library with the cDNA fragment as a probe resulted in the isolation of positive clones. We chose the clone with the largest insert for further analysis. Sequence analysis indicated that the total length of the cDNA was 2072 bp with an open reading frame of 1761 nucleotides that encodes a protein of 586 amino acids with a calculated molecular weight of 65.7 kDa and a pI of

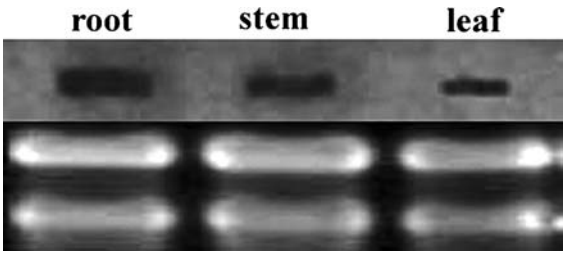

Fig. 2. Organ-specific expression of *ZmPLC* mRNA in maize. Total RNA was prepared from the roots, stems and leaves of 7-week-old maize plants. Thirty μg of total RNA was loaded in each lane. The ethidium bromide-stained rRNA is shown as a control for the amount of RNA loaded in each lane.

6.4. The cDNA sequence was deposited in the GenBank database with the accession no. AY536525.

Alignment of the deduced protein sequence of ZmPLC with PI-PLCs from other species was performed using the ClustalW program (Figure 1). The predicted protein sequence is highly homologous to the previously determined PI-PLC sequences, showing 60, 59 and 59% identity to the PI-PLC from tobacco, rice, and potato, respectively, and 58, 56 and 49% identity to the PI-PLC from pea, soybean, and *Arabidopsis*, respectively. The amino acid sequence of the predicted protein contains the X, Y and C2 domains that are reportedly conserved in the various PI-PLCs from plants.

Regulation of ZmPLC *expression*

Northern blot analysis indicated that the ZmPLC mRNA was expressed in the roots, stems and leaves from 7-week-old maize under normal growth conditions, however, the fully expanded leaves showed lower expression of ZmPLC than the roots (Figure 2). Then the expression of the ZmPLC gene in roots under stressful conditions was analyzed. The results showed that the gene expression of ZmPLC varied under different conditions of stress. ZmPLC accumulated to significant levels under dehydration, high salt, cold, and low osmotic stress conditions (Figure 3). The drought response was detected within 2 h of treatment and reached a peak at 5 h of treatment. Thereafter, the levels of the transcript gradually declined. *ZmPLC* expression increased within 2 h of cold treatment and reached a maximum level at 10 h. The induction of *ZmPLC* expression with high salt and low osmotic stress had nearly the same pattern with the mRNA levels reaching a maximum level at 2 h, and slowly declining after that. These results suggest that the expression of *ZmPLC* is up-regulated by these stress conditions, though in different patterns.

Expression of the recombinant protein, antibody preparation and immunoblotting

Recombinant ZmPLC protein was expressed in *E. coli* strain BL21(DE3). SDS-PAGE analysis indicated high levels of production of a protein with molecular weight of about 97 kDa which consisted of the tag protein and the ZmPLC protein (Figure 4). Nearly all of the recombinant

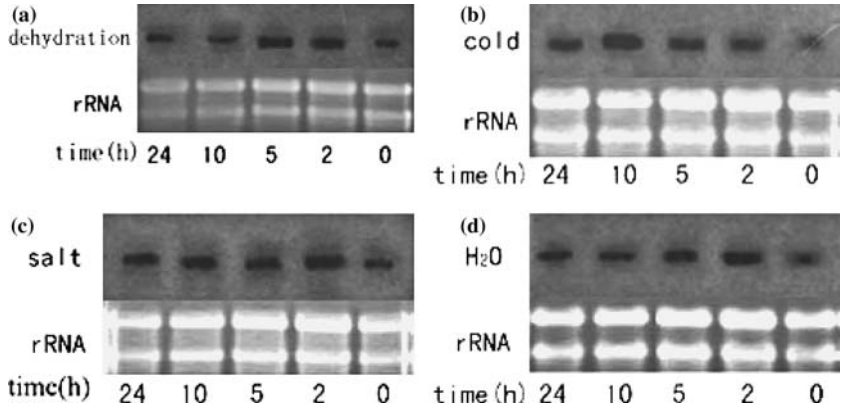

Fig. 3. Northern blot analysis of ZmPLC mRNA expression. Total RNA was prepared from 10-day-old maize seedlings. (a) Induction of *ZmPLC* by dehydration (30 mM PEG); (b) induction of ZmPLC by low temperature (4 °C); (c) induction of ZmPLC by high salt (200 mM NaCl); (d) induction of ZmPLC by low osmotic stress. Total RNA (30 μg) was loaded in each lane, and the ethidium bromide-stained rRNA is shown as a control for the amount of RNA loaded in each lane.

fusion protein was insoluble and therefore was purified under denaturing conditions. The purified ZmPLC fusion protein was used to immunize adult rabbits. To test the specificity of the prepared polyclonal antibody, a small amount of soluble ZmPLC fusion protein was purified by GST·Bind Resin chromatography (Merck). Western blotting analysis showed that not only the purified ZmPLC fusion protein, but also the protein digested with thrombin were detected with the antiserum (Figure 5), which demonstrates that the polyclonal antibodies were specific for ZmPLC.

Discussion

We have now cloned a novel cDNA for a phospholipase C from maize. The ZmPLC primary structure had the typical features of a PI-PLC enzyme. Northern blot analysis revealed that expression of the *ZmPLC* gene was higher in roots compared with leaves. Analysis of *ZmPLC* transcription showed that it was induced by dehydration, high salt, cold and low osmotic stress conditions. These results suggest that the *ZmPLC* gene may play an important role in signal transduction under stress conditions and its accumulation may enhance the ability of plant cells to adapt to these conditions.

We expressed the full-length coding region of ZmPLC in *E. coli* strain BL21(DE3) and prepared anti-ZmPLC polyclonal antibody. Experiments to optimize expression of the *ZmPLC* protein indicated that high levels of expression were only produced with 1 mM IPTG and 6 h of induction at 37 °C. However, most of the ZmPLC protein in the bacterial cells was in the insoluble fraction similar to the AtPLC1 fusion protein (Hirayama *et al.* 1995) and the PsPLC protein, which was expressed in inclusion bodies (Venkataraman *et al.* 2003). These results suggest that the physical properties of PI-PLCs affect their location in *E. coli*. Furthermore, previous studies have indicated that plant PI-PLCs play a key role in stress signaling transduction pathways, so the production of anti-ZmPLC polyclonal antibody is an important step in the study

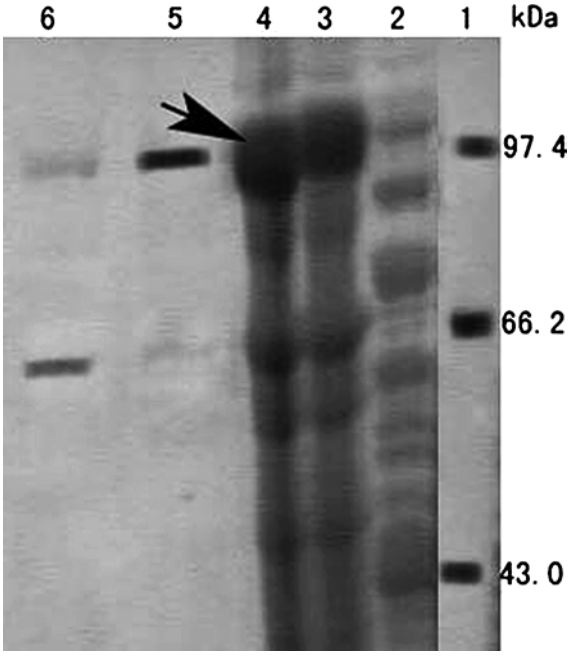

Fig. 4. SDS-PAGE analysis of the expression of recombinant *ZmPLC* fusion protein by *E. coli* transformants. Lane 1, protein marker; lane 2, bacterial proteins from BL21(DE3) transformed with pET42c–ZmPLC uninduced; lanes 3 and 4, bacterial proteins from BL21(DE3) transformed with pET42c–*ZmPLC* induced with 1 mm IPTG at 37 °C for 3 or 6 h respectively; lane 5, *ZmPLC* fusion protein purified by His·Bind Resin Chromatography; lane 6, thrombin-digested recombinant protein purified by GST·Bind Resin Chromatography. The expressed *ZmPLC* fusion protein is indicated with an arrow.

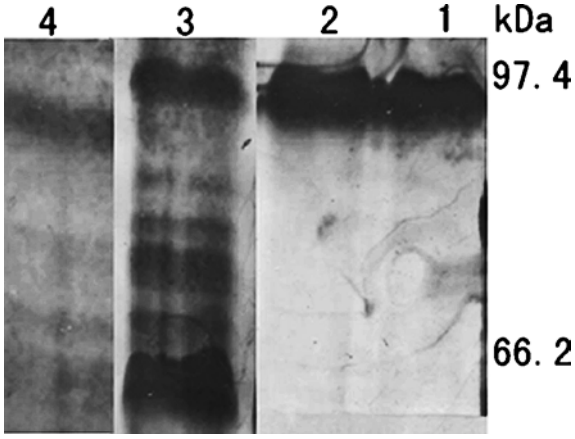

Fig. 5. Western blot analysis showing the specificity of the polyclonal antibody for *ZmPLC*. Lanes 1 and 2, purified recombinant *ZmPLC* fusion protein; lane 3, incompletely thrombin-digested recombinant fusion protein; lane 4, bacterial proteins from BL21(DE3) cells transformed with pET-42c(+) vector shown as a control. The membrane was probed with anti-*ZmPLC* fusion protein polyclonal antibody and the ZmPLC-antibody complex was visualized by staining with alkaline phosphatase conjugated to Goat anti-Rabbit IgG.

of these pathways and in understanding the function of ZmPLC.

Acknowledgement

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