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Structure Analysis, Prokaryotic Expression, and Subcellular Location of a New Protein Phosphatase 2C Protein from Maize

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ZmPP2C (AY621066) is a protein phosphatase 2C mRNA that we cloned from maize previously. TMpred program analysis indicated this protein contains a significant transmembrane helix of about 20 amino acid residues; however SVMtm predicted it is not a transmembrane protein. STRIDE, Modeller and RasMol program analysis suggested the ZmPP2C protein is a globular protein containing seven alpha helixes, fourteen beta sheets, twenty-two turns and one 3_{10} helix. The coding region of *ZmPP2C* mRNA was sub-cloned into expression vectors, pET30a-c(+), and introduced into *E. coli* BL21 (DE3) for expression. SDS-PAGE analysis indicated ZmPP2C was highly expressed at 37°C for 4 h with induction by 1 mM IPTG. Ultrasonic extraction experiment showed this recombinant protein was soluble in lysis buffer solution. The hexahistidine-tagged ZmPP2C fusion protein were purified and used to immunize rat for producing antibody. Protein gel blot analysis stated clearly that a specific polyclonal antibody against the protein was produced and can be used for further study about the *ZmPP2C* gene. Subcellular localization suggested that ZmPP2C protein was located in cell nucleus.

Key words: Subcellular location, Prokaryotic expression, Maize, Protein phosphatase 2C, Protein gel blot.

Protein [1] phosphatase 2C (PP2C) is one of the four protein serine/threonine phosphatases in both prokaryotes and eukaryotes(Cohen, 1989). It is encoded by different genes in plant species such as *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum* L.), beech (*Fagus sylvatica*), and alfalfa (*Medicago sativa* L.)(Kerk, *et al*., 2002). These PP2C proteins are involved in signaling pathways activated by stresses (including drought, salt, wounding and cold) and by plant hormones such as abscisic acid (ABA) and gibberellic acid (GA). These pathways cross-talk with each other and cover multiple steps in the cell-signaling network (Xiong *et al.*, 1999, 2002).PP2C-type phosphatases have also been reported to inactivate mitogen-activated protein kinase (MAPK) cascades in yeast and mammalian cells (Takezawa,2003, Warmka *et al*., 2001; Takekawa,*et al*., 1998).

The plant PP2Cs regulate many aspects of plant life, including inhibiting seed germination, promoting seed dormancy and plant senescence. It can also regulate plant responses to stresses negatively or positively(Schroeder *et al*.,2001; Finkelstein *et al*.,2001; Hetherington *et al*.,2003; Mumby *et al*.,1993). PP2Cs, including ABI1, ABI2, HAB1, AtPP2CA, RAB18 in *Arabidopsis* and FsPP2C1 in beech nut, all act as negative regulators of ABA signal transduction(Sheen,1998; Gosti *et al*., 1999; Kuhn *et al*.,2006; Saavedra *et al*., 2010). In vertebrate species, PP2Cs plays important roles in development of retina, regulation of cell trans-membrane signaling, fatty and sugar in liver(McGowan *et al*., 1988; McGowan *et al*., 2008;

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Schwarz *et al*., 2006; Barford, 1995; Barford, 1996). More and more type-2C protein phosphatases were cloned from maize(Broz *et al*., 2001). However, much less is known about the regulation mechanism of maize protein phosphatases 2C. Defining their molecular and physiological function will therefore enrich our understanding of signaling networks. The expression characteristics were very interesting, which was induced by Ca^{2+} but inhibited by ABA and Mg^{2+} (Xu *et al*., 2005). Our previous study demonstrated that over-expressed *ZmPP2C* in *Arabidopsis* could increase tolerance to salt and drought stress and decrease sensitivity to ABA (Liu *et al*., 2009). All the works emphasized the PP2Cs most in biology function but few in physical character. In recently years, along with the bioinformatics development, both bioinformatics programs and biology methods were used for studies about genes and proteins at the same time(Dai *et al*., 2007; Vlad *et al*., 2009). A conserved gate–latch–lock mechanism properly explained the relation of apo receptors to PP2C and ABA signaling: the apo receptors contain an open ligand-binding pocket flanked by a gate that closes in response to ABA by way of conformational changes in two highly conserved â-loops that serve as a gate and latch, tryptophan in the PP2C acts as a lock between the gate and latch(Melcher *et al*., 2009).

For further study, we analyzed some properties about ZmPP2C itself by some bioinformatics programs firstly, then characterized this protein localized at the nucleus by transient expression in onion epidermal cells. On the other hand, the *ZmPP2C* coding region was cloned into expression vectors, expressed as a fusion protein in *E. coli* strains, and purified to obtain the antigen. Protein gel blotting analysis indicated a specific polyclonal antibody against the ZmPP2C protein was produced, which can be used to study the expression characteristics and sub-cellular localization of *ZmPP2C* gene.

MATERIALS AND METHODS

Transmembrane, secondary and three dimensions structures analysis

Transmembrane regions and orientation of ZmPP2C polypeptide was predicted by the TMpred program online (http://www.ch.embnet.org/software/TMPRED_form.html). Amino acids sequence (ID or AC or GI) of ZmPP2C was input, the algorithm is based on the statistical analysis of TMbase, a database of naturally occuring transmembrane proteins. Secondary and tertiary structures were predicted by different methods(Rost *et al*., 1993). STRIDE, MODELLER and RasMol softwares were used for displaying macromolecules stucture.

Subcellular localization

For transient expression in onion epidermal cells, GFP-ZmPP2C fusions were constructed under the control of the 35S promoter in the pBI121 vector, GFP-fusion and control constructs were introduced into epidermal cells by particle bombardment (Bio-Rad, California, USA)(von Arnim *et al*.,1998, Saavedra *et al*., 2010). Epidermal peels were placed on solid MS medium for 16 h, 25oC in the dark, mounted on slides, and observed under a fluorescence microscope (Olympus BX51, Tokyo, Japan). All the vectors constructed were checked correct. And the primers are: 3'-ATGGTGAGCAAGGGCGAGGA-5' and 3'-TTACTTGTACAGCTCGTCCATGCCG-5'.

Expression and purification

The pET30a: ZmPP2C recombinants were transformed into E. coli BL21 (DE3). Positive clones were selected by PCR and restriction digestion with BamH I and Sal I, and then sequenced. The primers are: 3'-ATGGATCCATGGGGCTCGCCGGGGA-5' and 3' -ACGAGCTCCTAGGAGTGTTGGTCGTT-5'. Correct clones were cultured overnight at 37oC in 2×YT liquid medium suspended with 50 $mg.L^{-1}$ kanamycin, when OD_{600} reached 0.6, 1 mM IPTG (Isopropyl-â-D-thiogalactopyranoside) was added to induce expression of the fusion protein. The induced cells were harvested respectively at 0.5 h, 1 h, 2 h, 4 h, resuspended in PBS (phosphate-buffered saline) plus 1 mM PMSF (Phenylmethane sulfonyl fluoride) and sonicated on ice. The cell lysate was centrifuged, then the supernatant and sediment were examined by SDS-PAGE (polyacrylamide gel electrophoresis). The 6×his-tagged ZmPP2C protein was purified by Ni-NTA spin column (Novagen), as described in the pET System Manual.

Production of ZmPP2C antibodies and protein gel blot analysis

Crude extract of fusion protein was dialysed for concentration, and dried at -20°C, then

injected into rat with Freund's adjuvant four times for making primary antibody. The blood from rats was agglomerated and centrifuged (3000 rpm, 5 min) at 4°C, and the supernatant liquor was anti-ZmPP2C serum(Bradford *et al*., 1976). The antibody titer was determined by indirect ELISA(Govind , *et al*., 2000). Tissue samples from eight *Arabidopsis* lines, seven over-expressed *ZmPP2C* gene lines, one wild type lines, were homogenized in the ice-cold extraction buffer, then centrifugated at 4°C (12000 rpm, 20 min), The supernatant was collected and the protein content was determined by the Bradford method(Bradford *et al*., 1976). 20 mg total proteins were separated by SDS-PAGE and transfered onto a PVDF (Polyvinylidene Fluoride) membrane (Millipore Corporation, Billerica, MA, USA). After incubation with the primary antibody (ZmPP2C rat antiserum) and secondary antibody (sheep anti-rat IgG), protein blot signals were detected by ECL system (Amersham Bioscience, Uppsala, Sweden)(Ueda *et al*., 2008).

RESULTS AND DISCUSSIONS

Transmembrane analysis of ZmPP2C protein

ZmPP2C gene (AY621066) contained an open reading frame with 873 base pairs, encoding a 290 amino acid polypeptide. There was no report about ZmPP2C protein hydrophilic property. So we analysed its hydrophilic regions by the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). There was a significant transmembrane helix only about 20 amino acid residues: 170-189 from outside to inside, 167-185 from inside to outside (see Figure 1). These results suggested that most of the polypeptide hydrophilic.

Though there is a significant transmembrane peak in this protein, we defined it not a membrane-binding proteins. Firstly, the predicted transmembrane structure is very short, only 20 amino acid residues, 7.2% of the whole ZmPP2C polypeptide. For integral alpha-helical membrane proteins (cytoplasmic membrane proteins), it should be about 20 to 30%(Boyd *et al*., 1998a, 1998b). Secondly, the peak values (score) of the predicted transmembrane structure are only 621 (Figure 1a) and 741 (Figure 1b), they are not enough for a membrane protein(Hofmann *et al*., 1993). Furthermore, we predicted this protein

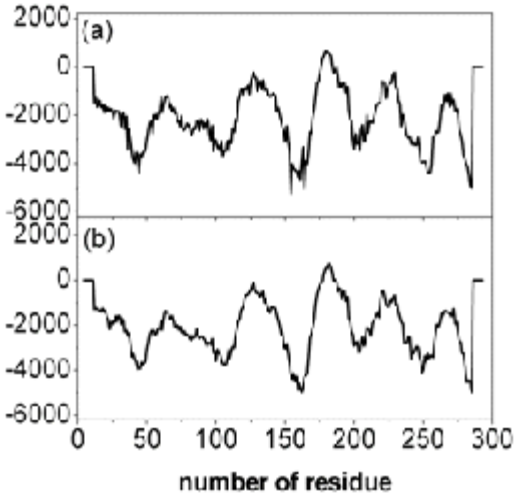
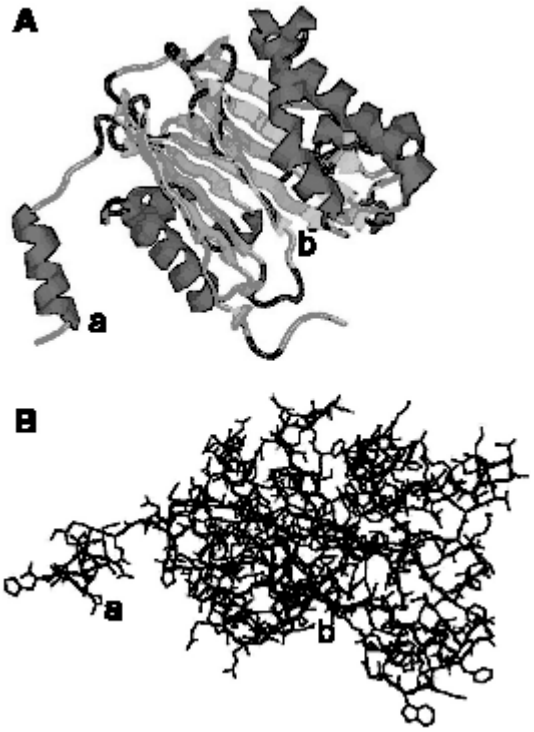


Fig. 1. Hydropathy plot analysis of the predicated ZmPP2C polypeptide by TMpred program



A, Secondary structures sketch map; B, Tertiary structures sketch map.
a, an amino arm out of the globular ZmPP2C protein; b, a pocket structure in the globular ZmPP2C protein

Fig. 2. Two- and three-dimensional structure sketch map of ZmPP2C protein

by SVMtm Transmembrane Domain Predictor on line server (http://ccb.imb.uq.edu.au/svmtm/SVMtm_Predictor.shtml) (Yuan *et al*., 2004). The result showed that the number of transmembrane domains is zero (data not show). Taken together, these results indicated ZmPP2C protein is not a transmembrane protein.

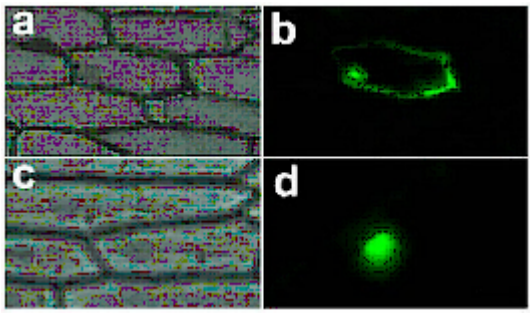
The hydrophobic and hydrophilic positions are plotted above and below the zero point of ordinate, respectively. (a) inside -> outside, (b) outside -> inside

Two- and three-dimensional structure analysis of ZmPP2C protein

To define the structure of ZmPP2C protein, we utilized STRIDE and MODELLER programs to analyze its two- and three-dimensional structure. The results indicated there were seven alpha helixes, fourteen beta sheets, twenty-two turns and one 3_{10} helix in the protein (Figure 2A), and there was a pocket structure in the 3-D image (Figure 2). From the 3-D image of the ZmPP2C protein, we can see there was a long polypeptide arm protruding out of the globular ZmPP2C protein (Figure 2Aa). We considered the pocket structure and the polypeptide arm may have something to do with its active center. In recent years, molecular biological methods were used widely to study genes and proteins. But little information about PP2Cs structure in maize is known. So which amino acid residue makes up the active site concretely and is concerned with the protein activity or not should be studied further. On the other hand, although there were some reports about PP2C proteins structure analysis, most of them were about iatrical and diseases of animal and human(Saito *et al*., 2008; Yamaguchi *et al*., 2006; Wang *et al*., 2002; Anantharaman V, *et al*., 2006). From the figure 2, we can see the predicted transmembrane helix (hydrophobic region) (see Figure 1)was located inside the ZmPP2C protein, and other predicted non-transmembrane helixes (hydrophilic region) (see Figure 1) were distributed around. This result suggested the ZmPP2C protein might be a soluble protein in plant cell.

Subcellular localization of ZmPP2C protein

To confirm ZmPP2C subcellular localization, we constructed a GFP-ZmPP2C recombinant under the control of a cauliflower mosaic virus (CaMV) 35S promoter. The expression vector pBI121 with GFP-ZmPP2C were transferred into onion epidermal cells by bombardment with DNA-coated gold particles. Transient expression in onion epidermal cells demonstrated that GFP alone was found in both membrane and nucleus (Figure 3b), whereas GFP-ZmPP2C fusion protein was found densely localized in nucleus (Figure3d). Those results indicated that GFP-ZmPP2C fusion protein located in intracellular components rather than in cell membrane. This is consistent with the prediction of Tmpred software that most of the polypeptide of the ZmPP2C protein are hydrophilic domains (Figure 1).

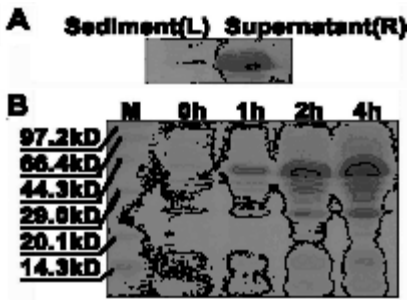


a and b, GFP alone expressed in onion epidermal cells; c and d, GFP-ZmPP2C fusion protein expressed in onion epidermal cells; a and c are corresponding bright-field images of b and d. The scale bar represents 50 mm

Fig. 3. Localization of ZmPP2C-GFP protein in onion epidermal cells

SDS-PAGE of ZmPP2C recombinant protein

The plasmid DNA was extracted from anti-kanamycin clony and purified. Then the purified plasmid DNA was restrictively digested with *Bam*H I and *Sal* I. The agarose gel electrophoresis result indicated ZmPP2C mRNA was inserted into the pET30a expression vector. The clony was sequenced and the results further indicated *ZmPP2C* is in frame in pET30a vector. The ZmPP2C protein was induced by 1 mM IPTG at 37°C. Supernatant and sediment were separated by SDS-PAGE after ultrasonic fragmentation of the cells. As Figure 4A shows, supernatant contains most fusion protein. This result indicated ZmPP2C protein was soluble which according with the prediction by TMpred software (see Figure 1). SDS-PAGE in course of time step results demonstrated the fusion protein has a highest production after induced 4 h with 1 mM IPTG (see Figure 4B), and



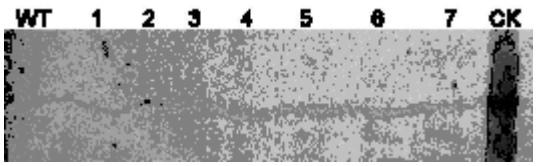
A: SDS-PAGE of ZmPP2C recombinant protein after ultrasonic fragmentation; B: SDS-PAGE of ZmPP2C recombinant protein collected after 0 h, 1 h, 2 h, and 4 h, respectively; M: low protein molecular weight marker

Fig. 4. SDS-PAGE of ZmPP2C recombinant protein

the production reduced after 4 h (data not show). In Figure 4B, the molecular weight of recombinant protein is bigger than predicted (about 33 kDa). The hexahistidine-tag and systematic errors maybe attributed to this(Cserzo, *et al*., 2002; Li *et al*., 2007).

Protein gel blotting analysis

The fusion protein 6×his-ZmPP2C was purified by Ni-NTA spin column and injected into rat for making primary antibody. To investigated whether the antiserum produced by ZmPP2C fusion protein can be used to examine protein in plant or not, wild type and over-expressed ZmPP2C *Arabidopsis* lines were used as materials for protein gel blotting analysis. There is no *ZmPP2C* gene in wild type *Arabidopsis* genome, except in the transgenic *Arabidopsis*, which was a perfect control. As Figure 5 showed, the signals were detected in over-expressed lines (1-7) and purified recombinant ZmPP2C fusion protein *in vitro*, but not in the wild type line. So the antiserum of ZmPP2C fusion protein was suitable for further study, such as expression characteristics in protein level in maize, sub-cellular localization of ZmPP2C protein by immunohistochemistry analysis, transgenic plant identification and so on.



WT: wild type *Arabidopsis*; Lane1-7, over-expressed ZmPP2C transgenic *Arabidopsis* lines; CK: purified recombinant ZmPP2C protein

Fig. 5. The identification of transgenic plants with western blotting

CONCLUSIONS

In this study we analyzed the structures, subcellular location, prokaryotic expression and purification of a maize protein phosphatase 2C protein (ZmPP2C). The results indicated that most of this protein was hydrophilic, and has seven alpha helixes, fourteen beta sheets, twenty-two turns and one 3_{10} helix. There was a pocket structure in and a long polypeptide arm protruded out of the globular ZmPP2C protein in the 3-D simulative structure. Subcellular localization suggested that ZmPP2C protein was located in cell nucleus. SDS-PAGE results implied that the ZmPP2C protein can be expressed in *E.coli* and it was soluble. We conjecture that ZmPP2C protein in maize cell is not a membrane protein, and the further study is carrying on. At last, the protein gel analysis proved the ZmPP2C protein expressed *in vitro* is effective for antibody production and for further study.

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