# Isolation and Expression of a Maize Type 1 Protein Phosphatase[1]

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## ABSTRACT

The dephosphorylation of phosphoproteins by protein phosphatases represents an important mechanism for regulating specific cellular processes in eukaryotic cells. The aim of the present study was to examine the structural and biochemical characteristics of a specific class of protein Ser/Thr phosphatases (type 1 protein phosphatases) which have received very little attention in higher plants. A cDNA clone (ZmPP1) was isolated from a maize (Zea mays L.) cDNA library. The deduced amino acid sequence is 80% identical with a 292-amino acid core region of rabbit and yeast type 1 protein phosphatase catalytic subunit. Southern blot analysis indicates that ZmPP1 may belong to a family of related genes in maize. ZmPP1 RNA was present in all maize tissues examined, indicating that it may play a fundamental role in cellular homeostasis. To demonstrate that ZmPP1 encodes an active protein phosphatase and, in an effort to characterize this gene product biochemically, high levels of ZmPP1 were expressed in Escherichia coli. Active ZmPP1 enzyme dephosphorylates rabbit phosphorylase a and is strongly inhibited by okadaic acid and by the mammalian inhibitor-2. These data show that ZmPP1 is structurally and biochemically very similar to the corresponding enzyme in animal cells. These results also suggest that the function and regulation of the higher plant type 1 protein phosphatases may be similar to the mammalian protein phosphatases.

The reversible phosphorylation of proteins is a primary mechanism by which metabolic and developmental processes are regulated in eukaryotic cells (14). The protein kinases and phosphatases that catalyze the phosphorylation and dephosphorylation of proteins, respectively, have been well characterized in animal cells (7). Less is known about these enzymes in plant cells, although a number of studies of plant protein kinases have recently received more attention (5). In contrast, plant protein phosphatases are poorly understood. Recent studies have identified protein phosphatases in higher plants that correspond to two of the four major protein phosphatase groups (PP1[2] and PP2A) present in animal cells. PP1 has been

[2] Abbreviations: PP1, 2A, 2B, and 2C, types 1, 2A, 2B, and 2C protein phosphatase(s); I-1 and -2, inhibitor-1 and -2; $IC_{50}$, concentration required for 50% inhibition; IPTG, isopropyl $\beta$-D-thiogalactoside; MCH1, maize 14S ribosomal protein; PCR, polymerase chain reaction; ZmPP1, maize PP1; PP1c, catalytic subunit of PP1; SSC, standard sodium citrate; bp, base pair(s); poly(A)+ RNA, polyadenylated RNA.

partially purified from Brassica napus seeds (18) and from wheat (20), and PP2A has been partially purified from B. napus seeds (18) and from Bryophyllum fedtschenkoi (6). In addition, partial cDNA clones of PP1 and PP2A have been isolated from B. napus (19). The deduced amino acid sequences of these clones show a high degree of similarity to mammalian PP1 and PP2A; however, it remains to be determined whether the Brassica cDNA clones encode active protein phosphatases. Other plant protein phosphatases have been described, but it is not known whether they bear any relation to the major groups of protein phosphatases from animal cells (11, 17, 23, 24). The functions of PP1 and PP2A in plant cells are not known, although recent studies indicate that PP2A may dephosphorylate spinach sucrose phosphate synthase (12, 26) and phospho*enol*pyruvate decarboxylase (6).

A comparison of the primary structure of mammalian PP1, PP2A, and PP2B reveals that they belong to a related family of protein phosphatases that are distinct from the PP2C family of protein phosphatases (8). Although each major group of protein phosphatases can be distinguished structurally, their classification is generally based on their biochemical characteristics (7). Mammalian PP1 selectively dephosphorylates the $\beta$ subunit of phosphorylase kinase and is specifically inhibited by the mammalian I-1 and I-2. PP2A, PP2B, and PP2C preferentially dephosphorylate the $\alpha$ subunit of phosphorylase kinase, are insensitive to I-1 and I-2, and are distinguished from each other by their requirements for divalent cations. PP2A activity requires no divalent cations for activity, whereas PP2B and PP2C have absolute requirements for $Ca^{2+}$ and $Mg^{2+}$, respectively. An additional parameter that is useful in distinguishing the different types of protein phosphatases is their sensitivity to the marine sponge toxin, okadaic acid (3). Okadaic acid has a greater specificity for PP2A ($IC_{50}$ = 1 nM) than for PP1 ($IC_{50}$ = 10 nM) and shows relatively no effect toward PP2B and PP2C in the nanomolar range (7).

The plant types 1 and 2A protein phosphatases that have been partially purified are biochemically nearly indistinguishable from the corresponding enzymes present in mammalian cells (18). The sensitivity of the plant PP1 to the mammalian I-1 and I-2 suggests that mammalian and plant PP1 are structurally conserved and raises the possibility that plants may contain endogenous proteins analogous to I-1 and I-2 in mammalian cells that inhibit the activity of PP1. The PP1c in mammalian cells is further regulated in vivo by targeting subunits that direct PP1c to specific subcellular targets (i.e. glycogen particles, myofibrils, and sarcoplasmic reticulum) and that modulate the activity of PP1 toward select substrates (7). Thus, free PP1c is not found in mammalian cells but is

normally complexed to various regulatory subunits and inhibitors. Forty percent of the PP1 activity extracted from *B. napus* seeds is recovered in a 20,000-g pellet, suggesting that PP1 may be localized in organelles or that it may be associated with various subcellular particles *in vivo*. Thus, plant cells may contain regulatory subunits that modulate the activity of PP1 as do regulatory subunits present in animal cells.

In an effort to gain a better understanding of the structural and biochemical characteristics of plant PP1, we have isolated a PP1 cDNA clone from maize and have synthesized high levels of this protein using an *Escherichia coli* expression system. Structural analysis of the deduced amino acid sequence of *ZmPP1*, and biochemical characterization of the recombinant ZmPP1 protein *in vitro*, demonstrates that PP1 from higher plants and animals is highly conserved.

## MATERIALS AND METHODS

### Genomic Southern Blot Analysis

Maize (*Zea mays* L. inbred line B73) genomic DNA was isolated from 7-d-old seedlings (25). The genomic DNA was digested with either *Eco*RI or *Hin*dIII, subjected to electrophoresis through 0.8% agarose gels, and transferred to nylon membranes by the method of Southern (27). Lanes were loaded with either 12.5 μg maize DNA or 7.5 μg *Arabidopsis* DNA. The filters were prehybridized and hybridized at 42°C in 50% formamide, 100 μg/ml sonicated salmon testes DNA, 100 μg/ml yeast RNA, 5× Denhardt's solution, 50 mM sodium phosphate (pH 6.5), 5× SSC, and 0.2% SDS. Filters were prehybridized for 4 h and hybridized for 16 to 20 h with about 1 × 10⁶ cpm/ml of an 800-bp cDNA clone encoding nearly the entire *ZmPP1* open reading frame. The filters were washed twice in 2× SSC and 0.2% SDS at room temperature, once in 2× SSC and 0.2% SDS at 65°C, and once in 0.2× SSC and 0.2% SDS at 65°C. The filters were then exposed to x-ray film (Kodak X-AR) with an intensifying screen.

The filters were subsequently striped with 0.1× SSC and 0.1% SDS at 100°C and rehybridized using the same probe under less stringent conditions. Prehybridization and hybridization conditions were the same as described above except that the hybridization buffer contained 25% formamide. Filters were rinsed three times with hybridization buffer (25% formamide) lacking probe at room temperature and exposed to x-ray film.

### Northern Blot Analysis

Poly(A)⁺ RNA was isolated from maize and maize Black Mexican Sweet suspension cell culture as previously described (30). The maize tissues included 6-d-old seedling roots and shoots (coleoptile, leaves, and stem), mature leaves, pollen-receptive silks, emerging tassels, unfertilized cobs, ear shoots, and husks. Poly(A)⁺ RNA (2 μg/lane) was electrophoresed on 6% formaldehyde/1% agarose gels and transferred to nylon membranes. Prehybridization, hybridization, and washing conditions for Northern blots were identical with those used for Southern blots. The filters were hybridized with about 1 × 10⁶ cpm/ml of the *ZmPP1* cDNA clone and subsequently rehybridized with about 1 × 10⁶ cpm/mL of an *MCH1* cDNA clone (16). The filters were exposed to x-ray film for 42 h.

Relative transcript levels were measured by scanning the developed x-ray film with a densitometer (Ultroscan XL, Pharmacia).

### Isolation, Cloning, and Sequencing of Protein Phosphatase cDNA Clones

Single-stranded cDNA was synthesized from poly(A)⁺ RNA isolated from maize roots using oligo(dT) as primer and subsequently used as a template for the PCR using degenerate oligonucleotides as primers as previously described (30). The PCR products were cloned into the *Sma*I site of pUC19, and the resulting recombinant plasmids were sequenced with Sequenase (US Biochemicals). The amino acid sequences deduced from the nucleotide sequences of the clones were compared with published amino acid sequences from mammalian and yeast type 1 protein phosphatases, and clones showing a high degree of amino acid identity were selected as probes to screen a λgt11 cDNA library synthesized from maize root RNA (30). A total of 1.6 × 10⁵ recombinants from the library were screened with the recombinant plasmids containing the putative protein phosphatase PCR products. Ten positive recombinants were selected for further analysis. Nucleotide sequences were determined by sequencing 100% of both strands.

### Construction of a Protein Phosphatase Expression Plasmid

A full-length *ZmPP1* cDNA clone was digested with *Bam*HI and *Nde*I, and the sites were filled in using the Klenow fragment of DNA polymerase I. The *Bam*HI-*Nde*I fragment was purified from an agarose gel and cloned behind the bacteriophage T7 promoter in the vector pT7-7 which had previously been digested with *Bam*HI and filled in with Klenow (29). The resulting pT7-ZmPP1 plasmid was sequenced to verify that the *Bam*HI-*Nde*I fragment was in the correct orientation and in-frame. The first amino acid (Met) of ZmPP1 was replaced by four amino acids (Met-Ala-Arg-Ile) from the pT7-7 vector. The remaining 315 amino acids corresponded to the maize protein phosphatase.

### Expression of the Protein Phosphatase in *E. coli*

The *E. coli* strain BL21(DE3) (28) containing the pT7-ZmPP1 plasmid was grown overnight at 37°C in LB media containing 40 μg/mL ampicillin. The overnight culture (5 ml) was diluted in 50 mL LB plus ampicillin and grown for 3 h at 37°C. Expression of ZmPP1 was induced by the addition of IPTG to a final concentration of 1 mM (29). The induced cells were incubated for an additional 5 h at 37°C and harvested by centrifugation for 10 min at 3000 rpm. Cell lysis using lysozyme was carried out as outlined by Martson (21). Inclusion bodies containing the recombinant ZmPP1 protein were purified by centrifugation at 12,000g for 10 min and washed three times with 9 volumes of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, and 0.5% (v/v) Triton X-100. Inclusion bodies were then resuspended to a final concentration of 200 μg protein/mL in 6 M urea (deionized), 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 2.5 mM EDTA, 0.2% 2-

mercaptoethanol, and 0.1% Tween-80 and incubated at room temperature for 1 to 2 h. Insoluble material was removed by centrifugation at 12,000g for 10 min.

Urea-solubilized maize protein phosphatase was renatured as outlined by Berndt and Cohen (2) with modifications. Denatured protein in urea buffer was rapidly diluted with 100 volumes of renaturation buffer: 50 mM Tris-HCl (pH 7.0), 30 mM 2-mercaptoethanol, 0.8 M NaCl, 1 mM $MnCl_2$, and 0.02% Tween-80 and incubated for 40 min at room temperature. The renatured protein was subsequently dialyzed overnight (4°C) against two changes of the renaturation buffer lacking NaCl. The samples were stored in 50% glycerol at −20°C and diluted 10-fold before assaying for phosphatase activity. Protein samples were analyzed by SDS-PAGE by the method of Laemmli (15). Proteins separated on SDS-PAGE were stained with Coomassie Brilliant Blue R-250 and scanned with a densitometer to estimate the relative abundance of ZmPP1 in the samples. Protein concentrations were measured using the method of Bradford (4).

## Protein Phosphatase Activity

$^{32}$P-labeled rabbit skeletal muscle phosphorylase $a$ ($10^6$ cpm/nmol) was prepared by incubating phosphorylase $b$ (10 mg/ml) with phosphorylase kinase (0.2 mg/ml) from rabbit skeletal muscle in the presence of $[\gamma$-$^{32}$P]ATP ($10^6$ cpm/nmol) and subsequently purified as described by Cohen et al. (9). Protein phosphatase reactions were carried out by preincubating 10 μL diluted enzyme (1:10) sample with an equal volume of reaction buffer, 50 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, and 0.03% Brij-35 for 10 min at 30°C (9). The reactions were initiated by the addition of 10 μL [$^{32}$P]phosphorylase $a$ (3 mg/ml) diluted in reaction buffer containing 15 mM caffeine and incubated for 10 min at 30°C. The reactions were terminated by adding 100 μL 10% (w/v) TCA, and the suspension was centrifuged at 12,000g for 5 min. An aliquot of the supernatant was added to a scintillation cocktail and counted to determine the amount of $^{32}$P released. One unit of activity is the amount of enzyme required to catalyze the dephosphorylation of 1.0 μmol phosphorylase $a$/min at 30°C. When okadaic acid (Moana Biochemicals, Honolulu, HI) or I-2 was included in the reactions, diluted enzyme samples were preincubated with these compounds for 15 min before initiating the reactions with substrate (10, 18).

## RESULTS

### Isolation and Characterization of cDNAs Coding for Type 1 Protein Phosphatases

Mixed oligonucleotides (Fig. 1) corresponding to two separate amino acid sequences conserved in PP1 (22) were used as primers in the PCR to amplify maize cDNA sequences (30) encoding putative PP1. PCR products of a single size range (350–370 bp) were obtained, cloned into plasmids, and sequenced. A clone (357 bp) was identified whose deduced amino acid sequence was 80% identical with the corresponding amino acid sequence of the rabbit skeletal muscle PP1 catalytic subunit (PP1α). This clone was used as a hybridization probe to isolate full-length clones from a maize root



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                         GAGACCCATTACCAATGCCAAGAACAAGAAACCAGAGA   -361
GGACAGCCGCGAATACAATCCGAATCCCCTCCAAAAACCCACCATACCGTCTCCCCCATC   -301
TGCAAGAACAAGATCAGGAGAGCATCCTAGGGGGAAGAAACAGGAGGGGGAAAAAGAAGC   -241
CCAGAAAGACCAACTGACCAAACAAAAAGGTGGCATCTTTGTGTCACCGAGGCACCGCCG   -181
ATCACAGACCAAAGCCCGCTCCTCTGGTCCATCCCAAGCGTACAGCAGCCGAGATTTCTT   -121
CGATCTGCGCTTGCGTTTTTTTTTCTTGTAAAGATTTTAGTTTTCCCCCCTTCAACTGTT   - 61
GCTAGCCTTGAGTCGCTGAGAAGTTTCGAGAAGGGTGGTGTGAGTGTCGGATAGCTCAAG   -  1
ATGGATCCGGCGTTGCTGGACGACGTCATACGCCGGCTTCTGGAGGTGAAGAATCTCAAG     60
M  D  P  A  L  L  D  D  V  I  R  R  L  L  E  V  K  N  L  K       20

CCTGGGAAGAACGCGCAGCTGTCAGAGTCGGAGATTAAGCAGCTCTGCGCTGCCGCCAAG    120
P  G  K  N  A  Q  L  S  E  S  E  I  K  Q  L  C  A  A  A  K       40

GAGATCTTCCTGCAGCAGCCCAACCTGCTGGAGCTCGAGGCCCCCATCAAAATCTGCGGT    180
E  I  F  L  Q  Q  P  N  L  L  E  L  E  A  P  I  K  I  C  G       60

GATGTCCATGGCCAGTACTCTGATCTCCTGAGGCTATTTGATTATGGTGGCTATCCGCCT    240
D  V  H  G  Q  Y  S  D  L  L  R  L  F  D  Y  G  G  Y  P  P       80

CAGGCCAACTACCTTTTCTTGGGTGATTATGTGGATCGTGGAAAGCAAAGCCTAGAAACA    300
Q  A  N  Y  L  F  L  G  D  Y  V  D  R  G  K  Q  S  L  E  T      100

ATATGTCTTCTTTTGGCTTACAAGGTCAAGTACCCGGAGAACTTCTTTCTTCTAAGGGGC    360
I  C  L  L  L  A  Y  K  V  K  Y  P  E  N  F  F  L  L  R  G      120

AACCATGAATGCGCATCAGTAAATCGCATCTATGGTTTTTATGACGAGTGCAAACGCAGA    420
N  H  E  C  A  S  V  N  R  I  Y  G  F  Y  D  E  C  K  R  R      140

TTCAGTGTAAAGCTGTGGAAAACATTCACAGACTGTTTTAACTGCTTGCCAGTGTCAGCA    480
F  S  V  K  L  W  K  T  F  T  D  C  F  N  C  L  P  V  S  A      160

TTGATAGATGAAAAGATTCTCTGTATGCATGGTGGTCTATCTCCGGAGTTGAACAAGCTT    540
L  I  D  E  K  I  L  C  M  H  G  G  L  S  P  E  L  N  K  L      180

GAGCAAATACTCAACCTGAATCGCCCCACAGACGTGCCTGATACTGGATTGCTCTGTGAT    600
E  Q  I  L  N  L  N  R  P  T  D  V  P  D  T  G  L  L  C  D      200

CTTCTTTGGTCTGATCCTTCCAATGAAGCAACAGGCTGGGCCATCAATGATCGTGGTGTT    660
L  L  W  S  D  P  S  N  E  A  T  G  W  A  I  N  D  R  G  V      220

TCATTCACATTTGGTCCTGATAAAGTTTCTGAATTTCTTGAGAAGCATGATTTGGACCTT    720
S  F  T  F  G  P  D  K  V  S  E  F  L  E  K  H  D  L  D  L      240

ATCTGCCGAGCACATCAGGTTGTCGAAGATGGATACGAGTTTTTCGCTAGCCGTCAACTC    780
I  C  R  A  H  Q  V  V  E  D  G  Y  E  F  F  A  S  R  Q  L      260

GTAACAATATTTTCAGCCCCTAACTACTGTGGAGAATTCGACAACGCTGGTGCCATGATG    840
V  T  I  F  S  A  P  N  Y  C  G  E  F  D  N  A  G  A  M  M      280

AGTGTAGATGATACATTGATGTGCTCGTTCCAAATATTGAAGCCTGCAAGGAAGATGATG    900
S  V  D  D  T  L  M  C  S  F  Q  I  L  K  P  A  R  K  M  M      300

GGTGGTTCAACTAATAACAAATCTGGCTTCAAGTCATTCAGGGGATGGTGACAACGGCCT    960
G  G  S  T  N  N  K  S  G  F  K  S  F  R  G  W  *            316

GATGGTGGGCACGCTGCGTTCGACAGGGGGTACATCTTACAGCGGCTGCAACTAACAGGC   1020
ATATGCATTTTCGCGGAAACCTGCTTGGGCCTTGGAAGGGCAAAGCAGATGTAGAACTGT   1080
GCATCTTCCATGTTGGATTAACACTTGATGTAATGTATTGTTATCGGATTTTTACTGTGC   1140
GGATCTCCTTATGATATGGGAGGATGTGAATGAGTACTGCCCTCCCCTTTGCGTCCTAAG   1200
ATTCGGAAAGAATCCATTCGCCAATAAAAATAGCCAGACTATTCT               1245
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**Figure 1.** Nucleotide and deduced amino acid sequences of the maize ZmPP1 cDNA clones. The deduced amino acid sequence is presented using the single letter code. Mixed oligonucleotides used in the present study (see "Materials and Methods") correspond to the underlined amino acid sequences.

cDNA library. The screening of 160,000 recombinants yielded 10 positive clones. Two of the clones (88-C and 8–17) with inserts of 1.1 and 1.5 kilobases, respectively, were sequenced. The clones were identical over a 950-bp overlapping sequence. The entire 1645-bp sequence (ZmPP1) contained an open reading frame that encodes a protein of 316 amino acids with a mol wt of 35,739 (Fig. 1). This sequence represents a nearly complete transcript; Northern blots indicate that the ZmPP1 mRNA is approximately 1700 nucleotides (Fig. 3). The sequence also contains 399-bp 5'- and 297-bp 3'-noncoding regions (Fig. 1). Although a polyadenylation site was not present in either of the two cDNA clones, a putative polyadenylation signal, AATAAA, is located 15 nucleotides upstream of the 3' end of the sequence (Fig. 1).

The deduced amino acid sequence of ZmPP1 shows significant sequence similarity to the rabbit skeletal muscle PP1α sequence (1) and to the deduced amino acid sequences of yeast (bws1+) and B. napus PP1 cDNA clones (19, 22). The sequence similarity is seen only within a core region of 292

amino acids (amino acids 7–298 of ZmPP1; Fig. 2). Within the core region, there is 80% identity and 90% conservation between ZmPP1 and both the rabbit and yeast amino acid sequences (Fig. 2) and 67% identity between the maize and rabbit nucleotide sequences (data not shown). The partial cDNA clone from *B. napus* shows 77% identity and 90% conservation between amino acids 63 and 298 of ZmPP1 (Fig. 2). The sequences on either side of the core region are highly variable, and both the carboxyl and amino termini of ZmPP1 are shorter than the corresponding ends of the rabbit and yeast sequences (Fig. 2).
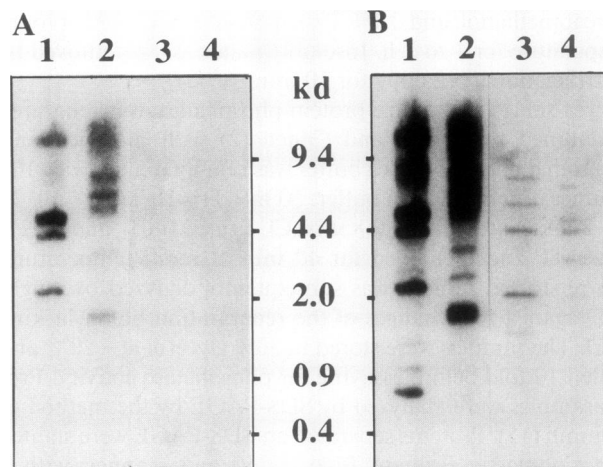
Southern blot analysis of maize genomic DNA was performed with the *ZmPP1* cDNA clone to determine whether *ZmPP1* belongs to a family of related genes in maize. Under high stringency hybridization conditions we observed four or five bands (Fig. 3A). Four additional bands were revealed under reduced stringency hybridization conditions (Fig. 3B), suggesting that one or more related genes are present in maize. We also performed a Southern blot analysis of *Arabidopsis thaliana* genomic DNA with the maize clone to determine whether related genes could be observed in this species. No bands were observed under high stringency hybridization conditions (Fig. 3A), but five or six bands were observed under low stringency (Fig. 3B).

## Expression of *ZmPP1* mRNA

Northern blots of RNA isolated from vegetative (dark-grown seedling roots and shoots, light-grown seedling shoots,
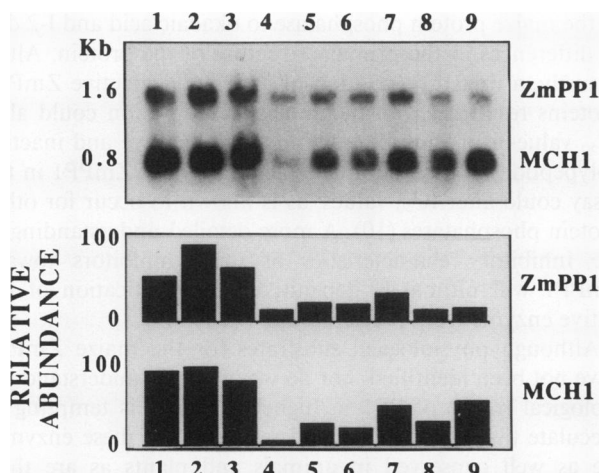


**Figure 3.** Hybridization of maize *ZmPP1* cDNA clone to maize and *Arabidopsis* genomic DNA. A, Southern blot of maize genomic DNA (lanes 1 and 2) and *Arabidopsis* genomic DNA (lanes 3 and 4) under high stringency hybridization conditions. Genomic DNA was digested with *Eco*RI (lanes 1 and 3) or *Hind*III (lanes 2 and 4). B, Southern blot of maize genomic DNA (lanes 1 and 2) and *Arabidopsis* genomic DNA (lanes 3 and 4) under reduced stringency hybridization conditions. Genomic DNA was digested with *Eco*RI (lanes 1 and 3) or *Hind*III (lanes 2 and 4).

husks, and mature leaves) and reproductive tissues (emerging tassels, pollen-receptive silks, and ear shoots), and suspension tissue culture cells were hybridized with the maize cDNA clone to determine the relative abundance of *ZmPP1* mRNA transcripts in various maize tissues. The 1.7-kilobase *ZmPP1* mRNA is expressed in all tissues examined (Fig. 4). The highest levels of *ZmPP1* expression were in seedling roots and shoots, and the lowest levels were found in mature and reproductive tissues and in tissue culture cells (Fig. 4). As a control, hybridization to a *MCH1* is shown (Fig. 4 and ref. 16).

## Expression of ZmPP1 in *E. coli*

To examine whether *ZmPP1* codes for an active PP1, we expressed ZmPP1 in *E. coli* to assay activity of the recombinant protein *in vitro*. The *ZmPP1* cDNA was cloned behind the bacteriophage T7 promoter in the *E. coli* expression vector pT7–7 (29). The expression of ZmPP1 was under the control of the T7 promoter, and the T7 RNA polymerase was under the control of the *lac* promoter. The expression of *ZmPP1* was induced by the addition of IPTG to the culture medium. Little ZmPP1 protein was synthesized in the absence of IPTG (data not shown), whereas a prominent 36-kD protein was synthesized in IPTG-induced cells carrying the pT7-ZmPP1 plasmid (Fig. 5, lane 5). The protein was not present in cells carrying the control plasmid pT7–7 (Fig. 5, lanes 2 and 3). Nearly all of the ZmPP1 protein was recovered in the insoluble fraction as inclusion bodies (Fig. 5, lane 5), whereas only a minor amount of the total protein was present in the soluble fraction (Fig. 5, lane 4). The ZmPP1 protein accounted for approximately 55% of the protein comprising the inclusion bodies based on a densitometric analysis of SDS-PAGE gels.



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                10        20        30        40        50        60
ZmPP1       MDPALLDDVIRRLLEVKNLKPGKNAQLSESEIKQLCAAAKEIFLQQPNLLELEAPIK
bws1+       MSNPDVD--SI-D-----RGSR--RQV----D--RF--NK-R---IS--I-------L-
PP-1        MSDSEKLN--SI-G-----QGSR----V--T-N--RG--LKSR----S--I-------L-
Brassica PP1

                70        80        90       100       110       120
ZmPP1       ICGDVHGQYSDLLRLFDYGGYPPQANYLFLGDYVDRGKQSLETICLLLAYKVKYPENFFL
bws1+       ----I----Y------E---F--E-----------------V--------I--------I
PP-1        ----I----Y------E---F--EF-------------------------I--------
Brassica PP1  --I----Q------E------S--F----------------------IR--SKIY-

               130       140       150       160       170       180
ZmPP1       LRGNHECASVNRIYGFYDECKRRFSVKLWKTFTDCFNCLPVSALIDEKILCMHGGLSPEL
bws1+       ---------I--------------YNI--------------IA-I-----FT-------D-
PP-1        ---------I--------------YNI--------------IA-IV----F-C------D-
Brassica PP1  ------D-KI--------------N-R---I----------A----D-------------

               190       200       210       220       230       240
ZmPP1       NKLEQILNLNRPTDVPDTGLLCDLLWSDPSNEATGWAINDRGVSFTFGPDKVSEFLEKHD
bws1+       -SMD--QRIM------------------DKDL---GD------------V--R--H---
PP-1        QSM---RRIM------Q----------DKDVQ--GE----------AEV-AK--H---
Brassica PP1  DN-NS-REIQ---EI-S-----------DQKIE---DS---I-C---A---A---D-N-

               250       260       270       280       290       300
ZmPP1       LDLICRAHQVVEDGYEFFASRQLVTIFSAPNYCGEFDNAGAMMSVDDTLMCSFQILKPAR
bws1+       M--V--------------SK-----L--------------------ES-L---------E
PP-1        ------------------K----L--------------------E------------D
Brassica PP1  ------G------------K-R----------G--------LL---ES-V---E-M---L

               310       320       330
ZmPP1       KMMGGSTNNKSGFKSFRGW
bws1+       -KQRYGYQGS-QNWHMTPPRKNKTGNSK
PP-1        -NK-KYGQLSGLNPGGRPITPPRNSAKAKK
Brassica PP1  ASSS-HPLK-VPKMGKS
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**Figure 2.** Amino acid sequence comparison among PP1 from maize, rabbit, yeast, and *B. napus*. From top to bottom, maize *ZmPP1*; yeast *bws1+*; rabbit *PP1α*; *B. napus PP1*. Hyphens indicate identical residues.

**Figure 4.** Expression of *ZmPP1* and *MCH1* in various tissues from maize. A, Northern blot analysis of *ZmPP1* RNA and *MCH1* RNA in different maize tissues. Transcript sizes are given on the left axis. Each lane contained 2 µg poly(A)$^+$ RNA. Lanes: 1, seedling roots; 2, dark-grown seedling shoots; 3, light-grown seedling shoots; 4, mature leaves; 5, husks; 6, emerging tassels; 7, silks; 8, ear shoots; 9, tissue culture cells. B, Relative abundance of *ZmPP1* and *MCH1* RNA transcripts in different maize tissues estimated by densitometry (see "Materials and Methods"). Relative abundance of *ZmPP1* is presented as a percentage of the amount of *ZmPP1* RNA in lane 2. Relative abundance of *MCH1* RNA is given as a percentage of the amount of *MCH1* RNA in lane 2. Lanes are same as in A.

Expression of ZmPP1 *in E. coli* yielded approximately 1 mg recombinant protein from a 50-ml culture (data not shown).

### Renaturation and Expression of Recombinant ZmPP1

To measure activity of the recombinant ZmPP1 protein, inclusion bodies were initially solubilized in 6 M urea, 0.5 M NaCl, 50 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 0.2% 2-mercaptoethanol, and 0.1% Tween 80. The denatured protein was subsequently renatured by rapidly diluting the sample in neutral buffer containing 1 mM Mn$^{2+}$ following the protocol of Berndt and Cohen (2). Optimal protein concentrations for proper refolding of ZmPP1 were found to be <1 µg protein/mL renaturation buffer (data not shown). Similar results were reported for optimal refolding of recombinant rabbit PP1 (*B-PP1α*; ref. 2). Renaturation of ZmPP1 from solubilized inclusion bodies resulted in active enzyme that catalyzed the dephosphorylation of phosphorylase *a* with a specific activity of approximately 7.2 units/mg. The activity was linear up to 20 min and resulted in a 23% dephosphorylation of the substrate (Fig. 6A). Protein extracted from *E. coli* carrying a control plasmid (pT7–7) that did not contain the *ZmPP1* cDNA insert exhibited no measurable phosphatase activity (Fig. 6A). Therefore, all of the activity measured from *E. coli* carrying the pT7-ZmPP1 plasmid was attributed to the maize ZmPP1 protein.

The activity of ZmPP1 was strongly inhibited by okadaic acid (Fig. 6B), which has been shown to selectively inhibit PP1 and PP2A (3), and also by I-2 (Fig. 6C), which is a specific inhibitor of type 1 protein phosphatases (7). The IC$_{50}$

of ZmPP1 was approximately 200 nM for okadaic acid (Fig. 6B) and approximately 0.1 nM for I-2 (Fig. 6C). Activity was fully inhibited by okadaic acid at concentrations >10 µM and by I-2 at concentrations >3 nM.

### DISCUSSION

We isolated a cDNA clone from maize (*ZmPP1*) that encodes a 35.7-kD protein with a deduced amino acid sequence that is 80% identical with a highly conserved core region of PP1 from mammals and yeast (1, 22). Expression of this cDNA clone in *E. coli* resulted in high yields of ZmPP1 protein that was able to catalyze the dephosphorylation of phosphorylase *a* and whose activity was sensitive to mammalian I-2, a specific inhibitor of type 1 protein phosphatases (7). These findings clearly identify ZmPP1 as a maize PP1. Southern blot analysis of genomic DNA from maize and *Arabidopsis* using the *ZmPP1* cDNA clone as a hybridization probe show that *ZmPP1* may belong to a family of related genes in maize and in other plant species analogous to the families of protein phosphatases described in animal systems (7, 8, 13).

Most of the ZmPP1 protein expressed in *E. coli* was recovered in insoluble inclusion bodies and accounted for approximately 55% of the total protein within these particles. Renaturation of ZmPP1 resulted in active protein with a high specific activity (7.2 units/mg total protein) toward rabbit phosphorylase *a*. Like other PP1, ZmPP1 activity was strongly inhibited by okadaic acid and I-2. Okadaic acid inhibited ZmPP1 activity with an IC$_{50}$ value (~approximately 200 nM) that is significantly higher than values reported for mammalian (IC$_{50}$ = 10 nM) and *B. napus* seed (IC$_{50}$ = 10 nM) PP1 (7, 18). Conversely, ZmPP1 activity was >1 order of magnitude more sensitive to I-2 (IC$_{50}$ = 0.1 nM) than the corresponding enzymes from mammalian cells (IC$_{50}$ = 2 nM) and *B. napus* (IC$_{50}$ = 2 nM). This may reveal differences in the sensitivity
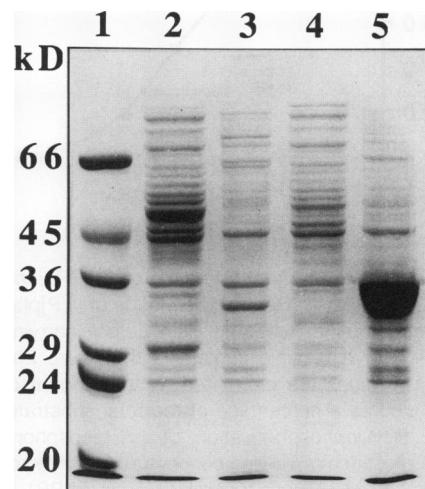


**Figure 5.** Expression of *ZmPP1* cDNA clone in *E. coli*. SDS-PAGE of soluble (lanes 2 and 4) and insoluble (lanes 3 and 5) proteins extracted from IPTG-induced *E. coli* carrying the control plasmid pT7–7 (lanes 2 and 3) and plasmid containing the *ZmPP1* cDNA clone (pT7-ZmPP1; lanes 4 and 5). Mol wt standards are in lane 1.
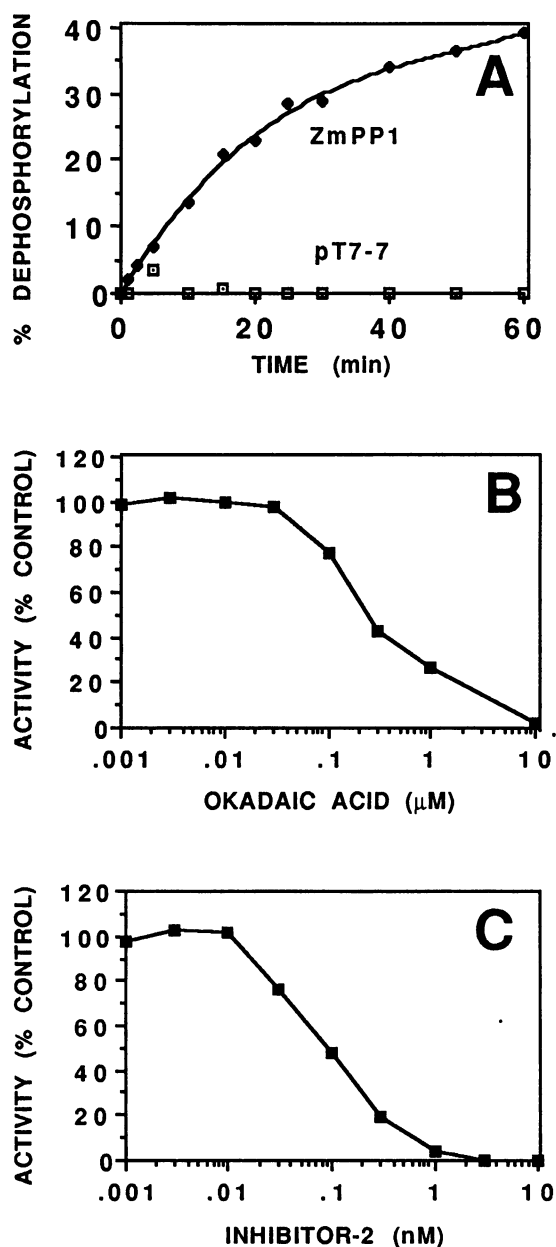
**Figure 6.** *In vitro* activity of renatured maize ZmPP1 and sensitivity to okadaic acid and I-2. A, Dephosphorylation of $[^{32}P]$phosphorylase *a* by renatured proteins extracted from *E. coli* carrying a control plasmid (pT7-7) or a plasmid containing the maize *ZmPP1* cDNA clone (ZmPP1). Values are presented as the amount of substrate dephosphorylated as a percentage of the total substrate present in the reaction. B, Dephosphorylation of $[^{32}P]$phosphorylase *a* by ZmPP1 in the presence of varying concentrations of okadaic acid. C, Dephosphorylation of $[^{32}P]$phosphorylase *a* by ZmPP1 in the presence of varying concentrations of the mammalian I-2.

of the maize protein phosphatase to okadaic acid and I-2 due to differences in the primary structure of the protein. Alternatively, a mixed population of active and inactive ZmPP1 proteins resulting from incomplete renaturation could alter $IC_{50}$ values if the inhibitors bind to both active and inactive polypeptides. Thus, the final concentration of ZmPP1 in the assay could alter $IC_{50}$ values, as is known to occur for other protein phosphatases (10). A more detailed understanding of the inhibitory characteristics of these inhibitors toward ZmPP1 will ultimately depend on the purification of the native enzyme from maize tissues.

Although physiological substrates for the maize ZmPP1 have not been identified, nor do we presently understand the biological role(s) of PP1 in higher plants, it is tempting to speculate that the function and regulation of these enzymes are as well conserved in animals and plants as are their structural and biochemical characteristics. If this is true, plant PP1 could be involved in the regulation of a number of cellular processes including mitosis, chromosome separation, transcription, and protein synthesis (8, 22). The function and regulation of mammalian PP1 is best characterized in skeletal muscle where it is associated with glycogen particles, myofibrils, and the sarcoplasmic reticulum (7). Targeting subunits regulate the activity of the phosphatase by directing it to specific subcellular locations and by selectively enhancing the activity toward their substrates (7). Analogous targeting subunits may also regulate the activity of ZmPP1 in maize. MacKintosh and Cohen (18) reported that 40% of the PP1 activity extracted from *B. napus* seeds is particulate, implying that PP1 in higher plants may be associated with subcellular particles and that regulatory subunits may selectively target and modulate the activity of these enzymes.

In summary, these findings show that a PP1 is expressed in maize tissues that is structurally and biochemically very similar to the corresponding enzymes in animals. Furthermore, these studies represent the first successful expression system for a PP1 using *E. coli*. Previous attempts to express a mammalian PP1 in *E. coli* were unsuccessful, although Berndt and Cohen (2) recently developed a protocol to renature insoluble recombinant rabbit PP1 expressed at high levels using the baculovirus/insect cell system. The renaturation protocol of Berndt and Cohen (2) was used in this study to renature ZmPP1 protein expressed in *E. coli*. These results lay the groundwork for future studies of the structural and functional characteristics of PP1 and will allow us to begin investigating the physiological function(s) and mechanisms for regulating the activity of these enzymes in higher plants.

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