The Plant Cell, Vol. 10, 849–857, May 1998, www.plantcell.org

Molecular Characterization of a Tyrosine-Specific Protein Phosphatase Encoded by a Stress-Responsive Gene in Arabidopsis

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Protein tyrosine kinases and phosphatases play a vital role in the regulation of cell growth and differentiation in animal systems. However, none of these enzymes has been characterized from higher plants. In this study, we isolated a cDNA encoding a putative protein tyrosine phosphatase (PTPase) from Arabidopsis (referred to as *AtPTP1*). The expression level of *AtPTP1* is highly sensitive to environmental stresses. High-salt conditions increased *AtPTP1* mRNA levels, whereas cold treatment rapidly eliminated the *AtPTP1* transcript. The recombinant AtPTP1 protein specifically hydrolyzed phosphotyrosine, but not phosphoserine/threonine, in protein substrates. Site-directed mutagenesis defined two highly conserved amino acids, cysteine-265 and aspartate-234, as being essential for the phosphatase activity of the AtPTP1 protein, suggesting a common catalytic mechanism for PTPases from all eukaryotic systems. In summary, we have identified AtPTP1 as a tyrosine-specific protein phosphatase that may function in stress responses of higher plants.

INTRODUCTION

Protein phosphorylation and dephosphorylation regulate numerous biological processes and are catalyzed by protein kinases and phosphatases (Boyer and Krebs, 1986; Hunter, 1995). In eukaryotes, these enzymes are categorized based on their substrate specificity. Protein kinases are classified into two major groups: serine/threonine kinases and tyrosine kinases, which phosphorylate serine/threonine and tyrosine, respectively. Accordingly, there are two major groups of phosphatases that remove the phosphate group from phosphoserine/threonine and phosphotyrosine. These are the phosphoprotein (serine/threonine) phosphatases (PPases) and the protein tyrosine phosphatases (PTPases). Although all protein kinases share extensive similarities in their sequences and three-dimensional structures (Hardie and Hanks, 1995; Johnson et al., 1996; Mohammadi et al., 1997; Sicheri et al., 1997), phosphatases represent a diverse family of enzymes (Cohen, 1989; Stone and Dixon, 1994; Neel and Tonks, 1997).

The PPases specifically dephosphorylate phosphoserine/threonine in protein substrates. These enzymes traditionally are classified into four subgroups (PP1, PP2A, PP2B, and PP2C) based on their biochemical and pharmacological properties (Cohen, 1989). PP1 and PP2A are inhibited by okadaic acid and calyculin A. PP2B is Ca^{2+} and calmodulin dependent, and PP2C requires Mg^{2+} for activity. Most enzymes from these subgroups share high sequence similarities (Cohen, 1989; Walton and Dixon, 1993), but they are very different from PTPases. All PTPases contain an active site signature motif, (I/V)HCXAGXXR(S/T)G, which harbors the catalytic cysteinyl residue involved in the formation of a phosphoenzyme reaction intermediate (Guan and Dixon, 1990). The PTPases are categorized into three subgroups: receptor-like PTPases, intracellular PTPases, and dual-specificity PTPases (Stone and Dixon, 1994). The common structural features of the receptor-like PTPases include an extracellular domain of variable length and composition, a single membrane-spanning region, and one or two cytoplasmic catalytic domains. The intracellular PTPases typically contain a single catalytic domain and various N- and C-terminal extensions. These extensions are believed to have targeting or regulatory functions (Mauro and Dixon, 1993). Dual-specificity PTPases constitute a special class of intracellular PTPases that are unique in their ability to hydrolyze both phosphoserine/threonine and phosphotyrosine residues.

PTPases play important roles in a number of signal transduction pathways in animal and yeast systems (Neel and Tonks, 1997). As a cell surface antigen, CD45 was the first receptor-like PTPase characterized and shown to function in both T cell and B cell signaling (Charbonneau et al., 1988; Ledbetter et al., 1988). Some intracellular PTPases have been shown to interact with activated growth factor receptors in mammalian cells (Kazlauskas et al., 1993; Lechleider et

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al., 1993). A PTPase with a Src-homology 2 (SH2) domain is required for normal development of anterior and posterior structure during embryogenesis of Drosophila (Perkins et al., 1992). Both intracellular PTPases and dual-specificity PTPases regulate the mitogen-activated protein kinases (MAPKs) involved in a variety of signaling pathways.

MAPKs are activated after mitogen stimulation or environmental stress in mammalian cells (Guan, 1994; Rosette and Karin, 1996). At the molecular level, MAPK activation is dependent on phosphorylation of a pair of neighboring tyrosine and threonine residues (Anderson et al., 1990). Dual-specificity PTPases dephosphorylate and inactivate MAPKs in mammalian cells (Keyse and Emslie, 1992; Sun et al., 1993). On the other hand, tyrosine-specific PTPases play a major role in the regulation of MAPK pathways in yeast. In *Shizosaccharomyces pombe*, two tyrosine-specific PTPases, Pyp1 and Pyp2, act on Spc1, a MAPK involved in the stress and cell cycle coupling process (Shiozaki and Russell, 1995). In *Saccharomyces cerevisiae*, tyrosine-specific PTPases Ptp2 and Ptp3, but not Ptp1, are major PTPases responsible for dephosphorylation and inactivation of Hog1, a MAPK-mediating osmosensing pathway (Wurgler-Murphy et al., 1997).

In higher plants, serine/threonine types of protein kinases and phosphatases have been characterized and are implicated in various cellular processes (Luan et al., 1993; Stone and Walker, 1995; Smith and Walker, 1996). However, whether plants contain tyrosine kinases and phosphatases remains controversial. Recent studies have identified a number of MAPKs from higher plants (Mizoguchi et al., 1997). In several cases, activation of plant MAPKs has been shown to accompany tyrosine phosphorylation (Suzuki and Shinshi, 1995; Stratmann and Ryan, 1997; Zhang and Klessig, 1997), as found with mammalian and yeast MAPKs. These studies imply that higher plants may also contain enzymes that dephosphorylate tyrosine residues in protein substrates, such as MAPKs. In fact, PTPase-like activities have been detected and partially purified from plant tissue extracts (Cheng and Tao, 1989; Guo and Roux, 1995). To date, however, a PTPase protein has not yet been purified to homogeneity, nor has a gene for any of these enzymes been identified from a higher plant. We report here the molecular characterization of a protein tyrosine phosphatase encoded by a stress-responsive gene in Arabidopsis.

RESULTS

Isolation and Sequence Analyses of the *AtPTP1* cDNA

As detailed in Methods, the *AtPTP1* cDNA from Arabidopsis was identified by a systematic polymerase chain reaction (PCR) approach followed by library screening. The cDNA sequence of *AtPTP1* is 1973 bp long, with an open reading frame of 1020 bp encoding a protein of 340 amino acids (Figure 1A). Sequence analyses showed that the AtPTP1 protein shares high homology with tyrosine-specific PTPase in other eukaryotic systems. An expressed sequence tag (H10F3T7) in the Arabidopsis database was found to be

A

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1    gttcctgacgtcgcgttagatcccgccgtcaatcggtcATGGCGACCGGTAAAACCTCTT   60
                                          M  A  T  G  K  T  S  S
61   CCGCCGCGAATCTTTTCACTGGCTCGACGCGTTTTGATTTATCATCCGCTGATTCGCCTC  120
       A  A  N  L  F  T  G  S  T  R  F  D  L  S  S  A  D  S  P  P
121  CTTCAAAACTCTCTCTCTCCTCCGATCAGCTCAACCACTGCCACCAAGCTCTCGGCGTTT  180
       S  K  L  S  L  S  S  D  Q  L  N  H  C  H  Q  A  L  G  V  F
181  TCCGGGGAAAGATCCAAAATCCTGACTCGATCGCTCATGAGTTTACTGGTTTACAGGCTA  240
       R  G  K  I  Q  N  P  D  S  I  A  H  E  F  T  G  L  Q  A  N
241  ATAGGATGTGGCCATCGGAGCTGCTGCTAAACAGTACAGTGGCTATGAACAGTGTCAATG  300
       R  M  W  P  S  E  L  L  L  N  S  T  V  A  M  N  S  V  N  V
301  TTGAGAAAAACAGATACAGTGATGTTGTTCCATTTGACAAGAACAGGATTGTTCTGAATC  360
       E  K  N  R  Y  S  D  V  V  P  F  D  K  N  R  I  V  L  N  P
361  CATGTAAAGACTCATCTGCAAAAGGATATGTGAATGCAAGCTTAATTAAGACGTCGGAGT  420
       C  K  D  S  S  A  K  G  Y  V  N  A  S  L  I  K  T  S  E  S
421  CTGAGAGTATTTCTCAGTTCATAGCTACGCAAGGTCCTTTACCACACACGATGGAGGCCT  480
       E  S  I  S  Q  F  I  A  T  Q  G  P  L  P  H  T  M  E  A  F
481  TCTGGGAGATGGTTATTCAGCAGCATTGCCCAATCATAGTGATGCTCACTCGATTGGTTG  540
       W  E  M  V  I  Q  Q  H  C  P  I  I  V  M  L  T  R  L  V  D
541  ATAATAATAGGACTGTTAAATGCGGGGACTATTTTCAAGACGAAGATGGACCTAGAGAAT  600
       N  N  R  T  V  K  C  G  D  Y  F  Q  D  E  D  G  P  R  E  F
601  TTGGCAACATATCTCTTACAACAAAGTGGATAAAGACTACTGACACTTCATTGATGTTAC  660
       G  N  I  S  L  T  T  K  W  I  K  T  T  D  T  S  L  M  L  R
661  GGAATCTTGAGGTTAACTACAAGGAGACAGAGGATCAGCCCATGTCCGTTTTGCATATTC  720
       N  L  E  V  N  Y  K  E  T  E  D  Q  P  M  S  V  L  H  I  Q
721  AGTATCCAGAATGGCCTGATCATGGAGTTCCCAAGGATACAGTGGCTGTCCGTGAAATTC  780
       Y  P  E  W  P  D  H  G  V  P  K  D  T  V  A  V  R  E  I  L
781  TAAAAAGACTATATCAAGTACCACCTAGTCTCGGCCCAATCATTGTGCACTGCAGTGCAG  840
       K  R  L  Y  Q  V  P  P  S  L  G  P  I  I  V  H  C  S  A  G
841  GTATAGGAAGAACTGGAACATACTGTGCGATACATAACACAATCCAAAGAATTCTTGCTG  900
       I  G  R  T  G  T  Y  C  A  I  H  N  T  I  Q  R  I  L  A  G
901  GCGATATGTCTGCGTTGGATCTTGCTAAAACCGTGGCACTATTTCGCAAGCAACGCATTG  960
       D  M  S  A  L  D  L  A  K  T  V  A  L  F  R  K  Q  R  I  G
961  GCATGGTTCAAACCATGGATCAATACTTCTTTTGCTACAATGCTATTGTTGATGAATTAG 1020
       M  V  Q  T  M  D  Q  Y  F  F  C  Y  N  A  I  V  D  E  L  E
1021 AAGATCTAACCGCGGGGACAAATGCTGGAACGAGTTCCTAAagctgaagggttcgtctgc 1080
       D  L  T  A  G  T  N  A  G  T  S  S  *
1081 ttagagagggaaaaaggcttctacccaactatatcaatttcgtaatgtgattcaagaaga 1140
1141 aacccggcgtgaatctctacattcaagacttttcaatttctgtaaatttccatatctgata 1200
1201 ggtttgttgttgttacttttgttgtgggaaatgctgattaaaaagcaaatgaagtcgccaatg 1260
1261 cgagattctcggagagatatcgagatttagtgggaagttgactcattacaacaaatgtccg 1320
1321 aacaaagaaacacacttgaaagataagttttgaaacaaaagggacaaaaacaaaagcacc 1380
1381 aaccaacttcataagaaaagattacatcccctaatgtgacatgtctaggcaaggtcaagac 1440
1441 aaaggagacggccatcaaagcgacggctagagcaacgactgcgtgaccatcctttgctga 1500
1501 agctgatcctccctctctcgtggacgtagctggagttgccggagactttcctgctggtgc 1560
1561 caccggtgttgttgtggcattgtttgtgaatacggctgcgtctggcgagctaggtgaaat 1620
1621 ccctagaagctttggacagttggtaatgctagcgttgtggagctgacaagaagttgggag 1680
1681 ttgaatgacttgtcttcttgaacaccaaatccttcaaggcttgtcctcctgtcttcgct 1740
1741 tgttgtataacgaaacacaaacacttggatctctttctttgatatcttcaacagcgtca 1800
1801 caacacttcttagacggaatttgttgccttcccggtcgcaaagtccaaacacaaagtcacc 1860
1861 ttttgaaaatcctggttgcattcatcagccagagctcctccgggagcagccggtgcagcg 1920
1921 gcggcgatggaggcaacgatcgtcatggtgactaggacgaggtggcggccgcg        1973
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B

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AtPTP1   44 ALGVFRGKIQNPDSIAHEFTGLQANRMWPSELLLNSTVAMNSVNVEKNRY 93
            .::   :.|:..:| |  : ::     :...  : : ||. . | ::|||
PTP1B     2 EMEKEFEQIDKSGSWAAIYQDI.....RHEASDFPCRVAKLPKNKNRNRY 46

           94 SDVVPFDKNRIVLNPCKDSSAKGYVNASLIKTSESESISQFIATQGPLPH 143
              .|| |||..|| |:.    ...:|:|||||| .|.::  . :| ||||||:
           47 RDVSPFDHSRIKLHQ....EDNDYINASLIKMEEAQ..RSYILTQGPLPN 90

          144 TMEAFWEMVIQQHCPIIVMLTRLVDNNRTVKCGDYF.QDEDGPREFGNIS 192
              | : ||||| :|.:: :|||.|:::.. .:||::|: |.|: .. |::..
           91 TCGHFWEMVWEQKSRGVVMLNRVMEKG.SLKCAQYWPQKEEKEMIFEDTN 139

          193 LTTKWIKTTDTS.LMLRNLEVNYKETEDQPMSVLHIQYPEWPDHGVPKDT 241
              |. .:|... .| . :|.||:: . |.:::...:||::|..||| |||...
          140 LKLTLISEDIKSYYTVRQLELE.NLTTQETREILHFHYTTWPDFGVPESP 188

          242 VAVREILKRLYQ...VPPSLGPIIVHCSAGIGRTGTYCAIHNTIQRILA. 287
              ... ::| :: :   :.|. ||::|||||||||.||:| : :|.  ::.
          189 ASFLNFLFKVRESGSLSPEHGPVVVHCSAGIGRSGTFC.LADTCLLLMDK 237

          288 .GDMSALDLAKTVALFRKQRIGMVQTMDQYFFCYNAIVDELEDLTAGTNA 336
                | |.:|: |.:  :|| |:|::|| ||. |:| |:::: . : ::...
          238 RKDPSSVDIKKVLLEMRKFRMGLIQTADQLRFSYLAVIEGAKFIMGDSSV 287

          337 GTSS 340
              ...
          288 QDQW 291
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Figure 1. Sequence Analyses of the *AtPTP1* cDNA.

(A) Nucleotide and deduced amino acid sequence of the *AtPTP1* cDNA. The conserved catalytic core motif is underlined. Lowercase letters indicate nucleotide sequence in the noncoding regions. The GenBank accession number is AF055635.

(B) Sequence alignment of AtPTP1 and human PTP1B proteins. Vertical lines indicate identical and colons indicate similar amino acids. The conserved regions critical for catalysis are underlined, and the two invariant amino acids are in boldface.

nearly identical to a region of *AtPTP1* cDNA and may represent the same or a highly related gene. There is little homology between AtPTP1 and dual-specificity PTPases, except in the catalytic signature motif present in all PTPases (underlined regions in Figure 1A). In addition, *AtPTP1* does not show sequence homology to the acid/alkali phosphatases or serine/threonine phosphatases.

The amino acid sequence identity between AtPTP1 protein and tyrosine-specific PTPases from other organisms is in the range of 30 to 40%. For example, it is 32% identical to the cytoplasmic region of human CD45, a receptor-type PTPase (Charbonneau et al., 1988). Higher homology is found between *AtPTP1* and intracellular PTPases, such as human PTP1B (35%; Charbonneau et al., 1989) and PTP1 from *Dictyostelium discoideum* (34%; Howard et al., 1992). In Figure 1B, the predicted amino acid sequence of the *AtPTP1* cDNA is aligned with human PTP1B, a typical intracellular tyrosine-specific PTPase (Charbonneau et al., 1989). Based on hydropathy analysis, the AtPTP1 protein does not have a transmembrane domain; therefore, it may be a member of the intracellular PTPase family.

DNA gel blot analysis confirmed that *AtPTP1* is present in the Arabidopsis genome (Figure 2). Based on this high-stringency blot, it is likely that only one copy of the *AtPTP1* gene exists in the Arabidopsis genome. Low-stringency hybridization revealed the presence of several related sequences in the genome (data not shown), suggesting the presence of other PTPase homologs in Arabidopsis.

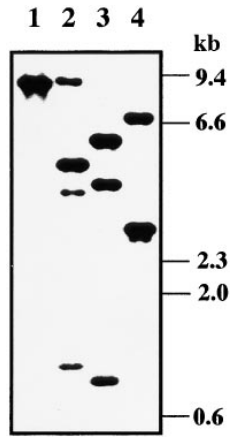

Figure 2. DNA Blot Analysis of *AtPTP1.*

Total genomic DNA from Arabidopsis plants (ecotype Columbia) was digested with BglII (lane 1), BamHI (lane 2), EcoRI (lane 3), and HindIII (lane 4), respectively, and analyzed by using the labeled *AtPTP1* cDNA as a probe. The lengths (in kilobases) of the DNA markers are shown at right.

Expression of the *AtPTP1* Gene Is Regulated by Stress Signals

To determine whether the *AtPTP1* gene is expressed in plant tissues, we performed RNA gel blot analyses by using total RNA isolated from various Arabidopsis tissues, including roots, flowers, leaves, and stems. Figure 3A shows that the *AtPTP1* gene is actively expressed in all tissues that were examined, although leaves appeared to accumulate a lower level of the *AtPTP1* transcript than did other tissues.

To gain further insight into the possible function of *AtPTP1* in plants, we studied its expression patterns under various stress conditions. We subjected plants to several stress factors, including drought, heat shock, wounding, high salt, and cold temperature. Among these factors, high-salt conditions were most effective in increasing the expression level of *AtPTP1.* As shown in Figure 3B, 24 hr after high-salt treatment, plants accumulated a significantly higher level of *AtPTP1* (lane 2 versus lane 3). The mRNA abundance returned to the control level in plants treated for 2 days (Figure 3B, lanes 4 and 5). This study suggests that *AtPTP1* expression is positively regulated by high-salt exposure.

More interestingly, the *AtPTP1* mRNA level rapidly declined in rosette plants challenged by cold temperature (Figure 3C). Within 2 or 3 hr of treatment at 4°C, the transcript level became undetectable (Figure 3C, lanes 3 and 4). After a longer period (⩾6 hr) of cold treatment, *AtPTP1* mRNA levels started to recover (Figure 3C, lanes 5 to 7). If the plants were moved back to the control temperature (21°C) after a 2-hr cold treatment, *AtPTP1* mRNA accumulated rapidly to the control level or higher (Figure 3C, lanes 8 to 10). This mRNA accumulation pattern indicates that the expression of the *AtPTP1* gene is downregulated by cold temperature and that this regulation is transient and immediately reversible upon temperature shift. Other stress factors such as heat shock, wounding, and drought did not appear to have a significant effect on *AtPTP1* expression (data not shown).

The *AtPTP1* Gene Encodes a Functional Phosphotyrosine-Specific PTPase

The *AtPTP1* gene is present in the Arabidopsis genome, and its expression in plants is regulated by high-salt and cold stress (Figures 2 and 3). This suggests that the *AtPTP1* gene product may be involved in plant response to environmental stresses. To determine whether the *AtPTP1* gene encodes a functional PTPase, we produced a recombinant AtPTP1 protein and characterized its enzyme properties.

We cloned the coding region of *AtPTP1* cDNA into the pGEX-4T-3 vector and expressed the recombinant protein in *Escherichia coli.* As shown in Figure 4, we induced overexpression of a glutathione *S*-transferase (GST)–AtPTP1 fusion protein by isopropyl β-D-thiogalactopyranoside (IPTG) (lane 2). The fusion protein was recovered in the supernatant of the bacterial lysate and purified by using glutathione Sepharose beads (Figure 4, lane 3). Thrombin cleavage of

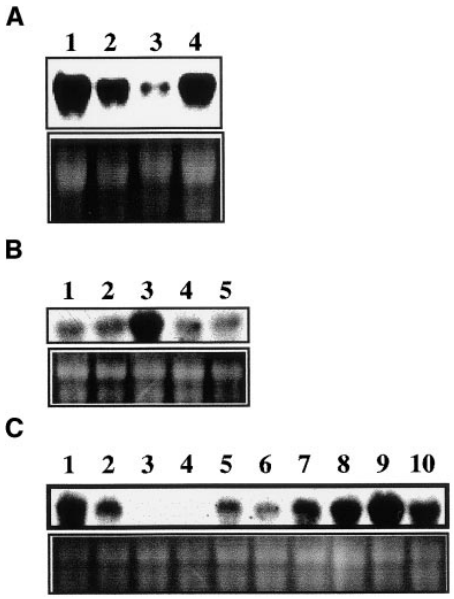

Figure 3. Stress-Regulated Expression of the *AtPTP1* Gene.

(A) RNA gel blot analysis of *AtPTP1* mRNA in roots (lane 1), flowers (lane 2), leaves (lane 3), and stems (lane 4).
(B) High-salt induction of *AtPTP1* expression. Lane 1, control before treatment; lane 2, control 24 hr later; lane 3, 24-hr salt treatment; lane 4, control 48 hr later; and lane 5, 48-hr salt-treated sample.
(C) Cold downregulation of *AtPTP1.* Plants were treated at 4°C for 0, 1, 2, 3, 6, 10, and 24 hr before RNA isolation (lanes 1 to 7, respectively). After 2 hr of cold treatment, portions of the plants were transferred back to room temperature for 1, 2, or 3 hr (lanes 8 to 10, respectively).
Each lane was loaded with 10 μg total of RNA, which was analyzed by an RNA gel-blotting procedure (Luan et al., 1994, 1996), using a ^{32}P-labeled *AtPTP1* cDNA as a probe. The gels at bottom in **(A)**, **(B)**, and **(C)** are nylon membranes showing rRNA bands stained with ethidium bromide, which indicate the amount of total RNA analyzed.

the fusion protein yielded the 38-kD AtPTP1 protein and the 26-kD GST protein (Figure 4, lane 4). Purification of the AtPTP1 protein was performed by "on-bead" cleavage that typically generates the AtPTP1 protein with a purity of 95% or higher (Figure 4, lane 5), which is sufficiently pure for most biochemical characterization.

After the recombinant AtPTP1 protein was purified, we performed phosphatase assay by using pyronitrophenyl phosphate (pNPP) as a substrate. Phosphatases cleave the phosphate from pNPP and generate a yellow nitrophenol product that can be quantitated by absorbance at 405 nm (A_{405}). The AtPTP1 protein hydrolyzed pNPP rapidly, and the activity was linear during the first 30 min of the reaction (Figure 5A). We also monitored the catalysis as the function of enzyme concentration. As more AtPTP1 protein was included in the reaction, pNPP hydrolysis increased proportionally (Figure 5B). These results show that the AtPTP1 protein is a highly active phosphatase.

To determine whether the *AtPTP1* gene product is a tyrosine-specific protein phosphatase, we conducted a PTPase assay, using substrates labeled by either tyrosine kinase or serine/threonine kinase. We used casein and the myelin basic protein as substrates for both kinases in the labeling process so that the phosphatase activity of the AtPTP1 protein (MBP) against phosphoserine/threonine or phosphotyrosine (in the same substrate) could be compared. As shown in Figure 6, the AtPTP1 protein dephosphorylated phosphotyrosine in p60–Src—labeled substrates (both casein and MBP). No phosphatase activity was detected against the phosphoserine/threonine in the same substrates labeled by the catalytic subunit of protein kinase A. This result demonstrates that the *AtPTP1* gene encodes a functional tyrosine-specific protein phosphatase.

Consistent with the above-mentioned observations, the phosphatase activity of the AtPTP1 protein was inhibited completely by vanadate, a PTPase inhibitor (Table 1). Inhibitors for phosphoserine/threonine phosphatases, such as okadaic acid, did not affect AtPTP1 activity (Table 1). Divalent cations (Mg^{2+} and Ca^{2+}) or their chelator EDTA had no significant effect on the AtPTP1 activity.

The Catalytic Core of PTPases Is Conserved in AtPTP1

Sequence alignment of PTPases from various organisms has revealed several invariant amino acid residues. At least

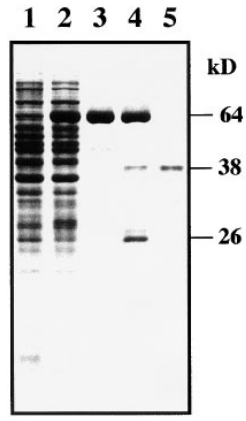

Figure 4. Expression and Purification of the Recombinant AtPTP1 Protein in *E. coli.*

Total protein was isolated from bacterial cells before (lane 1) and after (lane 2) IPTG induction. The fusion protein was purified using glutathione beads (lane 3) and partially cleaved by thrombin (lane 4). The purified recombinant AtPTP1 protein is shown as a single protein species (lane 5). Proteins were analyzed by SDS-PAGE and detected by Coomassie Brilliant Blue R 250 staining (Sambrook et al., 1989). The molecular masses of proteins are shown at right.

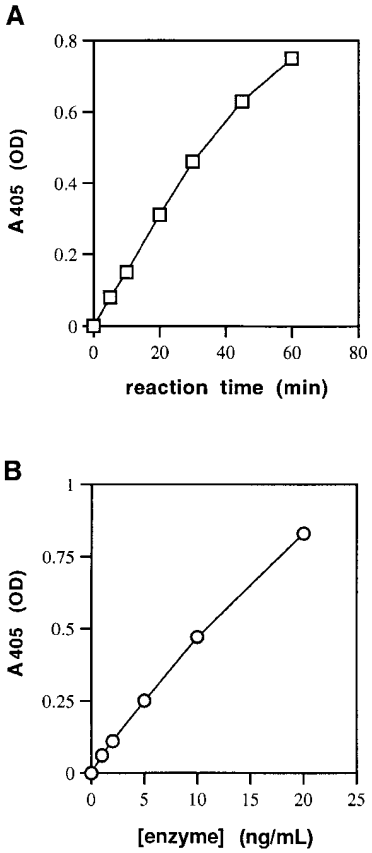

Figure 5. The Recombinant AtPTP1 Protein Is an Active Phosphatase.

(A) Time course of AtPTP1 phosphatase activity. Using pNPP as a substrate, AtPTP1 activity was measured as absorption at 405 nm (A_{405}), indicating the production of nitrophenol. The concentration of the AtPTP1 protein was 5 ng/mL.

(B) Enzyme activity as a function of enzyme concentration.

All experiments were repeated four times, and the data from one representative assay are shown here.

two of them have been shown to be critical for PTPase activity (Flint et al., 1997; Neel and Tonks, 1997). As shown in Figure 1B, these two invariant amino acids are conserved in AtPTP1 (Cys-265 and Asp-234 in boldface letters located in the underlined regions). To determine whether these conserved residues are important for the catalysis of AtPTP1 enzyme, we replaced Cys-265 by Ser and Asp-234 by Ala, respectively, using site-directed mutagenesis (Luan and Bogorad, 1992). As shown in Table 1, neither of the two mutant proteins had any detectable level of phosphatase activity. This study confirms that the two invariant amino acid residues are essential for catalytic activity of AtPTP1 phosphatase and implicates a similar catalytic mechanism for PTPases from all eukaryotes.

DISCUSSION

This report has identified AtPTP1 as a PTPase from a higher plant. The AtPTP1 cDNA encodes a typical PTPase that shares high homology with the core region of all tyrosine-specific PTPases in other systems. The most striking similarity is displayed by several regions that form specific structural domains in the PTPases. These include the active site signature motif (amino acids 263 to 271 in AtPTP1), the WPD motif upstream of the active site, and a region enriched in charged residues (amino acids 90 to 104) (Figure 1B). Recent structural studies of human PTP1B and several other PTPases have put the function of these conserved domains into perspective (Barford et al., 1994; Denu et al., 1996; Yuvaniyama et al., 1996).

The active site signature motif residues are found within a single loop, nestled at the base of a cleft on the surface of the protein. The essential cysteinyl residue (equivalent to Cys-265 in AtPTP1) is in position for nucleophilic attack on an incoming phosphate residue. For the thiophosphate intermediate to form efficiently, the phenolic oxygen of the tyrosyl leaving group must be protonated. Based on their mutagenesis results, Dixon and colleagues (Zhang et al., 1994) predict that an aspartyl residue (equivalent to Asp-234 in AtPTP1) would donate this proton, serving as a general acid. Therefore, these two domains (the signature motif and

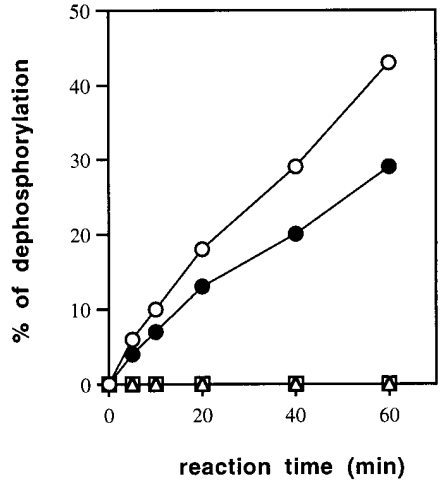

Figure 6. The Recombinant AtPTP1 Protein Is a Functional Tyrosine-Specific Protein Phosphatase.

The phosphotyrosine-containing casein (open circles) and myelin basic protein (filled circles) were hydrolyzed rapidly by the AtPTP1 protein. Phosphoserine/threonine–containing proteins (open squares for casein and open triangles for myelin basic protein) were not hydrolyzed. Experiments were repeated four times, and data from a typical assay are presented.

Table 1. Inhibitor and Mutation Analyses of AtPTP1 Enzyme Activity[a]

Chemicals	Concentration	% of Control
$MgCl_2$	5 mM	104
$CaCl_2$	1 mM	97
EDTA	10 mM	112
Okadaic acid	1 μM	101
Vanadate	100 μM	1
Mutations		
C265→S		0
D234→A		0

[a] Using pNPP as a substrate, we measured AtPTP1 activity as A_{405} in the presence of various chemicals and compared it with control activity (as 100%). The two mutant proteins were purified and subjected to the same assay.

the WPD motif) are required for catalytic activity of both tyrosine-specific and dual-specificity PTPases.

The highly charged region is present only in tyrosine-specific PTPases and has been shown to contribute to the overall depth of the active site cleft that can be accessed only by phosphotyrosine but not by phosphoserine/threonine. In addition, the charged amino acids are thought to be involved in phosphotyrosine binding (Yuvaniyama et al., 1996). These structural predictions are consistent with the results in this study. For example, the AtPTP1 protein contains the highly charged domain and specifically hydrolyzes phosphotyrosine in protein substrates. Mutation at either Cys-265 or Asp-234 in the AtPTP1 protein abolishes the catalytic activity. These studies also imply that the catalytic mechanism has been conserved for PTPases in eukaryotic systems, including yeast, plants, and mammals.

PTPases play a central role in a variety of signal transduction pathways in animal and yeast systems (Stone and Dixon, 1994; Neel and Tonks, 1997). In animals, PTPases target a wide range of protein substrates that are phosphorylated by either tyrosine-specific protein kinases or dual-specificity kinases. Tyrosine-specific kinases constitute a large family of enzymes that regulate cell growth and differentiation (Cantley et al., 1991; Hunter, 1995). As the counteracting enzymes, PTPases are critical for controlling the level of tyrosine phosphorylation in the cell under any given condition (Neel and Tonks, 1997). MAPK kinases are a major group of dual-specificity kinases that phosphorylate MAPKs at both threonine and tyrosine, thereby activating these enzymes. PTPases dephosphorylate MAPKs and inactivate them (Guan, 1994). Distinct from animals, yeast does not contain a tyrosine-specific protein kinase. The major phosphotyrosine proteins are those phosphorylated by dual-specificity kinases, such as MAPK kinases. As a result, PTPases in yeast are most important in the regulation of MAPK pathways (Shiozaki and Russell, 1995; Wurgler-Murphy et al., 1997).

In higher plants, research in protein tyrosine phosphorylation has lagged behind because none of the responsible enzymes (tyrosine kinase or phosphatase) has been characterized at the molecular level. Recent studies have shown that tyrosine phosphorylation is critical for the activation of MAPKs in higher plants (Suzuki and Shinshi, 1995; Zhang and Klessig, 1997) as well as in animals and yeast, suggesting the presence of protein kinases and phosphatases that are capable of phosphorylating or dephosphorylating tyrosines in protein substrates. Indeed, MAPK kinases with dual specificity for tyrosine and threonine have been identified recently from Arabidopsis (Mizoguchi et al., 1997). Studies described in this report have identified a PTPase in a higher plant.

In conjunction with the possibility of MAPK regulation by PTPases in plants, the finding of stress-regulated expression of the *AtPTP1* gene is significant. As shown in animal and yeast systems, a number of MAPKs are activated under stress conditions and inactivated by PTPases (Anderson et al., 1990; Guan, 1994; Rosette and Karin, 1996). In addition, expression of genes encoding PTPases is often upregulated by the MAPK pathway, forming a negative feedback loop for MAPK regulation (Sun et al., 1993; Brondello et al., 1997; Jacoby et al., 1997; Wurgler-Murphy et al., 1997). Because some MAPKs in higher plants have also been found to be activated by stress signals, including cold temperature, wounding, drought, pathogen elicitors, salicylic acid, and systemin (Bögre et al., 1997; Ligterink et al., 1997; Mizoguchi et al., 1997; Stratmann and Ryan, 1997; Zhang and Klessig, 1997), there may be a negative feedback loop similar to that in animal and yeast systems, that is, MAPK activation may upregulate expression of PTPase genes in higher plants. This hypothesis is consistent with the high-salt induction of *AtPTP1* expression. In contrast, *AtPTP1* expression is negatively regulated by cold temperature in Arabidopsis plants. Downregulation of PTPase expression by stress factors has not been reported in any other organism previously studied. This finding implicates AtPTP1 in a unique mechanism for plant response to environmental factors.

In summary, the molecular characterization of *AtPTP1* in this report provides a critical stepping stone for further exploration of the function of tyrosine phosphorylation in MAPK regulation and possibly in other unique signaling pathways yet to be identified in higher plants.

METHODS

Isolation of the *AtPTP1* cDNA

A systematic polymerase chain reaction (PCR) approach was used to isolate the *AtPTP1* cDNA. The PCR template was the phagemid DNA isolated from an Arabidopsis cDNA library (Kieber et al., 1993). PCR primers were designed based on the conserved regions in protein tyrosine phosphatases (PTPases) from a number of organisms, including

mammals, flies, and yeast. The amino acid sequences for deducing the degenerate oligonucleotides were NRNRYRD, TQGPLP, WPDXGVP, and (V/I)HCSAG. Two complementary oligonucleotides running in the opposite direction were synthesized for each of the four peptide sequences. Together with two vector primers (T3 and T7), 11 pairs of primers were used in PCR reactions with a Robocycler (Stratagene, La Jolla, CA). Each pair of primers was used in PCR reactions at six annealing temperatures, with the temperature gradient ranging from 34 to 54°C. The resulting PCR fragments were sequenced directly, and sequences were analyzed using BLAST programs provided by the National Center for Biotechnology Information (Bethesda, MD).

Among the sequenced cDNA fragments, two were highly homologous to animal PTPase sequences and overlapped with each other. One of the fragments was generated by a reaction using the T3 primer together with a reverse primer derived from TQGPLP (5′-GGIAG/AIGGICCCTGIGT-3′). The other fragment resulted from a reaction using the T3 primer with a reverse primer derived from (V/I)HCSAG (5′-CCIGCIG/CA/TA/GCAGTGIA-3′). In the partially degenerate oligonucleotides, I stands for inosine, and the two possible nucleotides are separated by a slash. The longer PCR fragment was used to screen the Arabidopsis cDNA library (Kieber et al., 1993) at high stringency (Sambrook et al., 1989). Eleven independent clones were isolated from $\sim 3 \times 10^5$ plaques. Partial sequencing results suggested that these clones may represent different lengths of an identical cDNA. One of the clones was fully sequenced after generating a series of deleted clones by using an ExoIII–mung bean nucleases deletion system (New England Biolabs, Beverly, MA). DNA and RNA gel blotting analyses were performed based on procedures described by Sambrook et al. (1989).

Plant Materials and RNA Analyses

Arabidopsis plants (*Arabidopsis thaliana* ecotype Columbia) were grown in a greenhouse under long-day conditions (16-hr-light/8-hr-dark cycle) to flowering before harvesting different tissues for RNA preparation. For the high-salt treatments, Arabidopsis plants were grown on Murashige and Skoog medium (Sigma) in Petri dishes for 2 weeks. Some plants were then treated with high salt by the addition of 20 mL of 300 mM NaCl to each plate. For cold treatments, plants were grown under short-day conditions for 5 weeks to induce the production of large rosettes. Some plants were then moved to a cold room kept at 4°C. Samples were taken at various time points, as indicated in Figure 3C. Some plants were treated at 4°C for 2 hr and then returned to room temperature for 1 to 3 hr before the samples were collected. The heat treatment was performed by moving plants to a warm room kept at 37°C. Wounding was conducted by puncturing plant leaves with a hemostat (~80% of the leaves were punctured). Drought conditions were as described by Yamaguchi-Shinozaki and Shinozaki (1994). Total RNA isolation was performed as described previously (Luan et al., 1994).

Overexpression of the Recombinant AtPTP1 Protein

The coding region of the *AtPTP1* cDNA was amplified with Pfu polymerase (Strategene) and subcloned into the pGEX-4T-3 vector (Pharmacia, Piscataway, NJ). The resulting clones were sequenced to ensure in-frame fusion of *AtPTP1* with glutathione *S*-transferase (GST) and to avoid clones that contained PCR-introduced mutations. The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) (Novagen, Madison, WI). Overexpression of the GST–AtPTP1 fusion protein was performed, as described previously (Luan et al., 1994, 1996), with some modifications. After inducing expression with 0.25 mM isopropyl β-D-thiogalactopyranoside (IPTG), bacterial cultures were pelleted and resuspended in a buffer containing 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2 μM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 2 mM EDTA before lysis by sonication. The cell lysate was pelleted at 15,000*g* to collect the supernatant containing the fusion protein that subsequently was purified by glutathione Sepharose 4B (Pharmacia). On-bead cleavage was performed using 0.5 μg/mL thrombin (Sigma).

PCR Site-Directed Mutagenesis

The mutagenesis primers contained single base changes that converted Cys-265 to Ser (from TGC to TCC) and Asp-234 to Ala (from GAT to GCT), respectively. The first round of PCR was performed using the mutagenesis primer and the 3′ pGEX sequencing primer, with the *GST–AtPTP1* fusion plasmid DNA as the template. The PCR product was used as a primer together with the 5′ pGEX sequencing primer to conduct the second round of PCR by using the same template. The second-round PCR product was cloned back into the pGEX-4T-3 vector and sequenced to confirm the specific mutation. The mutant proteins were produced as described for the wild-type protein.

Preparation of ^{32}P-Labeled Protein Substrates and Phosphatase Assays

Casein and the myelin basic protein were used as substrates for both tyrosine- and serine/threonine–specific protein kinase in the labeling reactions. The procedure was based on Tonks et al. (1988), with modifications. For tyrosine labeling, 25 units of human c-Src tyrosine kinase (Upstate Biotechnology Inc., Lake Placid, NY) was incubated with 50 μg of substrate protein and 50 μCi ^{32}P-γ-ATP (Du Pont–New England Nuclear, Boston, MA) in 100 μL of reaction buffer (25 mM Tris-HCl, pH 7.2, 5 mM $MnCl_2$, 0.5 mM EGTA, 0.05 mM Na_3VO_4, and 25 mM Mg-acetate) for 1 hr at 30°C. The reaction mixture was loaded onto a Sep-Pak-18 reverse phase column (Waters, Milford, MA), washed extensively with 0.1% trifluoroacetic acid, and eluted with acetonitrile. The eluted protein substrate was lyophilized and dissolved in the phosphatase buffer (50 mM Tris-HCl, pH 7.0, and 2 mM DTT) and stored in aliquots at −80°C if not immediately used. For ^{32}P-labeling of serine/threonine residues in protein substrates, 50 units of bovine heart protein kinase A (Sigma) were used in a similar reaction, as described for the Src kinase, except for the reaction buffer (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 mM NaCl, and 12 mM $MgCl_2$). Purification and storage of labeled protein were as described above for the Src-labeled substrate.

Protein phosphatase activity was determined by measuring the release of free phosphorus-32 from labeled substrates. Assays were performed as described previously (Tonks et al., 1988), with some modifications. The reaction mixture contained 5 ng/mL of enzyme, 50 mM Tris-HCl, pH 7.0, and 2 mM DTT in 100 μL of total volume. Reactions were initiated by adding 2×10^4 cpm ^{32}P-labeled protein substrate. After incubating the reaction mixture at 30°C for various periods of time, we terminated the reaction by adding 2 volumes of 25% trichloroacetic acid. Proteins in the reaction mixture were precipitated, and the supernatant was subjected to scintillation counting.

Blank incubations were performed without the AtPTP1 protein. The relative activity of the AtPTP1 protein is given as the percentage of total substrate that was hydrolyzed during the reaction.

When pyronitrophenyl phosphate (pNPP) was used as a substrate, 1 mM pNPP in the phosphatase buffer (50 mM Tris-HCl, pH 7.0, and 2 mM DTT) was included in all of the assays. For the time-course analysis of AtPTP1 activity, reactions were initiated by adding 5 ng of enzyme protein to the 1-mL reaction mixture and monitored by absorbance at 405 nm (A_{405}) at various time points. To analyze the phosphatase activity at various enzyme concentrations, 1, 2, 5, 10, and 20 ng of AtPTP1 protein was included in the 1-mL assay buffer, and the reaction mixture was incubated for 15 min.

ACKNOWLEDGMENTS

This study was supported by a Merit Award to S.L. from the University of California Division of Agriculture and Natural Resources. We are grateful for helpful discussions with colleagues in the Plant Phosphorylation Group sponsored by the U.S. Department of Energy, the U.S. Department of Agriculture, and the National Science Foundation.

Received January 21, 1998; accepted March 12, 1998.

REFERENCES

Anderson, N.G., Maller, J.L., Tonks, N.K., and Sturgill, T.W. (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature **343,** 651–653.

Barford, D., Flint, A.J., and Tonks, N.K. (1994). Crystal structure of human protein tyrosine phosphatase 1B. Science **263,** 1397–1403.

Bögre, L., Ligterink, W., Meskiene, I., Baker, P.J., Heberle-Bors, E., Huskisson, N.S., and Hirt, H. (1997). Wounding induces the rapid and transient activation of a specific MAP kinase pathway. Plant Cell **9,** 75–83.

Boyer, P.D., and Krebs, E.G., eds (1986). The Enzymes, Vol. 17. (New York: Academic Press).

Brondello, J.-M., Brunet, A., Pouyssegur, J., and McKenzie, F.R. (1997). The dual specificity mitogen-activated protein kinase phosphatase 1 and 2 are induced by the p42-p44-MAPK cascade. J. Biol. Chem. **272,** 1368–1376.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991). Oncogenes and signal transduction. Cell **64,** 281–302.

Charbonneau, H., Tonks, N.K., Walsh, K.A., and Fischer, E.H. (1988). The leukocyte common antigen (CD45): A putative receptor-linked protein tyrosine phosphatase. Proc. Natl. Acad. Sci. USA **85,** 7182–7186.

Charbonneau, H., Tonks, N.K., Kumar, S., Diltz, C.D., Harrylock, M., Cool, D.E., Krebs, E.G., Fischer, E.H., and Walsh, K.A. (1989). Human placenta protein tyrosine phosphatase: Amino acid sequence and relationship to a family of receptor-like proteins. Proc. Natl. Acad. Sci. USA **86,** 5252–5256.

Cheng, H.F., and Tao, M. (1989). Purification and characterization of a phosphotyrosyl protein phosphatase from wheat seedlings. Biochem. Biophys. Acta **998,** 271–276.

Cohen, P. (1989). Structure and regulation of protein phosphatases. Annu. Rev. Biochem. **58,** 453–508.

Denu, J.M., Stuckey, J.A., Saper, M.A., and Dixon, J.E. (1996). Form and function in protein dephosphorylation. Cell **87,** 361–364.

Flint, A.J., Tiganis, T., Barford, D., and Tonks, N.K. (1997). Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. Proc. Natl. Acad. Sci. USA **94,** 1680–1685.

Guan, K.-L. (1994). The mitogen activated protein kinase signal transduction pathway: From the cell surface to the nucleus. Cell. Signalling **6,** 581–589.

Guan, K.-L., and Dixon, J.E. (1990). Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia.* Science **249,** 553–556.

Guo, Y., and Roux, S.J. (1995). Partial purification and characterization of an enzyme from pea nuclei with protein tyrosine phosphatase activity. Plant Physiol. **107,** 167–175.

Hardie, G., and Hanks, S. (1995). The Protein Kinase Facts Book. (London: Academic Press).

Howard, P.K., Sefton, B.M., and Firtel, R.A. (1992). Analysis of a spatially regulated phosphotyrosine phosphatase identifies tyrosine phosphorylation as a key regulatory pathway in Dictyostelium. Cell **71,** 637–647.

Hunter, T. (1995). Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. Cell **80,** 225–236.

Jacoby, T., Flanagan, H., Faykin, A., Seto, A.G., Mattison, C., and Ota, I. (1997). Two protein-tyrosine phosphatases inactivate the osmotic stress response pathway in yeast by targeting the mitogen-activated protein kinase, Hog1. J. Biol. Chem. **272,** 17749–17755.

Johnson, N.L., Noble, M.E.M., and Owen, D.J. (1996). Active and inactive protein kinases: Structural basis for regulation. Cell **85,** 149–158.

Kazlauskas, A., Feng, G.-S., Pawson, T., and Valius, M. (1993). The 64-kD protein that associates with the platelet-derived growth factor receptor β subunit via Tyr-1009 is the SH2-containing phosphotyrosine phosphatase Syp. Proc. Natl. Acad. Sci. USA **90,** 6939–6943.

Keyse, S.M., and Emslie, E.A. (1992). Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. Nature **359,** 644–647.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). Ctr1: A negative regulator of the ethylene response pathway in Arabidopsis encodes a member of the Raf family of protein kinases. Cell **72,** 427–441.

Lechleider, R.J., Freeman, R.M., Jr., and Neel, B.G. (1993). Tyrosyl phosphorylation and growth factor receptor association of the human *corkscrew* homologue Sh-Ptp2. J. Biol. Chem. **268,** 13434–13438.

Ledbetter, J.A., Tonks, N.K., Fischer, E.H., and Clark, E.A. (1988). CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. Proc. Natl. Acad. Sci. USA **85,** 8628–8632.

Ligterink, W., Kroj, T., Nieden, U.Z., Hirt, H., and Scheel, D. (1997). Receptor-mediated activation of a MAP kinase in pathogen defense of plants. Science **276,** 2054–2057.

Luan, S., and Bogorad, L. (1992). A rice *cab* gene promoter contains separate *cis*-acting elements that regulate expression in dicot and monocot plants. Plant Cell **4,** 971–981.

Luan, S., Li, W., Rusnak, F., Assmann, S.M., and Schreiber, S.L. (1993). Immunosuppressants implicate protein phosphatase regulation of potassium channels in guard cells. Proc. Natl. Acad. Sci. USA **90,** 2202–2206.

Luan, S., Lane, W.S., and Schreiber, S.L. (1994). pCyP B: A chloroplast-localized, heat shock–responsive cyclophilin from fava bean. Plant Cell **6,** 885–892.

Luan, S., Kudla, J., Gruissem, W., and Schreiber, S.L. (1996). Molecular characterization of a FKBP-type immunophilin from higher plants. Proc. Natl. Acad. Sci. USA **93,** 6964–6969.

Mauro, L.J., and Dixon, J.E. (1993). 'Zip codes' direct intracellular protein tyrosine phosphatases to the correct cellular 'address.' Trends Biochem. Sci. **19,** 151–155.

Mizoguchi, T., Ichimura, K., and Shinozaki, K. (1997). Environmental stress response in plants: The role of mitogen-activated protein kinases. Trends Biotechnol. **15,** 15–19.

Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B.K., Hubbard, S.R., and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science **276,** 955–960.

Neel, B.G., and Tonks, N.K. (1997). Protein tyrosine phosphatases in signal transduction. Curr. Opin. Cell Biol. **9,** 193–204.

Perkins, L.A., Larsen, I., and Perrimon, N. (1992). *Corkscrew* encodes a putative protein tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase *torso.* Cell **70,** 225–236.

Rosette, C., and Karin, M. (1996). Ultraviolet light and osmotic stress: Activation of the JNK cascade through multiple growth factor and cytokine receptors. Science **274,** 1194–1197.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Shiozaki, K., and Russell, P. (1995). Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature **378,** 739–743.

Sicheri, F., Moarefi, I., and Kuriyan, J. (1997). Crystal structure of the Src family tyrosine kinase Hck. Nature **385,** 602–609.

Smith, R.D., and Walker, J.C. (1996). Plant protein phosphatases. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 101–125.

Stone, J., and Walker, J.C. (1995). Plant protein kinase families and signal transduction. Plant Physiol. **108,** 451–457.

Stone, R.L., and Dixon, J.E. (1994). Protein-tyrosine phosphatases. J. Biol. Chem. **269,** 31323–31326.

Stratmann, J.W., and Ryan, C.A. (1997). Myelin basic protein kinase activity in tomato leaves is induced systemically by wounding and increases in response to systemin and oligosaccharide elicitors. Proc. Natl. Acad. Sci. USA **94,** 11085–11089.

Sun, H., Charles, C.H., Lau, L.F., and Tonks, N.K. (1993). MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase *in vivo.* Cell **75,** 487–493.

Suzuki, K., and Shinshi, H. (1995). Transient activation and tyrosine phosphorylation of a protein kinase in tobacco cells treated with a fungal elicitor. Plant Cell **7,** 639–647.

Tonks, N.K., Diltz, C.D., and Fischer, E.H. (1988). Characterization of the major protein-tyrosine-phosphatases of human placenta. J. Biol. Chem. **263,** 6731–6737.

Walton, K.M., and Dixon, J.E. (1993). Protein tyrosine phosphatases. Annu. Rev. Biochem. **62,** 101–120.

Wurgler-Murphy, S.M., Maeda, T., Witten, E.A., and Saito, H. (1997). Regulation of the *Saccharomyces cerevisiae* HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases. Mol. Cell. Biol. **17,** 1289–1297.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994). A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, and high-salt stress. Plant Cell **6,** 251–264.

Yuvaniyama, J., Denu, J.M., Dixon, J.E., and Saper, M.A. (1996). Crystal structure of the dual specificity protein phosphatase VHR. Science **272,** 1328–1331.

Zhang, S., and Klessig, D.F. (1997). Salicylic acid activates a 48-kD MAP kinase in tobacco. Plant Cell **9,** 809–824.

Zhang, Z.-Y., Wang, Y., and Dixon, J.E. (1994). Dissecting the catalytic mechanism of protein-tyrosine phosphatases. Proc. Natl. Acad. Sci. USA **91,** 1624–1627.