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Phosphoprotein Phosphatase Genes of *Arabidopsis thaliana*

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Phosphoprotein phosphatases are highly conserved enzymes found in fungous and animal cells. These enzymes have multiple functions in those cells, but little is known about phosphoprotein phosphatases in plants. We have now studied what kinds of and how many phosphoprotein phosphatase genes are present in a model higher plant, *Arabidopsis thaliana*, by DNA hybridization, polymerase-chain-reaction, and gene cloning procedures. Genomic Southern-blot hybridization analysis suggested that the *A. thaliana* genome contains multiple copies of genes encoding serine/threonine-specific phosphoprotein phosphatases similar to those of fungi and animals: five to eight copies of the type 1 phosphatase (PP1) genes, two to three copies of the type 2A phosphatase (PP2A) genes, and probably one copy of the type 2C phosphatase (PP2C) gene. Besides, a putative tyrosine-specific phosphoprotein phosphatase gene appeared to exist, being considerably diverged from those of fungi and animals. Among these genes one PP1 cDNA and one PP2A cDNA were isolated from an *A. thaliana* cDNA library, and their sequences were determined and characterized.

KEY WORDS: Protein phosphatase / Signal transduction / Higher plant / Phosphorylation / Dephosphorylation

1. INTRODUCTION

Protein phosphorylation is a central regulatory mechanism for cellular functions in all eucaryotic cells.[1-4] Over the past decade it has become apparent that phosphoprotein phosphatases are as important as protein kinases in a variety of control mechanisms including signal transduction, metabolism, growth, and differentiation (for reviews see refs. 5 and 6). With regard to the substrate specificity, phosphoprotein phosphatases are categorized into two kinds, serine/threonine (Ser/Thr)-specific and tyrosine (Tyr)-specific phosphatases.

Ser/Thr-specific phosphatases are further divided into two types, type 1 (PP1) and type 2 (PP2) that are sensitive and insensitive, respectively, to the cellular inhibitors 1 and 2. The PP2 class of phosphoprotein phosphatases is subdivided into three groups termed PP2A, PP2B, and PP2C from their divalent cation dependency. PP2A requires no divalent cation for its activity, while PP2B and PP2C need Ca^{2+}/calmodulin and Mg^{2+}, respectively. All these phosphoprotein phosphatases except for PP2C appear to exist in a cell as hetero oligomers that consist of catalytic subunits (PP1c, PP2Ac, or PP2Bc) and regulatory subunits.[5] The primary structure of the catalytic subunits belonging to the same subfamilies is highly conserved among various organisms.[5] Furthermore, the catalytic subunits of the different subfamilies are significantly similar to one another. For instance, the amino acid sequence of PP1c is about 50% and 40% identical to those of PP2Ac and PP2Bc, respectively.[5]

Tyr-specific phosphatases (PTP) are classified for their architecture into two types, the

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membrane-spanning receptor-type and the non-receptor-type, the catalytic domain of which is highly conserved between two types and over species.[6] In addition, there is a novel type of PTP, Cdc25, that specifically dephosphorylates the cell-cycle-controlling Cdc2 kinase.[7] The similarity between Cdc25 and typical PTP is limited within very short regions probably essential for their activity.[7]

Biochemical and structural characterization of phosphoprotein phosphatases described above has been carried out chiefly with animals and more recently with fungi. Particularly, distinct evidence has been obtained exclusively with fungi, showing that PP1 and PP2A are essential because of their fundamental functions such as the regulation of cell division.[8,9] By contrast, little is known about phosphoprotein phosphatases in plants except for the presence in several plant species of phosphatase activities similar to PP1, PP2A, and PP2C of animals and fungi. Neither PP2B-like nor PTP-like activities have been detected in plants so far examined.[10-12] As a step toward shedding a light on features underlying cascades of protein phosphorylation/dephosphorylation in higher plants, we have now studied what kinds of and how many phosphoprotein phosphatase genes are present on the *Arabidopsis thaliana* chromosome using DNA hybridization, polymerase-chain-reaction (PCR), and gene cloning procedures.

2. MATERIALS AND METHODS

General methods. As described were procedures for transformation with *Escherichia coli*, preparation of plasmid and phage DNA, DNA cleavages with restriction endonucleases, repair synthesis, ligation, ^{32}P-labeling of DNA with random primers, gel electrophoresis with agarose and polyacrylamide, and gel blotting to a membrane filter.[13]

DNA sequencing. Long DNA fragments to be sequenced were separately inserted into the M13mp18 vector,[14] and their unidirectional deletions were generated by cleavages with restriction endonucleases. Each deletion derivative was sequenced by the chain-termination method with T7 DNA polymerase (United States Biochemical Corp.).[15] Sequencing of short DNA fragments were done by cloning in the pUC18 vector.[15]

Polymerase-chain-reaction (PCR). Oligodeoxyribonucleotides used for PCR primers were synthesized by a Beckman System 1 Plus DNA Synthesizer. Their nucleotide sequences were 5'-AAGTACCC(T/A)GAGAACTTCTT-3' (forward) and 5'-TC(A/G)TCGAACTC(A/T)CC(A/G)CAGTA-3' (reverse) that correspond to internal portions of a rabbit PP1 (KYPENFF at amino acid residues 113-119 and YCGEFDN at amino acid residues 272-278).[16] Other sets of primers specific for PTP were 5'-GG(A/T)GACTTCTGGCGTATG-3' (forward), 5'-CCAGC(A/G)GA(A/G)CA(A/G)TGAAC-3' (reverse 1), and 5'-GCAGCGGATGAAGATGGTTC-3' (reverse 2) that correspond to internal portions of human LAR (GDFWRM at amino acid residues 1406-1411 and VHCSAG at amino acid residues 1520-1525) and *Schizosaccharomyces pombe pyp1* (QRMKMV at amino acid residues 517-523), respectively.[16-18] PCR reaction mixtures (total volume of 100 μl) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin (w/v), 0.1 μg of an *A. thaliana* cDNA mixture, 0.2 mM each of the four dNTPs, 2.5 units of *Thermus aquaticus* DNA polymerase (Takara Shuzo Co.), 50 picomoles each of the forward and the reverse primers. The reactions were done by two consecu-

tive modes in a thermal cycler (Perkin Elmer/Cetus Corp.): the first mode was 10 cycles of 1 min at 92℃ (denaturation), 1 min at 42℃ (annealing), and 1 min at 70℃ (extension); and the second mode was 25 cycles under the same conditions except annealing temperature at 54℃.

Cloning of PCR fragments for PP1. After treatment with 0.4 units/ml of Klenow fragment of DNA polymerase I (Takara Shuzo Co.) at 37℃ for 30 min, PCR reaction products were separated by polyacrylamide gel electrophoresis. DNA fragments of about 480 base-pair (bp) long were eluted from the gels, and then inserted at the *Hin*cII site of pUC18.[14]

Isolation of plant DNA and RNA. The plant used was *Arabidopsis thaliana* (Columbia ecotype). Its cultivation was done under the standard growth conditions at 22℃ where 18-h illumination and 6-h darkness alternated. Plant DNA and RNA were isolated from whole tissues using cetyltrimethylammonium bromide, and poly(A)$^+$RNA was separated from poly(A)$^-$RNA through oligo(dT)-cellulose (Pharmacia) column chromatography.[19]

Yeast and animal phosphoprotein phosphatase clones. The following clones were used as probes in Southern-blot hybridization and library screening experiments. PP2A, the 1.2-kilobase-pair (kbp) *Eco*RI fragment of *Brassica napus* PP2A cDNA from pKS-M13$^+$PP2A[20]; PP2C, the 2.35 kbp *Sma*I fragment of rat PP2C cDNA from pST-11[21]; *pyp1*, the 1.5 kbp PCR-amplified cDNA of *S. pombe pyp1* (corresponding to the amino acid residues 24-523)[18]; *cdc*25, the 0.95 kbp *Bam*HI-*Bgl*II fragment of pCDC25[22]; and HPTP α, the 2.7 kbp *Bam*HI-*Hin*dIII fragment of pSP65.HPTP α.f1,[23] respectively.

Membrane hybridization. Four conditions of different stringency (high, moderate, low, and poor stringency) were used for hybridization. Under the high-stringency conditions, hybridization was performed at 42℃ for 20 h in a solution containing 50% (v/v) formamide, 5X SSC (1 X SCC = 150 mM NaCl-15 mM sodium citrate), 5 X Denhardt's solution,[15] 100 μg/ml calf thymus DNA, 0.5% SDS, and a probe DNA (about 5 X 10^8 cpm/μg of DNA). The filter was washed at room temperature, twice for 30 min in 2X SSC-0.1% SDS, once for 30 min at room temperature in 0.1X SSC-0.1% SDS, and once for 30 min at 65℃ in the same solution. For three other conditions, the formamide concentration and the last washing conditions were changed as follows: moderate stringency, 40% formamide, washing once at 50℃ in the same solution; low stringency, 20% formamide, washing once at room temperature in the same solution; and poor stringency, 10% formamide, washing twice at room temperature in 0.5X SSC-0.1% SDS.

Northern-blot hybridization. Plant total RNA was fractionated on a 1.2% agarose gel containing 1.8% formaldehyde with running buffer of 20 mM MOPS Good's buffer (pH 7.0)-5 mM EDTA-8 mM sodium acetate that was previously treated with diethyl pyrocarbonate. RNA was transferred to an Immobilon-N PVDF membrane filter (Millipore Corp.), and hybridization was carried out under the conditions of moderate stringency. The autoradiograms were generated by a Fujix BA 100 Bio-Image Analyzer (Fuji Photo Film).

Screening of cDNAs encoding PP1 and PP2A. An *A. thaliana* cDNA library constructed with the λgt10[13] vector was screened under the hybridization conditions of either high or low stringency.

Transformation of A. thaliana *root explants. A. thaliana* root explants were prepared by the standard procedures,[24] and then infected with *Agrobacterium tumefaciens* LBA4404[25] carrying the pBI121 binary vector[26] or its derivatives. Km^r transformant calli were selected, and then mature plants were regenerated as described.[24]

3. RESULTS

Type 1 phosphoprotein phosphatase genes of A. thaliana

To see if the *A. thaliana* genome contains PP1-like genes, PCR was run on a mixture of cDNA derived from whole plant tissues. The set of PCR primers used had degenerate sequences corresponding to the two highly conserved regions of PP1 (see Materials and methods). Gel electrophoresis of the PCR products gave the major band at about 480 bp position that roughly coincides with the size predicted from the primer locations (493 bp). These PCR fragments were inserted into pUC18, and the resulting clones were sequenced. Two out of them (PCR-1 and PCR-2) contained open reading frames intimating phosphoprotein phosphatases. Their putative translation products were close to each other (82% identity), and also to the corresponding positions of member enzymes of animal and fungous PP1c (78%-80%; see below). Thus these two PCR fragments appeared to be portions of *A. thaliana* PP1 cDNAs. To know the entire structure of PP1, an *A. thaliana* cDNA library constructed with the λgt10 vector was screened with a PCR-2 probe. From about 5.5×10^5 recombinants, six positive clones were obtained and sequenced. The longest clone contained an open reading frame of 957 bp, predicting a protein of 318 amino acid residues (35 kDa) (Fig. 1-A). The amino acid identity in its translation product with animal and fungous PP1c, PP2Ac, and PP2Bc occurred at 80%, 45%, and 40%, respectively, as shown in Fig. 2. The hydropathy profile[27] revealed this protein is a soluble one (Fig. 3). These results implied that the cloned DNA fragment is derived from an *A. thaliana* counterpart of PP1c, and thereby named AT-PP1. Northern-blot hybridization analysis using the PCR-2 segment as a probe and total RNA from whole tissues gave a unique hybridization signal at about 1.3-kilobase (kb) position (Fig. 4). Therefore, it seems that the majority of AT-PP1 transcripts are matured to mRNA in a unique manner, probably without alternative splicings.

Existence of a multi-gene family of type 1 phosphoprotein phosphatase

To find out whether AT-PP1 gene homologs are dispersed over the *A. thaliana* genome or not, genomic Southern-blot hybridization analysis (*Bam*HI and *Eco*RI digests) was done with PCR-1 and PCR-2 probes. Under the conditions of high stringency, each probe made one or two specific DNA fragments visible. Under the conditions of low stringency, on the other hand, several additional weak bands were detected. One such band probed with PCR-1 corresponded to the clear band probed with PCR-2 under the high-stringency conditions, and *vice versa* (Fig. 5-A). These results support the view that the *A. thaliana* genome contains multiple genes of PP1 with structural variations: presumably five to eight copies of PP1 genes including pseudogenes if any.

Type 2A phosphoprotein phosphatase genes of A. thaliana

Although an attempt was made to isolate PP2A cDNA clones by the PCR method as in the case of PP1, no candidate clone was obtained. As a next effort, using a truncated cDNA clone derived from the putative PP2A gene of *B. napus* as a probe, genomic Southern-blot hybridization analysis (*Bam*HI and *Eco*RI digests) was done under the three different conditions. Although no signal band appeared under the high-stringency conditions, a unique signal band was detected under the moderate-stringency conditions, and two or more signal

bands under the low-stringency conditions (Fig. 5-B). These results are interpreted as that at least two to three copies of PP2A-like genes are present on the *A. thaliana* genome.

To confirm it, an *A. thaliana* cDNA library was screened under the conditions of low stringency with the *B. napus* partial PP2A cDNA probe. By screening about 5.5×10^5 recombinants, four positive clones were obtained. From their nucleotide sequence analysis, it was found that the longest clone (787 bp insert) had an open reading frame whose translation product was 93% identical to that of the used probe, and about 80% indentical to fungous and animal PP2Ac members (Fig. 1-B and Fig. 2). This product also shared about 50% identity with PP1c members and 45% identity with a rat PP2Bc enzyme. Therefore, this product is likely to be an *A. thaliana* PP2A homolog (AT-PP2A).

Other types of phosphoprotein phosphatase genes of A. thaliana

To know if the *A. thaliana* genome contains type 2C phosphatase gene homologs, we carried out genomic Southern-blot hybridization analysis with a rat PP2C cDNA probe (Fig. 5-C). Only under the conditions of low stringency we detected a unique hybridization signal, intimating there is at least one PP2C-like gene in the *A. thaliana* genome.

The presence or absence of tyrosine-specific phosphoprotein phosphatases (PTP) was tested by the PCR method using various sets of PCR primers corresponding to the well-conserved regions among fungous and animal PTP members (see Materials and methods). However, no PCR fragment encoding PTP enzymes was produced. As a next effort, using yeast *pyp1*, yeast *cdc25*, and human PTPα clones as probes, genomic Southern-blot hybridization was done as above. We detected faint signal bands only with the yeast probes and only under the hybridization conditions of poor stringency. In addition, both yeast probes visualized a common restriction fragment as can be seen in Fig. 5-D. Since the similarity between *pyp1* and animal PTPs is slightly higher than that between *cdc25* and animal PTPs,[7] these results imply that the observed signal is derived from a novel type of PTP, a *cdc25* homolog, and that the *A. thaliana* genome contains no gene encoding typical types of PTP enzymes, though considerably diverged PTP-related genes may be present.

Trial for constructing transgenic plants carrying an antisense AT-PP1 gene

A large portion of AT-PP1 cDNA (corresponding to amino acid residues 19-221) was cloned into pBI121 in the antisense orientation under control of the cauliflower mosaic virus 35S promoter, and the resulting plasmid was named pBI121-PP1. *A. thaliana* root explants were infected with LBA4404(pBI121-PP1) and LBA4404(pBI121), and Km^r transformant calli were regenerated to mature plants. The transformation efficiency and degree of greening of calli were almost the same for both strains. However, shoot induction from calli occurred at a significantly lower efficiency with pBI121-PP1 than with pBI121. Almost all calli derived from pBI121-PP1 represented abnormal morphology showing strikingly tufty hairy roots, and their green portions gradually disappeared without further shoot induction (data not shown). We obtained only two regenerated mature plants after extensive efforts. Therefore, overexpression of the antisence AT-PP1 gene appeared toxic to plants, and the regenerated plants might be silent upon expression of the introduced gene.

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(A)   1    A AAT CTC CGT TCG TTT TCT ACC TCG TTT AGA TCG GAG ATG GCG GAG      46
      1                                                   M   A   E        3

     47  AAG CCG GCG CAA GAG CAA GAG CAG AAG CGA GCG ATG GAA CCT GCA GTT      94
      4   K   P   A   Q   E   Q   E   Q   K   R   A   M   E   P   A   V      19

     95  CTC GAC GAT ATT ATT CGT CGT TTG GTT GAG TTT CGG AAC ACG AGA CCT     142
     20   L   D   D   I   I   R   R   L   V   E   F   R   N   T   R   P      35

    143  GGA TCG GGG AAG CAA GTT CAT CTC AGT GAA GGT GAA ATT CGT CAG CTT     190
     36   G   S   G   K   Q   V   H   L   S   E   G   E   I   R   Q   L      51
              PKC                         CKII
    191  TGT GCT GTC TCC AAA GAA ATA TTT CTT CAA CAG CCC AAT CTG CTT GAA     238
     52   C   A   V   S   K   E   I   F   L   Q   Q   P   N   L   L   E      67

    239  TTG GAA GCT CCC ATC AAG ATC TGC GGT GAT ATT CAT GGG CAG TAT TCA     286
     68   L   E   A   P   I   K   I   C   G   D   I   H   G   Q   Y   S      83

    287  GAT CTA TTG AGG CTT TTT GAG TAT GGA GGG TTC CCT CCC GAA GCT AAT     334
     84   D   L   L   R   L   F   E   Y   G   G   F   P   P   E   A   N      99

    335  TAT TTG TTC TTG GGT GAT TAT GTT GAC CGT GGC AAG CAA AGC TTG GAA     382
    100   Y   L   F   L   G   D   Y   V   D   R   G   K   Q   S   L   E     115

    383  ACA ATA TGT CTT CTT CTA GCT TAC AAA ATC AAG TAC CCT GAG AAC TTC     430
    116   T   I   C   L   L   L   A   Y   K   I   K   Y   P   E   N   F     131

    431  TTC TTG TTG AGA GGG AAT CAT GAA TCT GCT TCC ATT AAT CGT ATT TAC     478
    132   F   L   L   R   G   N   H   E   S   A   S   I   N   R   I   Y     147

    479  GGT TTC TAT GAT GAG TGC AAA CGC AGG TTC AAT GTC AGA CTC TGG AAA     526
    148   G   F   Y   D   E   C   K   R   R   F   N   V   R   L   W   K     163

    527  ATA TTC ACC GAT TGC TTT AAC TGT CTT CCT GTG GCC GCC TTA ATT GAT     574
    164   I   F   T   D   C   F   N   C   L   P   V   A   A   L   I   D     179

    575  GAC AGA ATA CTA TGT ATG CAT GGT GGG ATT TCC CCA GAG CTG AAA AGT     622
    180   D   R   I   L   C   M   H   G   G   I   S   P   E   L   K   S     195

    623  TTG GAC CAG ATT AGA AAT ATT GCA CGG CCG ATG GAT ATT CCG GAG TCT     670
    196   L   D   Q   I   R   N   I   A   R   P   M   D   I   P   E   S     211

    671  GGT TTG GTA TGT GAT TTA CTA TGG TCG GAT CCT AGT GGA GAC GTA GGC     718
    212   G   L   V   C   D   L   L   W   S   D   P   S   G   D   V   G     227

    719  TGG GGC ATG AAT GAT CGT GGT GTT TCA TAC ACT TTT GGA GCT GAC AAA     766
    228   W   G   M   N   D   R   G   V   S   Y   T   F   G   A   D   K     243

    767  GTC GCA GAG TTC TTG GAG AAA CAT GAC ATG GAC CTT ATC TGT CGT GCC     814
    244   V   A   E   F   L   E   K   H   D   M   D   L   I   C   R   A     259

    815  CAC CAG GTT GTT GAA GAT GGG TAT GAG TTC TTT GCA GAA AGA CAA CTT     862
    260   H   Q   V   V   E   D   G   Y   E   F   F   A   E   R   Q   L     275

    863  GTT ACA GTA TTT TCA GCT CCC AAC TAT TGC GGG GAA TTT GAC AAC GCT     910
    276   V   T   V   F   S   A   P   N   Y   C   G   E   F   D   N   A     291

    911  GGC GCA ATG ATG AGC ATT GAT GAG AGC TTA ATG TGC TCA TTC CAG ATT     958
    292   G   A   M   M   S   I   D   E   S   L   M   C   S   F   Q   I     307
                            CKII
    959  CTA AAG CCG TCA GAA AAG AAG TCG CCT TTT CTG TGA CAC CGA TTA TCC    1006
    308   L   K   P   S   E   K   K   S   P   F   L   *                   319
                        PKC
   1007  AGT GAC CA                                                       1017
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(B) 1    T ATT CCG ACG GAC GCA ACC ATT GAT CTT GAT CTT GAT GAG CAG ATC     46
  1        I  P  T  D  A  T  I  D  L  D  L  D  E  Q  I                   15

 47  TCG CAG CTC ATG CAG TGC AAG CCT CTC TCG GAG CAA CAG GTT AGA GCA      94
 16   S  Q  L  M  Q  C  K  P  L  S  E  Q  Q  V  R  A                    31

 95  TTA TGC GAG AAA GCC AAG GAG ATC TTA ATG GAT GAA AGC AAT GTT CAG     142
 32   L  C  E  K  A  K  E  I  L  M  D  E  S  N  V  Q                    47

143  CCT GTG AAA AGC CCT GTG ACA ATC TGC GGT GAT ATT CAT GGA CAG TTC     190
 48   P  V  K  S  P  V  T  I  C  G  D  I  H  G  Q  F                    63

191  CAT GAT CTT GCA GAG CTT TTC CGT ATA GGG GGA ATG TGC CCT GAT ACC     238
 64   H  D  L  A  E  L  F  R  I  G  G  M  C  P  D  T                    79

239  AAT TAC CTG TTT ATG GGA GAC TAT GTG GAC CGT GGT TAT TAT TCT GTT     286
 80   N  Y  L  F  M  G  D  Y  V  D  R  G  Y  Y  S  V                    95

287  GAA ACT GTT ACG CTG TTA GTC GCC TTA AAG ATG CGA TAT CCT CAG CGA     334
 96   E  T  V  T  L  L  V  A  L  K  M  R  Y  P  Q  R                   111

335  ATC ACT ATT CTT AGA GGA AAC CAT GAA AGT CGT CAG ATT ACT CAG GTT     382
112   I  T  I  L  R  G  N  H  E  S  R  Q  I  T  Q  V                   127

383  TAT GGA TTT TAT GAA TGT CTA CGA AAG TAC GGC AAC GCA AAT GTT TGG     430
128   Y  G  F  Y  E  C  L  R  K  Y  G  N  A  N  V  W                   143

431  AAA ATC TTT ACA GAC CTC TTC GAC TAT TTT CCT CTG ACA GCC TTG GTT     478
144   K  I  F  T  D  L  F  D  Y  F  P  L  T  A  L  V                   159
      Tyr-kinase
479  GAG TCA GAA ATA TTT TGC CTT CAT GGT GGA TTA TCT CCA TCT ATC GAG     526
160   E  S  E  I  F  C  L  H  G  G  L  S  P  S  I  E                   175

527  ACC CTT GAC AAC ATA AGG AAT TTT GAT CGA GTT CAA GAA GTG CCC CAT     574
176   T  L  D  N  I  R  N  F  D  R  V  Q  E  V  P  H                   191

575  GAA GGG CCG ATG TGT GAC TTA TTA TGG TCT GAC CCC GAT GAC CGA TGT     622
192   E  G  P  M  C  D  L  L  W  S  D  P  D  D  R  C                   207
                                  CKII
623  GGT TGG GGC ATC TCT CCT CGG GGT GCC GGA TAT ACA TTT GGT CAG GAT     670
208   G  W  G  I  S  P  R  G  A  G  Y  T  F  G  Q  D                   223
               PKC
671  ATA TCT GAA CAA TTC AAT CAC ACA AAC AAC TTA AAG CTG ATC GCC CGA     718
224   I  S  E  Q  F  N  H  T  N  N  L  K  L  I  A  R                   239
                  Asn-glycosylation
719  GCT CAC CAG TTG GTT ATG GAT GGA TAC AAC TGG GCT CAC GAG CAA AAA     766
240   A  H  Q  L  V  M  D  G  Y  N  W  A  H  E  Q  K                   255

767  GTG GTT ACT ATT TTC AGT GCG                                        787
256   V  V  T  I  F  S  A                                              262
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Fig. 1. Nucleotide sequences and the deduced amino acid sequences of AT-PP1 (A) and AT-PP2A (B) cDNA, respectively. The amino acid sequences are given below the nucleotide sequences. The LRGNHE and RAH regions are shaded, respective potential phosphorylation sites and the corresponding kinases are underlined and cited, and a potential Asn-glycosylation site is boxed. *CKII*, casein kinase II; *PKC*, protein kinase C.

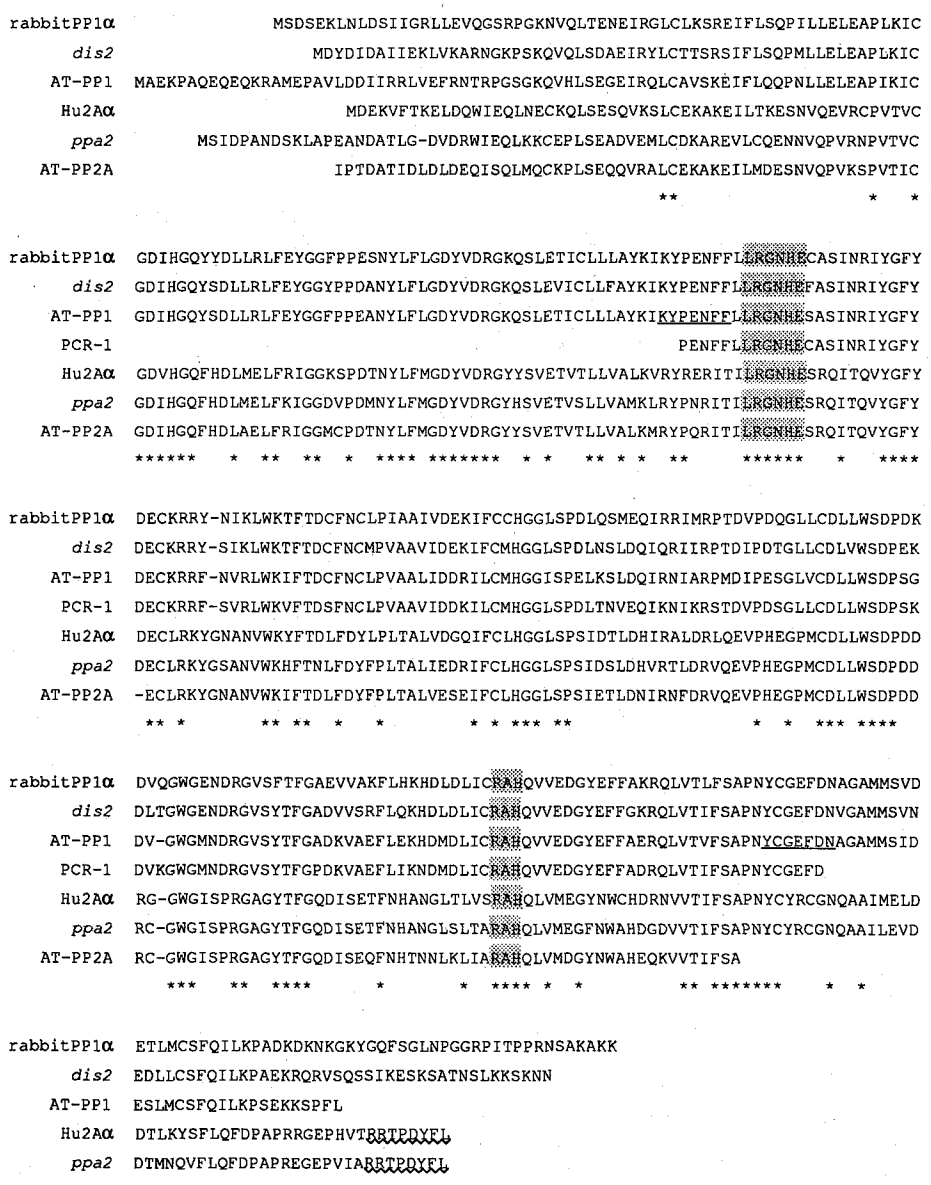

Fig. 2. Amino acid sequence comparison of Ser/Thr-specific phosphoprotein phosphatases. From upper to lower rows, rabbit PP1 α,[16] *S. pombe dis2* (PP1),[8] AT-PP1, PCR-1 translation product, human PP2A α,[39] *S. pombe ppa2* (PP2A),[9] AT-PP2A. The regions for PP1 PCR primers are underlined. Asterisks under the sequences represent highly conserved amino acid residues among PP1 and PP2A enzymes, and hyphens represent spacing for the best match. The LRGNHE and RAH regions and the RRTPDYFL region are indicated by shading and wavy lines, respectively.

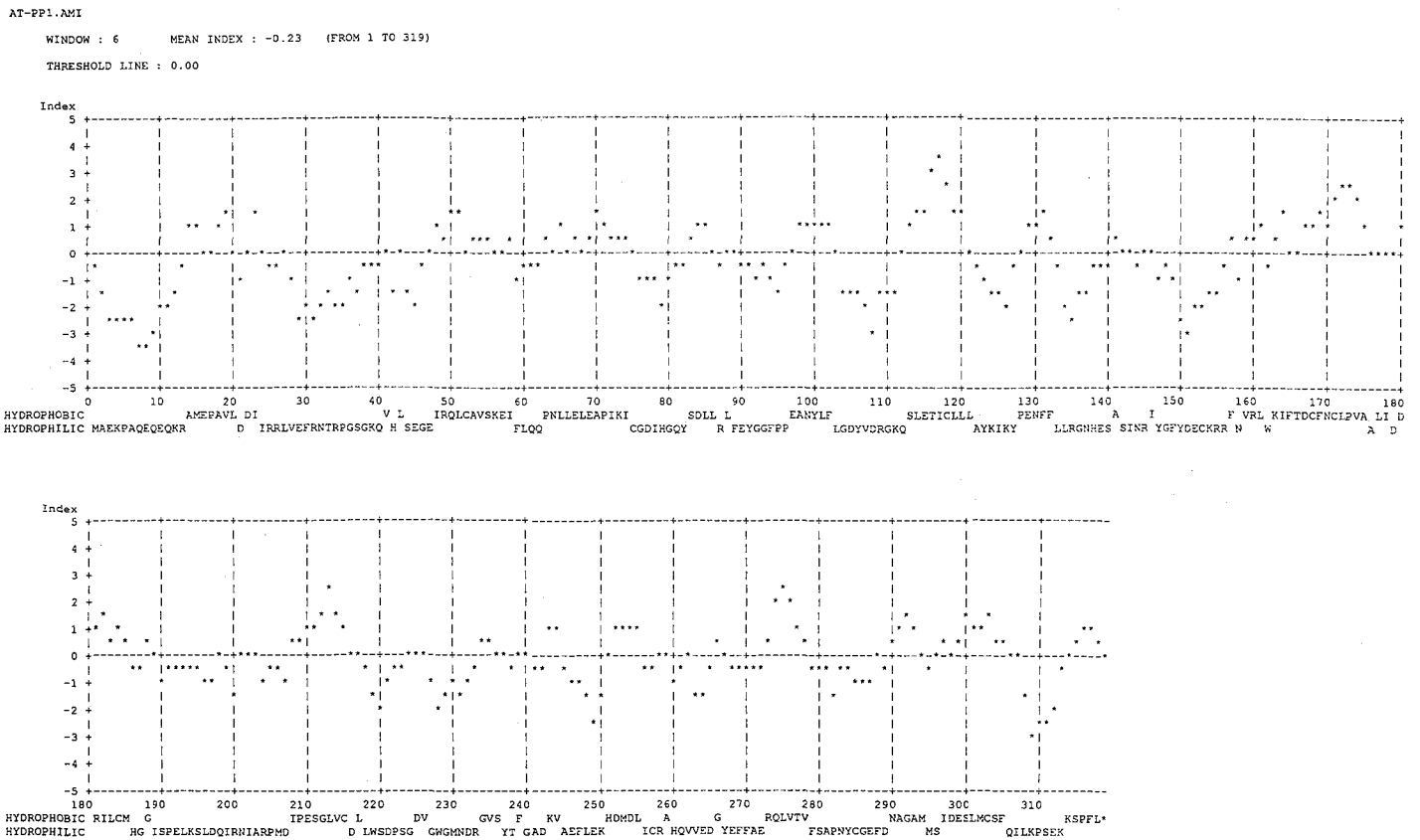

Fig. 3. Hydropathy profile of AT-PP1. Positive and negative values indicate hydrophobic and hydrophilic characters, respectively.[27]

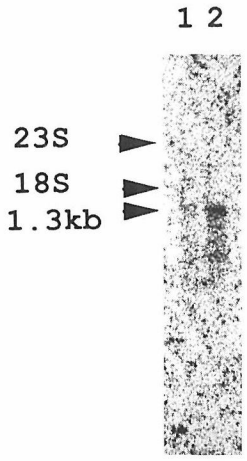

Fig. 4. Northern-blot hybridization analysis of AT-PP1 mRNA. Total plant RNA (lane 1, 0.8 μg RNA; lane 2, 4.0 μg) was fractionated by gel electrophoresis, blotted to a membrane filter, and hybridized with the ^{32}P-labeled PCR-2 segment. The position of ribosomal 28S and 18S RNAs are indicated by arrowheads.

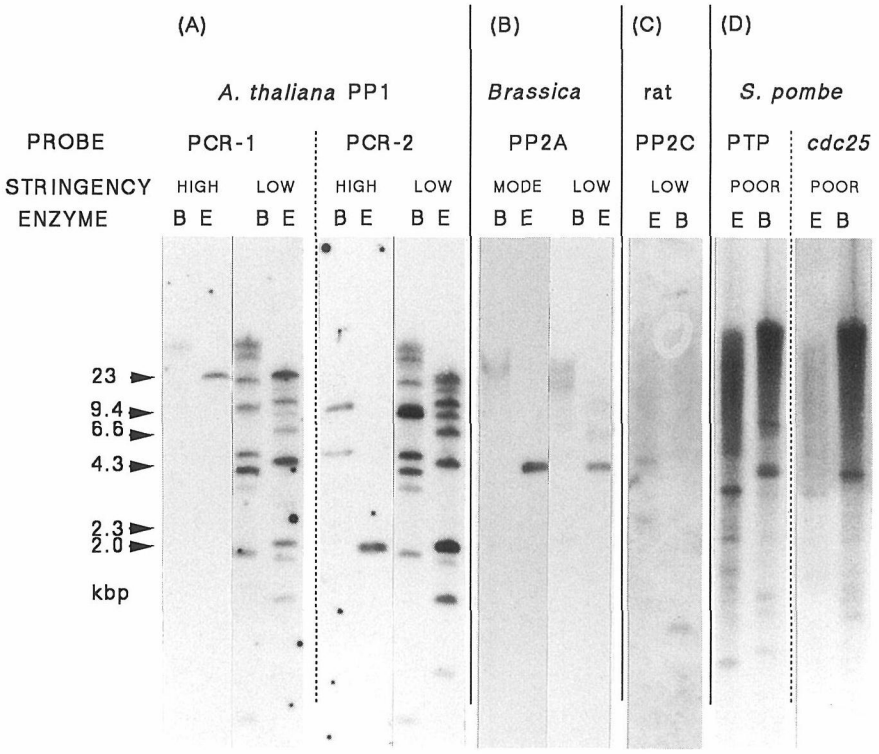

Fig. 5. Genomic Southern-blot hybridization analysis of the *A. thaliana* phosphoprotein phosphatase genes. *A. thaliana* DNA (5 μg/lane) was digested with *Bam*HI (lane B) or *Eco*RI (lane E) followed by gel electrophoresis and then by blotting. The probes used are indicated at the top. HIGH, MODE, LOW, and POOR are the high, moderate, low, and poor stringency for hybridization, respectively (see Materials and methods). The band positions of the λ *Hin*dIII fragments are indicated at the left for a size marker.

4. DISCUSSION

Type 1 phosphoprotein phosphatases of A. thaliana

The extremely conserved regions common to PP1c, PP2Ac, and PP2Bc are LRGNHE of residues 134-139 and RAH of residues 258-260, and are presumed to play critical roles in the phosphatase activity and/or interaction with regulatory subunits[8,28] (Fig. 2). In fact, a yeast dominant-negative mutant (*dis2-11*) contains a substitution by Q for R in the RAH sequence.[9] AT-PP1 completely conserved all of these sequences, implying that AT-PP1 actually encodes an *A. thaliana* Ser/Thr-specific phosphoprotein phosphatase.

From motif analysis of AT-PP1 with the PROSITE program,[29] we found SEGE (at amino acid residues 44-47) and SIDE (at amino acid residues 296-299) for potential casein kinase II targets, and SGK (at amino acid residues 37-39) and SEK (at amino acid residues 311-313) capable of being phosphorylated by protein kinase C (Fig. 1-A). Since the former two sequences are preserved among PP1 member enzymes (Fig. 2), the PP1 activity itself is likely to be regulated through phosphorylation by Ser/Thr-specific protein kinases (Fig. 1-A and Fig. 2).

Genomic Southern-blot hybridization analysis with the PP1 PCR segments indicated that five to eight PP1 genes including pseudogenes if any, are present on the *A. thaliana* genome, constituting a multi-gene family (Fig. 5-A). Rabbit and yeast (*S. cerevisiae* and *S. pombe*) have only two PP1 loci,[8] and *Drosophila melanogaster* contains three PP1 loci.[30] Therefore, PP1 genes seem to generally comprise a multi-gene family. It is known that two *S. pombe* PP1 genes (*dis2* and *sds21*) have overlapping functions[9]: a mutation within either gene is viable, while their simultaneous disruptions lead to defect in sister chromatid separation. By analogy, multi PP1 genes of *A. thaliana* presumably have overlapping functions critical for proliferation of plant cells. During the preparation of this manuscript, clonings of *A. thaliana* PP1 genes including the same one as described here have been reported, and one of them has been shown to complement the *S. pombe* cold sensitive *dis2-11* mutation.[31,32]

Type 2A phosphoprotein phosphatases of A. thailana

Genomic Southern-blot hybridization analysis with a *B. napus* partial PP2A cDNA probe revealed the existence of two to three copies of PP2A genes in the *A. thaliana* genome (Fig. 5-B). Similarly, *S. pombe* has two PP2A genes (*ppa1* and *ppa2*) highly homologous to each other, and also to the two genes of rabbit PP2A.[9] Their double disruptions result in the lethal phenotype, while either disruption does not, as in the case of the PP1 genes. However, no overlapping has been observed between PP1 and PP2A functions.[9]

We isolated a cDNA clone similar to *B. napus* and mammalian PP2A genes (Fig. 1-B). Its motif analysis revealed the LRGNHE and RAH regions characteristic of Ser/Thr-specific phosphoprotein phosphatases are completely conserved as in AT-PP1, intimating that this clone (AT-PP2A) is derived from one of *A. thaliana* PP2A genes (Fig. 2). Recently, similar clones have been reported.[33] AT-PP2A contains a conserved sequence of KIFTDLFDY at amino acid residues 144-152 that is a potential target of protein tyrosine kinase as predicted by the PROSITE analysis.[29] Besides, there are a possible Asn-glycosylation site at amino acid residues 229-232 and latent target sites for both casein kinase II and protein kinase C (at amino acid residues 201-204 and 212-214, respectively). Another conserved sequence, RRTPDYFL at the C-terminal portion,[9] though lacking in the AT-PP2A clone, contains a

probable target of protein tyrosine kinase [34] (Fig. 2). Thus, the AT-PP2A activity as well as AT-PP1 seems to be controlled by post-translational modifications including phosphorylation.

Type 2B and 2C phosphoprotein phosphatases of A. thaliana

Biochemical analyses with *B. napus* seed extract, wheat and pea leaf extracts, and carrot suspension culture have failed to identify PP2B-like enzymatic activities.[10-12] Genomic Southern-blot hybridization analysis with a rat PP2C probe indicated the presence of one PP2C gene on the *A. thaliana* genome (Fig. 5-C). PP2C is distinctive from other protein phosphatase families not only structurally but also characteristically: it acts as a monomer, being insensitive to okadaic acid.[5,21] Thus PP2C member enzymes may have quite different category of functions in cells.

Tyrosine-specific phosphoprotein phosphatases of A. thaliana

Though in animal cells reversible phosphorylation/dephosphorylation on tyrosine residues has been thought to play critical roles in the control of cellular functions, plants and yeast seem to carry no protein tyrosine kinase genes similar to those of animals.[13] Since the cell-cycle-controlling Cdc2 kinase (or its structural gene) whose activity is modulated by phosphorylation/dephosphorylation on its Tyr-15[35] has been identified in several plant species,[19,36-38] Tyr-specific kinase and Tyr-specific phosphatase for the Cdc2 kinase (yeast Wee1 and Cdc25 equivalents, respectively) should also be present in plant cells. We provided evidence to support this prediction that genomic Southern-blot analysis with either yeast *pyp1* or *cdc25* probe identified the same DNA fragment (Fig. 5-D). However, a human receptor type PTP probe did not visualize *A. thaliana* homologs in genomic Southern-blot hybridization analysis, nor were PCR fragments similar to animal PTP enzymes obtained. Therefore, the observed signal probed with *pyp1* and *cdc25* appears to correspond to the *A. thaliana* counterpart of *cdc25*. The results further support the view that *A. thaliana* PTP gene(s) are structurally diverged from those found in fungi and animals.

5. CONCLUSIONS

We revealed the presence of several types of *A. thaliana* phosphoprotein phosphatase genes similar to fungous and animal counterparts, being consistent with enzymatic activities previously identified in higher plants. The isolated clones described here provide a useful tool for functional analysis of PP1 and PP2A by over- and under-expressing the PP1 and PP2A genes in transgenic plants, through which it is expected to obtain new insight into the molecular mechanisms underlying protein phosphorylation/dephosphorylation regulation.

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REFERENCES

(1) A.M. Edelman, D.K. Blumenthal and E.G. Krebs, *Annu. Rev. Biochem.*, **56**, 567(1987).
(2) L.C. Cantley, K.R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller and S. Soltoff, *Cell*, **64**, 281(1991).
(3) Y. Nishizuka, *Nature*, **334**, 661(1988).
(4) G.F. Sprague Jr, *Trends in Genetics*, **7**, 393(1991).
(5) P. Cohen, *Annu. Rev. Biochem.*, **58**, 453(1989).
(6) E.H. Fischer, H. Charbonneau and N.K. Tonks, *Science*, **253**, 401(1991).
(7) J. Gautier, M.J. Solomon, R.N. Booher, J.F. Bazan and M.W. Kirschner, *Cell*, **67**, 197(1991).
(8) H. Ohkura, N. Kinoshita, S. Miyatani, T. Toda and M. Yanagida, *Cell*, **57**, 997(1989).
(9) N. Kinoshita, H. Ohkura and M, Yanagida, *Cell*, **63**, 405(1990).
(10) C. MacKintosh and P. Cohen, *Biochem. J.*, **262**, 335(1989).
(11) G. Siegl, C. MacKintosh and M. Stitt, *FEBS Lett.*, **270**, 198(1990).
(12) C. MacKintosh, J. Coggins and P. Cohen, *Biochem. J.*, **273**, 733(1991).
(13) T. Hirayama and A. Oka, *Plant Mol. Biol.*, **20**, 653(1992).
(14) C. Yanisch-Perron, J. Vieira and J. Messing, *Gene*, **33**, 103(1985).
(15) J. Sambrook, E.F. Fritsch and T. Maniatis, "Molecular Cloning: A Laboratry Manual", Cold Spring Harbor Laboratory Press, New York(1989).
(16) N. Berndt, D.G. Campbell, F.B. Caudwell, P. Cohen, E.F. da Cruz e Silva, O.B. da Cruz e Silva and P.T.W. Cohen, *FEBS Lett.*, **223**, 340(1987).
(17) M. Streuli, L.R. Hall, Y. Saga, S.F. Schlossman and H. Saito, *J. Exp. Med.*, **168**, 1523(1988).
(18) S. Ottilie, J. Chernoff, G. Hannig, C.S. Hoffman and R.L. Erikson, *Proc. Natl. Acad. Sci. USA*, **88**, 3455(1991).
(19) T. Hirayama, Y. Imajuku, T. Anai, M. Matsui and A. Oka, *Gene*, **105**, 159(1991).
(20) R.W. MacKintosh, G. Haycox, D.G. Hardie and P.T.W. Cohen, *FEBS Lett.*, **276**, 156(1990).
(21) S. Tamura, K.R. Lynch, J. Larner, J. Fox, A. Yasui, K. Kikuchi, Y. Suzuki and S. Tsuiki, *Proc. Natl. Acad. Sci. USA*, **86**, 1796(1989).
(22) P. Russell and P. Nurse, *Cell*, **49**, 559(1987).
(23) N.X. Krueger, M. Streuli and H. Saito, *EMBO J.*, **9**, 3241(1990).
(24) D. Valvekens, M. van Montagu and M.V. Lijsebettens, *Proc. Natl. Acad. Sci. USA*, **85**, 5536(1988).
(25) A. Hoekema, P.R. Hirsch, P.J.J. Hooykaas and R.A. Schilperoort, *Nature*, **303**, 179(1983).
(26) R.A. Jefferson, T.A. Kavanagh and M.W. Bevan, *EMBO J.*, **6**, 3901(1987).
(27) J. Kyte and R.F. Doolittle, *J. Mol. Biol.*, **157**, 105(1982).
(28) H. Ohkura and M. Yanagida, *Cell*, **64**, 149(1991).
(29) A. Bairoch, *Nucleic Acids Res.*, **20**, *Supplement*, 2013(1992).
(30) V. Dombrádi, J.M. Axton, N.D. Brewis, E.F. da Cruz e Silva, L. Alphey and P.T.W. Cohen, *Eur. J. Biochem.*, **194**, 739(1990).
(31) K. Nitschke, U. Fleig, J. Schell and K. Palme, *EMBO J.*, **11**, 1327(1992).
(32) R.D. Smith and J.C. Walker, *Plant Mol. Biol.*, **21**, 307(1993).
(33) J. Ariño, E. Pérez-Callejón, N. Cunillera, M. Camps, F. Posas and A. Ferrer, *Plant Mol. Biol.*, **21**, 475(1993).
(34) J. Chen, B.L. Martin and D.L. Brautigan, *Science*, **257**, 1261(1992).
(35) P. Nurse, *Nature*, **344**, 503(1990).
(36) J. Colasanti, M. Tyers and V. Sundaresan, *Proc. Natl. Acad. Sci. USA*, **88**, 3377(1991).
(37) A. Hemerly, C. Bergounioux, M. van Montagu, D. Inzé and P. Ferreira, *Proc. Natl. Acad. Sci. USA*, **89**, 3295(1992).
(38) S. Hata, H. Kouchi, I. Suzuka and T. Ishii, *EMBO J.*, **10**, 2681(1991).
(39) Y. Khew-Goodall, R.E. Mayer, F. Maurer, S.R. Stone and B.A. Hemmings, *Biochemistry*, **30**, 89(1991).