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Complementation of the cs *dis2*-11 cell cycle mutant of *Schizosaccharomyces pombe* by a protein phosphatase from *Arabidopsis thaliana*

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The activities of type I protein phosphatases play a central role in eukaryotic cell cycle control. Here, we report the cloning and characterization from the flowering plant *Arabidopsis thaliana* of a cDNA clone named PP1-At which is highly homologous to protein phosphatase 1. The deduced amino acid sequence of PP1-At shows that the PP1-At protein is 318 amino acid residues long and has a molecular weight of 35 298 Da. The PP1-At protein has strong similarity to all other known protein phosphatase type 1 catalytic subunits. Approximately 62% of the amino acids are identical to type 1 protein phosphatases of rabbit, mouse, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. RNA blot analysis revealed a single mRNA species of approximately the same size as the cDNA isolated. The PP1-At-encoded mRNA of 1.3 kb is abundant in most vegetative *Arabidopsis* tissues, with the lowest level of expression in leaves. When transferred to the fission yeast *S.pombe*, the PP1-At-encoded protein can rescue a semidominant mutant, cold sensitive (cs) *dis2*-11, which under nonpermissive conditions is unable to complete chromosome disjunction.

Key words: *Arabidopsis thaliana*/complementation/cs *dis2*-11 mutant/protein phosphatase 1/yeast

Introduction

Elucidation of the molecular mechanisms by which hormones and other extracellular signals produce defined responses in specific target cells is of major interest in developmental biology. A considerable variety of extracellular signals regulate diverse metabolic and physiological responses by influencing the state of phosphorylation of specific substrate proteins in target tissues. The large number of diverse molecular signalling pathways leading to protein phosphorylation support the view that in apparently all eukaryotic organisms, protein phosphorylation is a main pathway for the transduction of information to particular intracellular target(s). Thus, protein phosphorylation is a major regulatory mechanism controlling cellular metabolism, growth and differentiation in a variety of eukaryotic organisms (for references see Hunter, 1987; Ranjeva and Boudet, 1987). Although the level of phosphorylation of a particular protein depends on the relative activities of protein kinases and protein phosphatases, the latter enzymes have received much less attention, most likely due to the difficulty in obtaining well defined substrates for analysis.

Most known serine/threonine-specific phosphatases have been shown to have only limited substrate specificity in *in vitro* assays. Using biochemical criteria, four major classes of serine/threonine specific protein phosphatase catalytic subunits were identified in eukaryotic cells: protein phosphatase types 1, 2A, 2B and 2C (for review see Cyert and Thorner, 1989; Cohen, 1989a; Cohen and Cohen, 1989). Although their precise functions are not yet known, several mutants in a variety of organisms, including *Drosophila*, point to a particular role of protein phosphatase 1 in mitotic control (Ohkura *et al.*, 1989; Booher and Beach, 1989; Doonan and Morris, 1989; Dombradi *et al.*, 1990). The functional similarity of type 1 protein phosphatases in different organisms is reflected in the structural identity of their cDNAs and deduced amino acid sequences. Amino acid sequence comparisons indicate that this class of enzymes has changed very slowly through evolution (Dombradi *et al.*, 1989, 1990; Okhura *et al.*, 1989; Doonan and Morris, 1989; Cohen *et al.*, 1989b, 1990; MacKintosh *et al.*, 1990b).

Interest in the role played by protein phosphatases in cellular regulation has been kindled by a number of recent discoveries. (i) Identification of integral membrane proteins with topological features of receptor proteins having intrinsic protein phosphatase activity revealed a new transmembrane signal transduction mechanism (Ostergaard *et al.*, 1989; Pingel and Thomas, 1989; Mustelin *et al.*, 1989). (ii) Suppressor mutations allowing transcription of the *HIS4* gene of *Saccharomyces cerevisiae* led to the isolation of the *SIT4* gene, a novel member of the protein serine/threonine phosphatase family (Arndt *et al.*, 1989). (iii) Genetic analysis of lower eukaryotes revealed several cell cycle control genes sharing homologies with mammalian protein phosphatases. The gene products of $dis2^+$ and $sds21^+$ from the fission yeast *Schizosaccharomyces pombe* are involved in the control of chromosome condensation, formation of spindle morphology and sister chromatid exchange and are required for the completion of mitosis (Ohkura *et al.*, 1989; Booher and Beach, 1989). Furthermore, mutations in the *bimG* gene from *Aspergillus nidulans*, which has 86% similarity to type 1 protein phosphatase from mammals, led to a reduction in phosphatase activity, an increase of nuclear phosphoproteins and subsequently to a rise in mitotic index. Furthermore, anaphase separation of daughter nuclei is inhibited (Doonan and Morris, 1989). The pleiotropic phenotypes exerted by the products of these genes may point to multifunctional enzymes with broad substrate specificities.

Recently, enzymatic activities of several protein phosphatases from plants were shown to be virtually indistinguishable from those of corresponding mammalian enzymes, including substrate specificity and sensitivity to inhibitors such as okadaic acid or mycrocystin LR (MacKintosh and Cohen, 1989; MacKintosh *et al.*, 1990a).

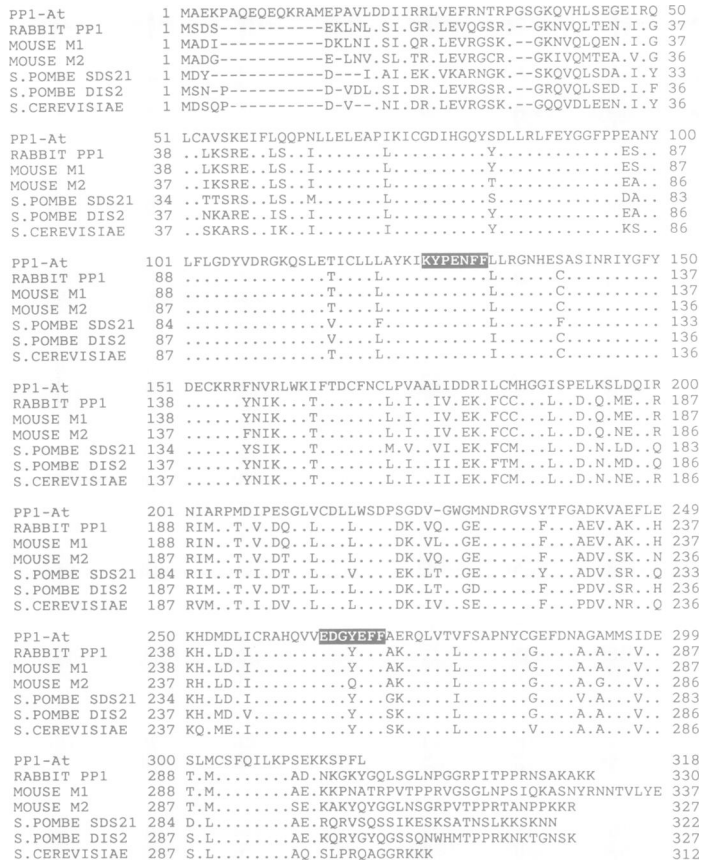

Fig. 1. Aminc acid sequence comparison of type 1 protein phosphatases. Shaded boxes identify areas used for construction of PCR primers. Black dots represent identical amino acid residues, bars represent missing amino acid residues at the particular position. The PP1-At sequence has been deposited in the EMBL database (accession number X64328).

It is therefore expected that genes coding for these enzymes may play an important role in the control of growth of higher plants.

In the present study we have searched for protein phosphatase 1 related genes in *Arabidopsis thaliana*. We describe the isolation and structural analysis of a cDNA clone from *A.thaliana* encoding a protein phosphatase 1 related protein. The amino acid sequence predicted from the DNA sequence of PP1-At is highly homologous to those of type 1 protein phosphatases from other eukaryotic organisms including *S.pombe* and mouse. The protein phosphatase 1 related gene from *A.thaliana* is transcribed differentially. Complementation of a fission yeast mutant, cs *dis2*-11, suggests that yeast and plant protein phosphatase 1 are functionally related.

Results

Isolation of PP1-At

To select suitable regions from which to generate phosphatase 1 specific oligonucleotide primers, we identified two of the most highly conserved regions within the phosphatase family. Figure 1 shows a comparison of seven type 1 protein phosphatase specific amino acid sequences, including *Arabidopsis* PP1-At. Sequences corresponding to amino acids KYPENFF and EDGYEFF, at positions 113−119 and 252−258 respectively of rabbit PP1 (Figure 1, shaded boxes), were chosen for derivation of oligonucleotide primers. The oligonucleotide probes synthesized (PP1A and PP1B) were mixtures of 128 different oligonucleotide sequences. Each oligonucleotide was additionally designed to include a restriction enzyme-specific linker at the 5′-end flanking the phosphatase consensus sequence, thereby facilitating directional cloning of amplified fragments into pUC119. After 40 cycles of amplification, a band of ~440 bp was detected in a size range predicted from the initial sequence comparison (438 bp). The isolated DNA fragment was subcloned into pUC119 and four of the clones obtained were sequenced. Analysis of the open reading frames of these clones revealed that two of the open reading frames were similar to protein phosphatases. Clone $4a_3$ from *A.thaliana* showed 77% amino acid sequence identity to both mouse and rabbit PP1, thus representing a homologue to protein phosphatase 1.

This clone was used to screen an *Arabidopsis* specific cDNA library. Approximately 2×10^5 recombinants from a λgt10 cDNA library were screened with this 440 bp phosphatase 1 specific PCR fragment, resulting in identification of 20 positive signals. *Eco*RI digestion of plaque purified phages revealed inserts ranging from 0.8 to 1.4 kb. A λ clone containing a cDNA insert of 1350 bp, named PP1-At for protein phosphatase 1 catalytic subunit from *A.thaliana*, was chosen for further analysis.

DNA sequence of PP1-At cDNA

The DNA sequence was determined for both strands after generating subclones using exonuclease III. A large open reading frame, beginning at the first translation initiation codon and ending at a TGA stop codon, is 954 nucleotides long. The predicted protein has 318 amino acid residues and

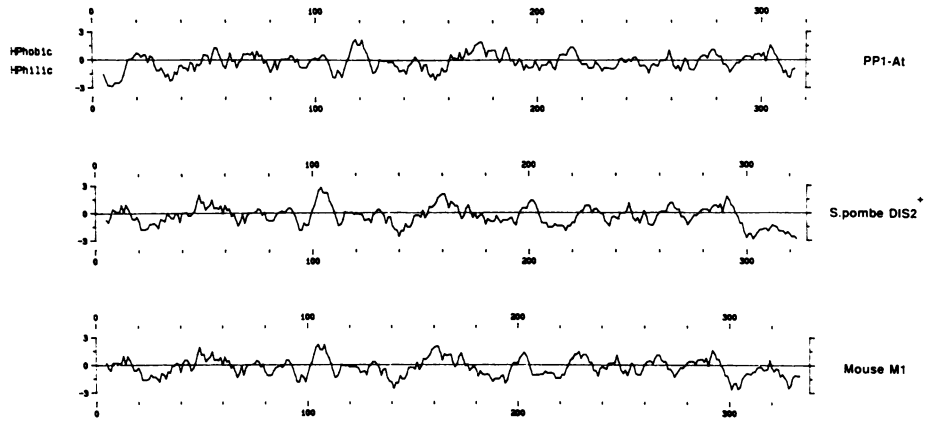

Fig. 2. Hydropathy profile of protein phosphatase 1 from *Arabidopsis thaliana*. Each value was calculated as the average hydrophobic index of a sequence of nine amino acids and plotted at the middle residue of each sequence. Positive and negative values indicate hydrophobic and hydrophilic regions of the protein (Kyte and Doolittle, 1982).

a mol. wt of 35 298 kDa. The DNA sequence flanking the ATG does not match the plant translation initiation sequence (Lütcke *et al.*, 1987). No canonical polyadenylation signals typical for other eukaryotic genes are present in the cDNA (Joshi, 1987). The motif AATAATATA located 72 nucleotides upstream of the poly(A) tail could act as a polyadenylation signal.

The PP1-At protein shows structural similarity to other protein phosphatase catalytic subunits

Figure 1 displays amino acid sequence alignments of PP1-At with protein phosphatase catalytic subunits from rabbit, mouse, *S.pombe* and *S.cerevisiae*. Amino acid similarity is significant from amino acid residue 58 to 310, whereas the N-terminal and C-terminal regions of the PP1-At protein have diverged as in the case for other type 1 protein phosphatases. Further comparison shows that 62% of the amino acid residues were identical and 29% similar to type 1 protein phosphatases from rabbit, mouse, *S.pombe* and *S.cerevisiae*. Structural similarity is further revealed in the hydropathy profiles showing a striking match of both hydrophobic and hydrophilic areas between PP1-At, *S.pombe dis2*$^+$ and mouse M1 (Figure 2). The PP1-At protein is slightly shorter at the C-terminal end than the mouse M1 and the *S.pombe dis2*$^+$ protein. The hydrophilic C-terminal amino acid residues (between 7 and 31) present in other eukaryotic type 1 phosphatases are missing in the PP1-At protein. The amino acid sequence comparison further reveals that the PP1-At protein contains 10 additional hydrophilic amino acids not present in other protein phosphatases 1 at the N-terminal end (Figures 1 and 2).

Northern and Southern blot analyses

Southern analysis of *A.thaliana* genomic DNA cleaved with various restriction enzymes revealed bands from 1.8 to 7 kb hybridizing with the PP1-At cDNA probe (Figure 3). The strongly hybridizing band at 3.5 kb obtained after digestion of *Arabidopsis* DNA with *Hin*dIII indicates that most of the gene is probably located within this genomic fragment. In addition, low stringency hybridizations revealed several weak bands. The sizes of the various fragments suggest that the gene identified here is probably a member of a small gene family.

A transcript of 1.3 kb was detected in Northern blot analysis of total RNA from various organs of an *Arabidopsis* plant (Figure 4). The highest levels of expression were observed in flowers and roots, whereas the lowest levels of PP1-At transcript were observed in young and old leaves.

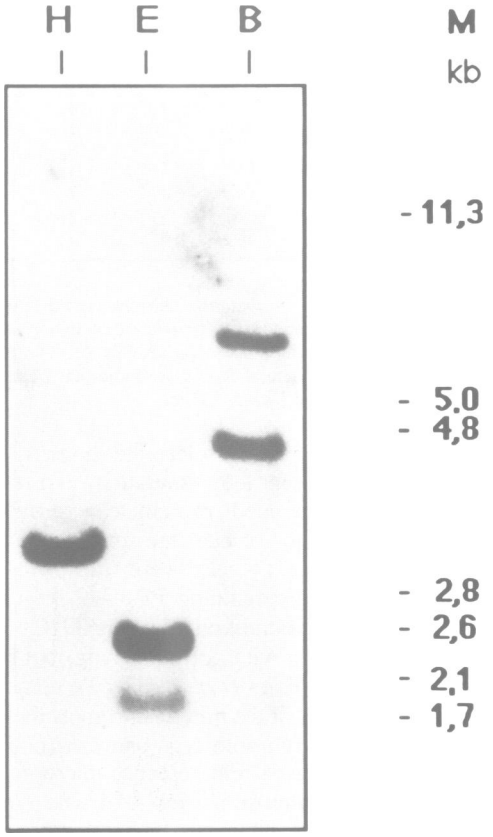

Fig. 3. Southern blot analysis of the *Arabidopsis* protein phosphatase 1 gene. Ten micrograms of genomic DNA from *A.thaliana* were digested with *Hin*dIII (lane H), *Eco*RI (lane E) and *Bam*HI (lane B). Genomic DNA was electrophoresed in a 1% agarose gel and transferred to nylon. The *Arabidopsis* blot was probed with phosphatase cDNA PP1-At, which is described in detail in Materials and methods.

Rescue of a fission yeast cs dis2$^-$ *mutant by complementation with the Arabidopsis PP1 homologue*

The PP1-At coding region was cloned into a multicopy yeast expession vector, pART1 (McLeod *et al.*, 1987), resulting in the plasmid shown in Figure 5. This plasmid contains the

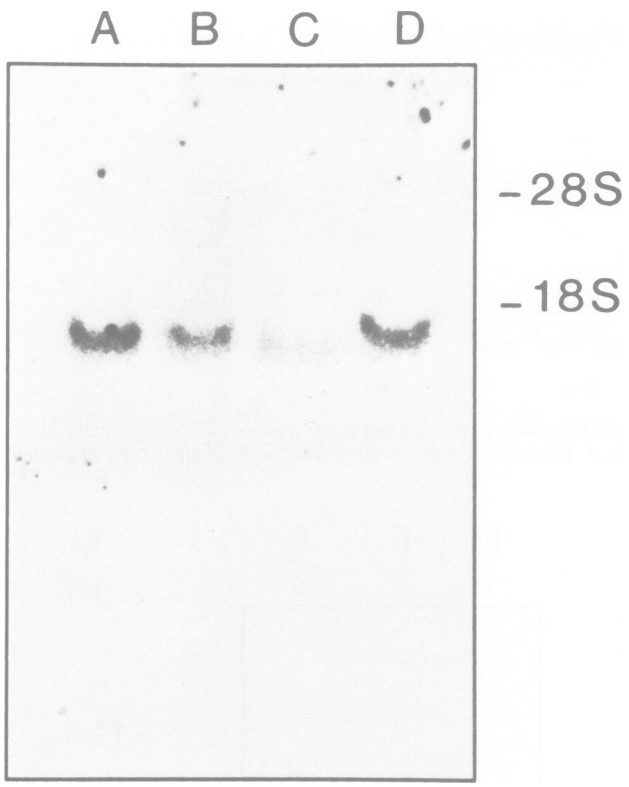

Fig. 4. Expression analysis of *Arabidopsis* phosphatase mRNA. RNA was fractionated in a formaldehyde gel, transferred to nylon and hybridized with ^{32}P-labelled phosphatase cDNA PP1-At. The position of ribosomal 28S and 18S structural RNAs are indicated. Lane a, root; lane b, stem; lane c, leaf; and lane d, flower.

S.cerevisae LEU2 gene as a selectable marker, which complements an *S.pombe* leu$^-$ mutation. To examine whether the PP1-At-encoded protein can provide the function(s) necessary to rescue the cs *dis2*$^-$ mutant phenotype, *S.pombe dis2*-11 cells were transformed with either vector or plasmid containing PP1-At.

Under nonpermissive conditions (i.e. at 20°C) *dis2*-11 mutants can enter mitosis with normal timing, but become lethal during mitosis (Ohkura *et al.* 1988). To demonstrate the anomalous behaviour of the mitotic chromosomes in this mutant, we compared both wild type and cs *dis2*-11 cells at the restrictive temperature. Fluorescence micrographs are shown in Figure 6. Chromosomal DNA of wild type cells (Figure 6A) and *dis2*-11 cells transformed with pART1 (Figure 6B) DNA was stained with DAPI. In *dis2*$^-$ mutant cells the mitotic chromosomes condense but do not disjoin. After transformation of *dis2*-11 cells with plasmid pART-PP1-At, transformants were able to form colonies at the restrictive temperature (20°C) (Figure 6C). In contrast, *dis2*-11 cells transformed with plasmid pART1 alone were unable to grow at 20°C and arrested with condensed chromosomes as indicated in Figure 6B.

Discussion

The importance of protein phosphorylation–dephosphorylation in the control of mitosis and cell proliferation has been particularly well documented. When cells enter mitosis, phosphorylation of many substrate proteins results in reorganization of the cytoskeleton, breakdown of the nuclear envelope and condensation of the chromosomes (Gerace and Blobel, 1980; Wu *et al.*, 1986; Arion *et al.*, 1988; Labbé *et al.*, 1988; Bailly *et al.*, 1989; Booher *et al.*, 1989; Verde *et al.*, 1990). Compared to the role of protein kinases, protein phosphatases are expected to contribute significantly to the regulation of the cell cycle (Cohen and Cohen, 1989).

The identification of cold sensitive mutations in *S.pombe* blocking mitotic chromosome disjunction, led to the isolation of protein phosphatase 1 encoding genes by complementation. These genes (*SDS21* and *dis*) play an essential role in cell cycle control in *S.pombe*. A single amino acid residue exchange from Arg to Gln at position 245 was found to cause dominant mitotic defects in fission yeast. In addition, it was found that a mutation in a protein phosphatase 1 encoding gene from *Drosophila melanogaster* resulted in the death of larvae at the transition of larvae to pupae (Doonan and Morris, 1989; Ohkura *et al.*, 1989; Axton *et al.*, 1990; Kinoshita *et al.*, 1990). Furthermore, a deletion in the promoter region of the *Drosophila* gene resulted in a reduction of expression of its mRNA and subsequently to an 80% reduction of enzymatic activity. The reduction in enzymatic activity was found to be responsible for the dominant disruption of normal larval development, preventing neuroblast and imaginal cell proliferation (Axton *et al.*, 1990; Dombradi *et al.*, 1990; Kinoshita *et al.*, 1990). These data indicate that loss of a single isoform of type 1 phosphatase results in a mitotic defect (Dombradi *et al.*, 1990).

Protein phosphatase 1 related enzymes have been detected in all eukaryotic cells tested. Thus, it was not unexpected that protein phosphatases would be detected in higher plants (Bennett, 1980; Lin *et al.*, 1980; Polya and Haritou, 1988; MacKintosh and Cohen, 1989). It was found that of the four major protein phosphatase catalytic subunits, only type 1 and type 2A activities were present in plant cell extracts at particularly high levels (MacKintosh and Cohen, 1989). This correlates well with the surprisingly rapid turnover of phosphate bound to plant proteins as compared with proteins from animal cells (Dietrich *et al.*, 1990; Palme *et al.*, unpublished). Analysis of *Brassica napus* seed extracts revealed that enzymatic activities of both type 1 and 2A protein phosphatases were virtually indistinguishable from the corresponding enzymes from animal cells. For example, type 1 phosphatase activity dephosphorylated the β-subunit of phosphorylase kinase selectively and was inhibited by protein inhibitors 1 and 2 from rabbit muscle in a concentration range similar to that of mammalian enzymes (MacKintosh and Cohen, 1989). Similar activities were also found in carrot, maize and pea leaf extracts. Interestingly, both enzymes are present in particulate and in cytosolic extracts, accounting for 77% and 23% of phosphatase activity, respectively. Both enzymes are apparently not involved in regulation of chloroplast metabolism (MacKintosh *et al.*, 1991). In addition to protein phosphatases 1 and 2A, a Mg^{2+}-dependent and okadaic acid insensitive protein phosphatase resembling protein phosphatase 2C was also identified in extracts from carrots, pea and wheat (MacKintosh *et al.*, 1991). While protein phosphatase 2A most likely plays an important role in regulation of plant specific metabolic events, the role of protein phosphatase 1 in higher plants is not yet understood. However, in analogy with *Drosophila* and mammalian cells, it might be expected that the latter enzyme could play an essential role in cell cycle control, specifically for the completion of mitosis in plant cells.

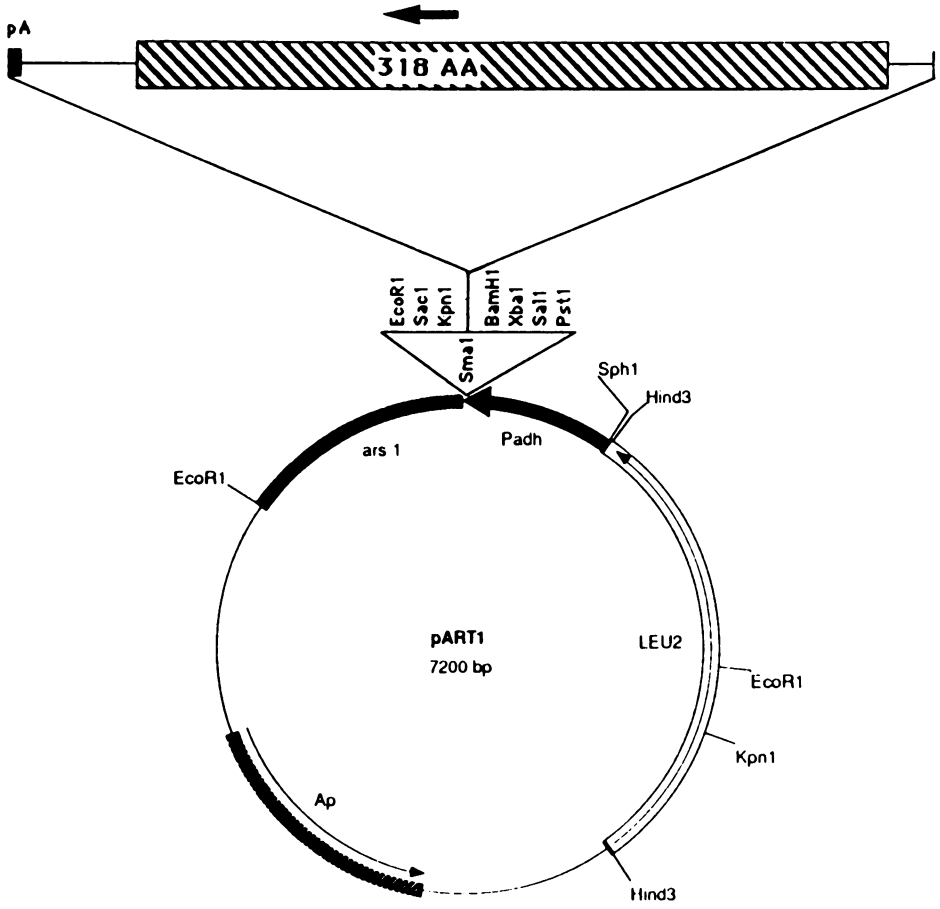

Fig. 5. Vector for expression of PP1-At protein in *S.pombe*. Padh, alcohol dehydrogenase promoter flanked by polylinker sequence. The orientation of the inserted PP1-At cDNA is indicated.

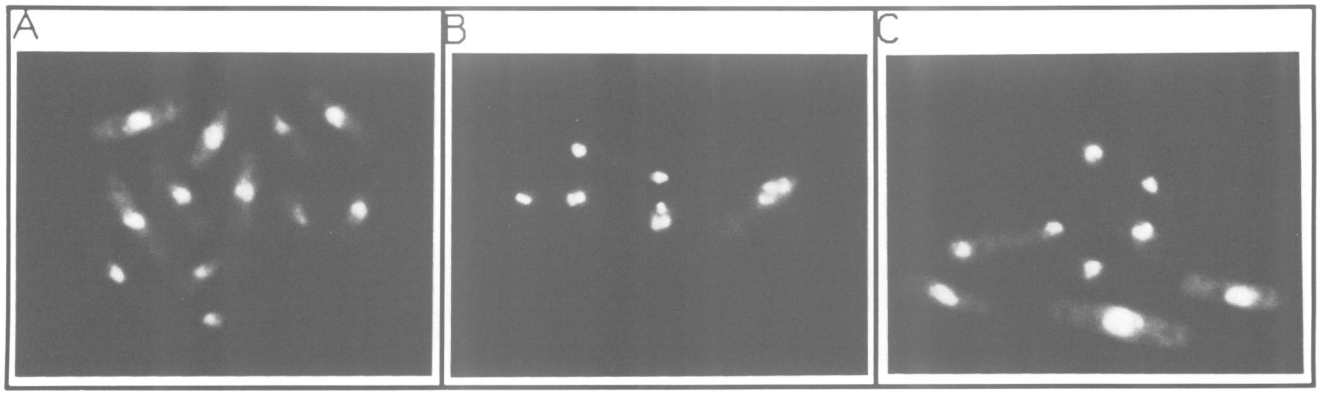

Fig. 6. Complementation of the cs phenotype of the *S.pombe* cs *dis2*-11 cells by the *A.thaliana* PP1-At protein. **A**, *S.pombe* wild type cells; **B**, *S.pombe* cs *dis2*-11 mutant cells transformed with plasmid pART1; **C**, *S.pombe* cs *dis2*-11 mutant cells transformed with plasmid pART1-PP1-At.

We have shown that a protein phosphatase 1 from *A.thaliana* is remarkably similar to other type 1 protein phosphatases (e.g. mouse, rabbit, *S.pombe* and *S.cerevisiae*, Figure 1). Table I illustrates the strong similarity between the amino acid sequences of PP1-At and various type 1 phosphatases. There is significant similarity from amino acid residue 58 to 310, whereas the N-terminal and the C-terminal regions of the PP1-At protein have diverged as is the case for other type 1 protein phosphatases. Structural similarity was further revealed in the hydropathy profiles, showing a striking match of both hydrophobic and hydrophilic areas between PP1-At, *S.pombe dis2*$^{+}$ and mouse M1. This striking conservation of amino acid sequences in different phyla suggests that an extremely strong selective pressure has maintained the general structure of this protein during evolution. This may also point to an essential function of this protein in higher plants.

To study whether the cDNA clone isolated from *A.thaliana* encodes a functional protein that is able to rescue a fission yeast cs *dis2*$^{-}$ mutant by complementation, we cloned the PP1-At coding region into a multicopy yeast expession vector, pART1 (McLeod *et al.*, 1987). Besides the *S.cerevisae LEU2* gene as a selectable marker, this plasmid contains the alcohol dehydrogenase (*adh*) promoter, thus

Table I. Level of homology between protein phosphatase 1 catalytic subunits from *A.thaliana*, rabbit, mouse and yeast

Percent homology	PP1-At	Rabbit PP1	Mouse M1	*S.pombe* sds21$^+$	*S.pombe* dis2$^+$	*S.cerevisiae* DIS2S1
PP1-At	–	85.3	86.6	86.7	86.2	85.3
Rabbit PP1		–	94.7	86.3	90.5	91.0
Mouse M1			–	86.0	90.0	89.4
S.pombe sds21$^+$				–	91.6	88.0
S.pombe dis2$^+$					–	91.4
S.cerevisiae DIS2S1						–

allowing high level expression of foreign proteins. To examine whether the PP1-At encoded protein can provide the function(s) necessary to rescue the cs *dis2*$^-$ mutant, *S.pombe dis2*-11 cells were transformed with either vector or plasmid containing PP1-At. Under nonpermissive conditions (i.e. at 20°C) *dis2*-11 mutants can enter mitosis with normal timing, but die during mitosis. In *dis2*-11 mutant cells the mitotic chromosomes condense, but do not disjoin. After transformation of *dis2*-11 cells with plasmid pART-PP1-At, transformants were able to form colonies at the restrictive temperature, whereas *dis2*-11 cells transformed with plasmid pART1 alone were unable to grow at 20°C. These cells where arrested with condensed chromosomes as indicated in Figure 6B.

The cs phenotype of the semidominant *S.pombe dis2*-11 mutant made it possible to test for rescue of this defect by the PP1-At protein. As indicated by the hydrophobicity plot (not shown), the lack of complementation by the mouse PP1 protein (Ohkura *et al.*, 1989) may be due at least in part to the nine amino acid residues C-terminal extension of the mouse M1 polypeptide. We noticed that in the PP1-At protein, this region differed from the mouse M1. We were therefore pleased to observe colony formation after high level expression of PP1-At under control of the efficient *adh* promoter. Thus, a high dosage of the PP1-At protein is able to rescue *S.pombe dis2*$^-$ mutant cells. This result might further point to a previously unrecognized functional significance of the C-terminal area in determining cellular substrate specificity for the various members of this protein family. As already pointed out by the extent of similarity suggesting possible conservation of function, our data indicate that PP1-At might be involved in mitotic control pathways in higher plants. Further analysis of this gene will contribute to the analysis of cell cycle control in higher plants.

After this work was completed, MacKintosh *et al.* (1990b) published the characterization of two partial cDNA clones from *Brassica napus* coding for protein sequences similar to mammalian protein phosphatases 1 and 2A, demonstrating 72% and 79% overall identity respectively. Since neither clone contained an initiating methionine codon, they apparently represent rearrangements in the 5′-area during construction of the cDNA library. Comparison with the PP1-At from *A.thaliana* demonstrates remarkable similarity (89.34%) to a related protein from *Brassica napus*.

Materials and methods

Materials

The Columbia wild type cultivar of *Arabidopsis thaliana* was the source of plant tissue

Recombinant DNA techniques

These were performed, with minor variations, according to Maniatis *et al.* (1982) and Sambrook *et al.* (1989).

Polymerase chain reaction

PCR primer sequences were derived from two internal peptides, pep1 (amino acid residues 113–119, KYPENFF) and pep2 (amino acid residues 252–258, FFEYGDE) from rabbit phosphatase 1; these regions are strongly conserved in different isolated PP1 genes. The oligonucleotide sequences were for PP1A (pep1) 5′-ACGAATTCAAR$_1$TAR$_2$CCR$_3$GAR$_4$AAR$_5$TT-R$_6$TT-3′(R$_1$: G,A; R$_2$: C,T; R$_3$: C,G,A; R$_4$: A,G; R$_5$: C,T; R$_6$: C,T) and for PP1B (pep2) 5′-CCTCTAGAR$_1$AAR$_2$AAR$_3$TCATAR$_4$CCR$_5$TCR$_6$T-C-3′ (R$_1$: A,G; R$_2$: A,G; R$_3$: C,T; R$_4$: G,T,A; R$_5$: G,A; R$_6$: C,T). Oligonucleotides were synthesized on an Applied Biosystems synthesizer (model 380B) and used after deprotection and NAP G-25 chromatography (Pharmacia) without further purification. The reaction mix for PCR contained 1 μl of amplified phage lysate of an *A.thaliana* λgt10 cDNA library (10^6-10^8 p.f.u./μl), *Taq* polymerase buffer (50 mM KCl, 10 mM Tris–HCl (pH 9.5), 1.5 mM $MgCl_2$, 0,01% gelatin (w/v) 0.1% Triton X-100), oligonucleotides PP1A and PP1B at 50 pM each and water. After DNA denaturation for 10 min at 100°C, 200 μM dNTPs (final concentration) and 2.5 U of *Thermus aquaticus* DNA polymerase (*Taq* polymerase, Promega) were included in the reaction mixture. PCR was performed in a total volume of 100 μl in a thermocycler (LEP Scientific, UK) for 40 cycles: 1 min at 92°C (denaturation), 1 min at 40°C (annealing) and 1 min at 70°C (extension).

Cloning of PCR fragments

After amplification, 1 U of Klenow polymerase (Boehringer) was added and the mixture was incubated at room temperature for 30 min. DNA was precipitated with ethanol, resuspended in 20 μl water and digested with *Eco*RI and *Xba*I (5 U each) for 2 h at 37°C. PCR products were separated by agarose gel electrophoresis (Seakem, FMC). Bands of the expected size (438 bp) were excised from the gel. Approximately 500 ng of this DNA was eluted and ligated into an *Eco*RI/*Xba*I cleaved pUC119 vector. DNA sequences were determined by the dideoxynucleotide method using T7 polymerase (Pharmacia) on double-stranded plasmid DNA isolated from miniprep cultures. A fragment, containing both PP1A and PP1B primer sequences as well as additional sequences conserved within protein phosphatases, was selected to probe an *A.thaliana* specific cDNA library.

Isolation and sequence analysis of protein phosphatase 1 cDNA clone from Arabidopsis thaliana

2×10^5 recombinants of an amplified *Arabidopsis* cDNA library, plated at a density of 35 000 λ phages per plate, were used for screening. Hybridization of replica filters was performed with a digoxygenin labelled probe, following the manufacturer's protocol (Boehringer). Nylon filters (Hybond-N, Amersham) were washed twice in 2×SSC, 0.1% SDS at room temperature for 30 min and then with 0.1×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS for 20 min at 65°C.

Twenty positive recombinants were identified; ten of these were plaque-purified and selected for further analysis. PCR was used to determine the insert length of each of the isolated recombinants using oligonucleotides complementary to the imm 434 region of the phage λgt10. These oligonucleotides flank the left and right borders of a unique *Eco*RI site located on phage λ. After selection of recombinant phages containing the longest inserts, the phage DNA was isolated and cleaved with *Eco*RI. Insert DNA was subcloned into *Eco*RI digested pUC119 vector. Nested deletions of both strands were generated by exonuclease III digestion. Double-stranded DNA was used as a template for sequencing. All sequencing reactions were performed by the dideoxy chain termination method with modified T7 DNA polymerase and both strands were sequenced (Sanger *et al.*, 1977; Tabor

and Richardson, 1989). Sequences were determined using fluorescent primers, T7 polymerase and an automated DNA sequencer (Pharmacia, model ALF).

Nucleic acid gel blot analysis

Total RNA was isolated from different organs of *Arabidopsis* plants, according to Chomczynski and Sacchi (1987). Aliquots of RNA samples (20 μ) were subjected to electrophoresis in 0.8% agarose–formaldehyde gels. RNA was transferred to nylon (Hybond-N, Amersham) in 20×SSC according to the manufacturer's recommendations. After baking, membranes were prehybridized overnight at 42°C in a solution containing 50% (v/v) formamide, 5×SSC, 0.5×Denhardt's solution, 0.1% SDS and boiled salmon sperm DNA at 200 μg/ml. Hybridization was carried out at 42°C for 12 h and ^{32}P-labelled probe (6×10^6 c.p.m./ml) was added to the same solution used for prehybridization. The radioactive probe was prepared by random priming according to the manufacturer's specifications (Boehringer). High stringency washes of hybridized blots were performed in 0.1×SSC, 0.1% SDS at 65°C; low stringency washes were in 2×SSC, 0.1 SDS at 42°C. Filters were subjected to autoradiography using intensifier screens.

Genomic DNA was isolated according to Dellaporta *et al.* (1983) and digested with various restriction enzymes before agarose gel separation. After transfer to nylon (Hybond-N, Amersham), the blot was hybridized with a DNA probe which was radioactively labelled using the Boehringer Multiprime Kit. After washing, blots were exposed to Kodak XAR-5 film as described above.

Microscopy

Formaldehyde fixed cells were stained with the fluorochrome DAPI as described (Moreno *et al.*, 1991) and photographed with Kodak Tmax 400ASA film using a Zeiss Axioskop microscope.

Yeast transformation

A *dis2*$^-$ strain (*dis2*-11, *leu1*-32, *h*$^-$) (Ohkura *et al.* 1988) was transformed (Moreno *et al.* 1991) with plasmids pART1 or pART1-PP1-At and incubated at the permissive temperature (33°C). Leu$^+$ transformants were streaked out for single colonies on selective minimal medium plates (Gutz *et al.*, 1974) and incubated at the restrictive temperature (20°C) for 10 days. Only cells carrying pART1-PP1-At were able to form colonies at this temperature.

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