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Cloning of cDNAs from *Arabidopsis thaliana* that encode putative protein phosphatase 2C and a human Dr1-like protein by transformation of a fission yeast mutant

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ABSTRACT

We characterized three *Arabidopsis thaliana* cDNA clones that could rescue the sterile phenotype of the *Schizosaccharomyces pombe pde1* mutant, which is defective in cAMP phosphodiesterase. The first clone had a coding capacity of 399 amino acids that is 35% identical with rat protein phosphatase 2C (PP2C). The second had a coding capacity of 159 amino acids that is 41% identical with human Dr1. Dr1 has been shown to interact with TATA-binding protein (TBP) and block its ability to activate transcription. The third encoded *Arabidopsis* TBP itself. *Saccharomyces cerevisiae* TBP also could suppress the sterile phenotype if expressed in *S.pombe pde1* cells, but overexpression of *S.pombe* TBP could do so very poorly. These observations suggest preliminarily that PP2C may counteract cAMP-dependent protein kinase in fission yeast cells, and that the heterologous TBPs and Dr1 may interfere with the general transcription factors of *S.pombe* so that the gene expression in the host cell becomes affirmative of sexual development. Furthermore, the identification of a Dr1-like protein in *A.thaliana* strongly argues for the ubiquity of this protein among eukaryotic genera and for a conserved mechanism to regulate transcription initiation that involves Dr1.

INTRODUCTION

Transcomplementation of yeast mutants is a useful strategy to clone genes of higher organisms that are functionally related to the mutated yeast genes. Two genetically well-characterized yeast species, namely the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, are used as suitable hosts. Human genes involved in cell cycle control have been isolated according to this strategy (1–5). A similar strategy appears to be applicable to the flowering plant *Arabidopsis thaliana*, because an *Arabidopsis* gene encoding protein phospatase 1 has been demonstrated to rescue a cold-sensitive mutation in the *S.pombe dis2* gene, which encodes the same enzyme (6).

Genes encoding components of the cAMP cascade have been well characterized in both *S.cerevisiae* and *S.pombe*. In *S.cerevisiae*, cAMP positively regulates cell growth via the cAMP-dependent protein kinase (PKA) and cells do not survive without this kinase activity (7,8). In contrast, *S.pombe* cells can grow even if they do not have a measurable level of intracellular cAMP (9,10), and disruption of the *pka1* gene, which encodes *S.pombe* PKA, is not lethal (11). However, *S.pombe* cells lacking cAMP or the PKA activity are highly derepressed for sexual development. They readily mate, undergo meiosis and form spores in the presence of rich nutrition, whereas wild-type *S.pombe* cells do not enter sexual development under the same conditions (9–11). The level of cAMP in the wild-type cells decreases when they are placed under poor nutrition, suggesting that the reduction of the cAMP level is indeed a critical signal for the initiation of sexual development in *S.pombe* (12).

Aiming to facilitate the study of physiological roles for cAMP in plant cells, we set out to isolate *Arabidopsis* genes that can transcomplement a null mutation in the *S.pombe pde1* (also called *cgs2*) gene, which encodes cAMP phosphodiesterase (12,13). *S.pombe* cells in which *pde1* is disrupted contain cAMP at the level of 12 pmol/mg protein, which is four times as high as the wild-type level (12). Because of this high level of cAMP, *pde1* mutant cells are defective in mating and sporulation.

We isolated a series of cDNA clones from *A.thaliana* that have the ability to rescue the sterile phenotype of the *S.pombe pde1* mutant. Analysis of these clones, however, suggested that none of their gene products is directly involved in the cAMP cascade in *Arabidopsis*. Although these results are away from our original aim, some of the *Arabidopsis* genes thus isolated are novel and apparently intriguing. We describe here three *Arabidopsis* genes, two of which have not been reported previously. We will discuss the possible mechanisms for the observed suppression of *S.pombe pde1* by these genes.

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MATERIALS AND METHODS

Yeast and *Arabidopsis* strains

A homothallic haploid *S.pombe* strain JZ666 (*h*^{90} *ade6-M216 leu1 ura4-D18 pde1::ura4*$^{+}$) was used as the host for transcomplementation. JY450 (*h*^{90} *ade6-M216 leu1*) was used as the *pde1*$^{+}$ control. *Arabidopsis thaliana* (Columbia ecotype) was used as the source for construction of a cDNA library.

Sporulation assay

Homothallic *S.pombe* cells that are competent in sexual development mate and sporulate when they are starved for nutrition. On SSA medium (14), which contains a limited amount of nitrogen, the cells propagate to form small colonies and then start to mate and sporulate in each colony. If a colony contains many sporulated cells, it is stained dark-brown by iodine vapor (15), and hence can be distinguished easily from unsporulated colonies, which are colored yellow. We used the intensity of iodine staining as a measure of sporulation. If a more quantitative assay was desired, the numbers of sporulated and unsporulated cells were determined microscopically and the sporulation frequency was calculated for each strain.

Arabidopsis cDNA library and transformation of *S.pombe*

RNA was extracted from the above-ground parts of developed *A.thaliana* plants by the GTC-method, and poly(A) RNA was fractionated by oligo(dT) column chromatography, as described (16). cDNA was prepared using the SuperScript lambda system (GIBCO BRL) according to the protocol supplied by the manufacturer. cDNA was then ligated to the expression vector pREP3 (17), which carries the *S.pombe nmt1* promoter. The size of the library thus obtained was roughly 4.5×10^{5}. Transformation of *S.pombe* cells was carried out by electroporation as described (18). The *nmt1* promoter is repressible by thiamine (17). It is used as a constitutive promoter in this study, omitting thiamine from the relevant media.

S.pombe and *S.cerevisiae* TBP clones

To clone the coding sequence of *S.pombe* TBP (19,20), a pair of PCR primers was designed. One primer was 5′-ATTGGT-CGACTGCA**ATG**GATTTCGCTTTAC-3′, which encompasses the initiation codon (indicated in bold). The three nucleotides underlined were altered from the authentic sequence to create a *Sal*I cutting site. The other was 5′-CTGTGCGGCCG-CC**TTA**ATGTTTTCGAAATT-3′, which encompasses the termination codon (indicated in bold). The six nucleotides underlined were altered from the authentic sequence to create a *Not*I cutting site. These primers were used to amplify the *S.pombe* TBP sequence from a cDNA library constructed in our laboratory. Another pair of PCR primers was prepared to clone the *S.cerevisiae* TBP sequence (21−24). One primer was 5′-C-TAGGTCGACATAAACAGGTGTATCAAGAG-3′, which precedes the initiation codon by 15 nucleotides. The three nucleotides underlined were altered from the authentic sequence to create a *Sal*I cutting site. The other primer was 5′-TTT-CGCGGCCGCTACTCCTTCCCCA**TCA**CA-3′, which covers the termination codon (indicated in bold). The six nucleotides underlined were altered from the authentic sequence to create a *Not*I cutting site. These primers were used to amplify the *S.cerevisiae* TBP sequence from its genomic DNA. Each amplified DNA was cloned into the expression vector pREP3 (17). Three independent clones were examined for both *S.pombe* and *S.cerevisiae* TBPs to exclude the possibility to pick up a mutant clone generated incidentally during the PCR procedure.

Southern blot analysis and DNA sequencing

Southern blotting was performed essentially as described (25). DNA was transferred to a membrane Hybond-N^{+} (Amersham). Hybridization was performed in 5×SSC containing 50% formamide, 0.02% SDS, 0.1% *N*-lauroylsarcosine sodium salt and 5% Blocking Reagent (Boehringer Mannheim) at 42°C for 14 h. The membrane was washed with 2×SSC−0.1% SDS at room temperature for 1 min three times and then at 60°C for 30 min. For sequence analysis, DNA fragments cloned from *Arabidopsis* were recloned in pBluescript KS(+) and SK(+) (Stratagene). Unidirectional deletion of DNA fragments was performed with exonuclease III and S1 nuclease (Takara Shuzo), according to the method of Henikoff (26). Single-stranded template DNA was prepared by using the helper M13KO7 bacteriophage. Nucleotide sequencing was performed by the chain termination method of Sanger *et al.* (27) using a DNA sequencer (Applied Biosystems 370A). All parts of the sequences reported in this paper have been determined in both directions at least once.

Northern blot analysis

To detect transcripts of the *ste11* gene, RNA was extracted from *S.pombe* cells either growing in the presence of rich nutrition or starved of nitrogen for 4 h. Cells of JY450 (*pde1*$^{+}$) carrying pREP1 (positive control), cells of JZ666 (*pde1*$^{-}$) carrying pREP3 (negative control), and cells of JZ666 carrying each clone of interest were analyzed. pREP1 and pREP3 have different restriction sites for cloning but are otherwise the same (17). RNA blotting and subsequent hybridization analysis were carried out

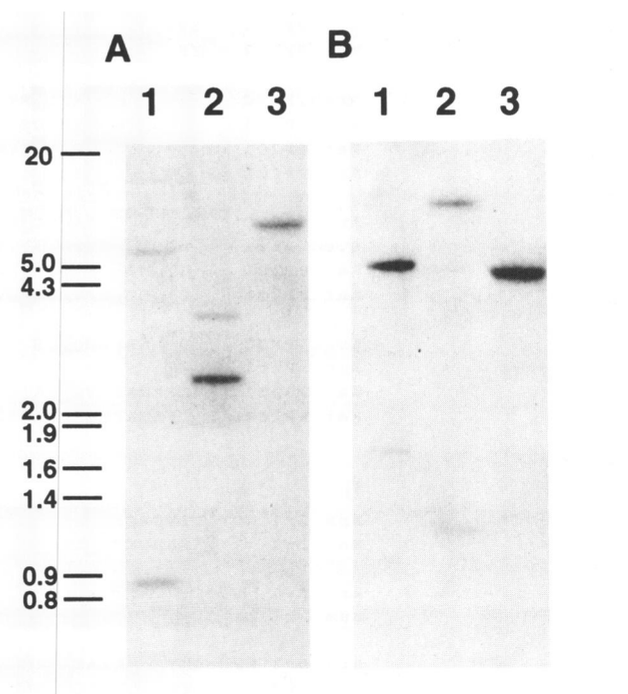

Figure 1. Southern blot analysis of *Arabidopsis* genomic DNA. *Arabidopsis* genomic DNA was completely digested by each of restriction endonucleases *Xba*I (lane 1), *Eco*RI (lane 2) and *Hin*dIII (lane 3). The digests were run in an agarose gel (1%), blotted on to the membrane, and probed by either the insert of pAME63 (panel A) or the insert of pAME67 (panel B).

according to a standard protocol (28). A 1.4 kb *Pvu*II fragment that covers most of the *ste11* ORF (34) was used as the probe.

RESULTS

Isolation of *Arabidopsis* cDNA clones that transcomplement *S.pombe pde1* mutant

An *S.pombe* homothallic haploid strain JZ666, which is defective in *pde1*, was transformed with an *Arabidopsis* cDNA library constructed as described in Materials and Methods. Of 35 000 transformants, seven were found to be able to mate and sporulate. Plasmids were recovered from them. These plasmids, named pAME61 through pAME67, were able to convert JZ666 cells to proficiency in mating and sporulation upon retransformation. pAME62 and pAME64 were found to carry the same DNA fragment.

pAME63, pAME66 and pAME67 were further analyzed in this study. The length of the insert in pAME63 was 1.4 kb, that in pAME66 was 1.1 kb and that in pAME67 was 0.9 kb, respectively. Preliminary sequence analysis of these clones suggested that pAME66 carries an *Arabidopsis* gene already reported (see below) whereas the other two carries new genes. To confirm that the inserts in pAME63 and pAME67 are derived from the plant, Southern blot analysis of *Arabidopsis* genomic DNA was performed using the inserts as probes. Both probes gave intense hybridization bands, indicating that they indeed originate from *Arabidopsis*. The pAME63 probe gave one hybridization band in the *Hin*dIII digest, and two bands in the *Xba*I and *Eco*RI digests (Fig. 1A). Because this probe carries

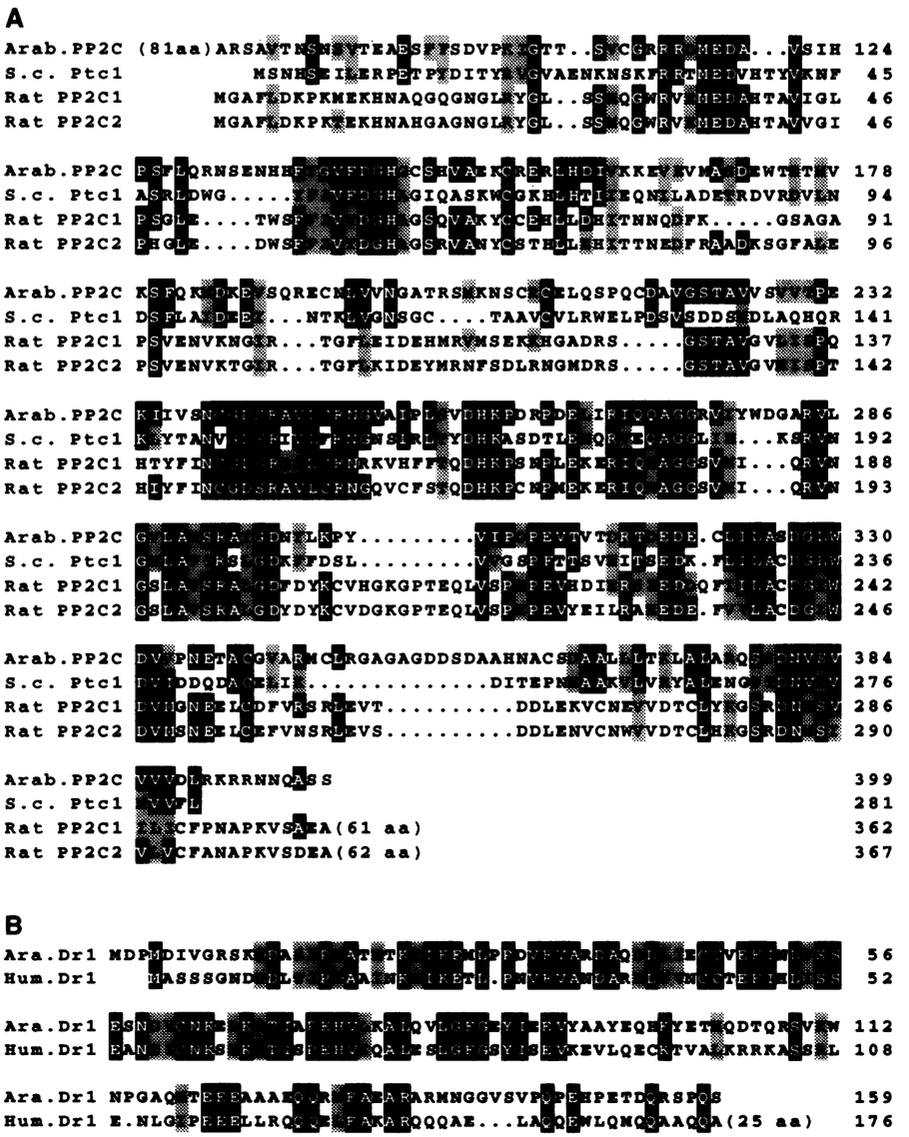

Figure 2. (**A**) Comparison of the deduced amino acid sequence of *Arabidopsis* PP2C with the *S.cerevisiae PTC1* gene product (31), rat PP2C1 (29) and PP2C2 (30). Amino acids identical between *Arabidopsis* PP2C and at least one of the other three are shown in white against black and those conserved between them are shaded. (**B**) Comparison of the deduced amino acid sequence of the *Arabidopsis* Dr1 with human Dr1 (32). Amino acids identical between them are shown in white against black and those conserved are shaded.

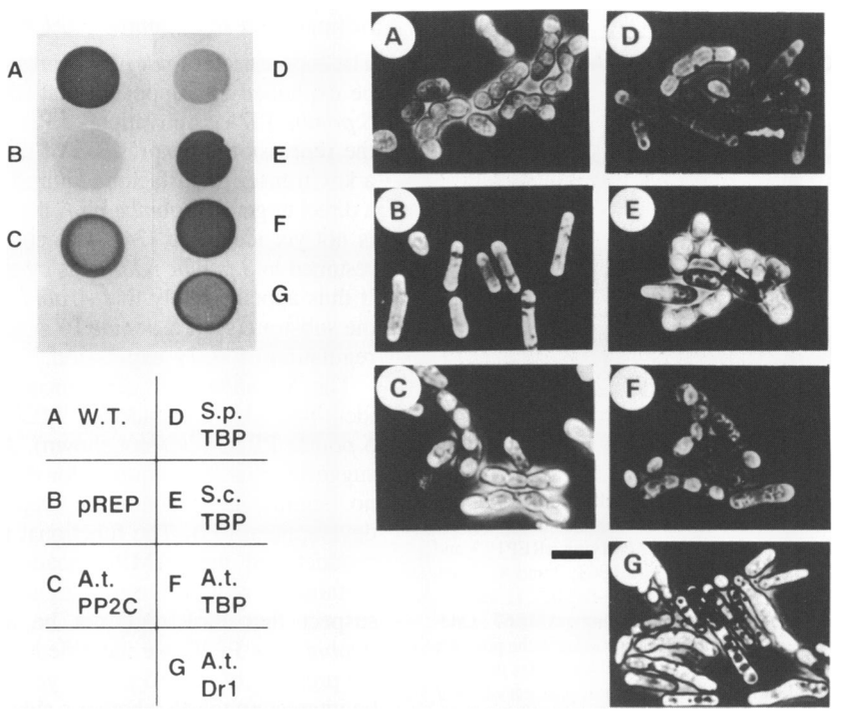

Figure 3. Suppression of the sterility of the *S.pombe pde1* mutant by *Arabidopsis* and *S.cerevisiae* clones. The experimental procedure is described in the text. (Left panel) Iodine staining assay. (**A**), JY450 (*pde1*$^+$) carrying pREP1 shown as a mating- and sporulation-positive control. (**B**), JZ666 (*pde1*$^-$) carrying pREP3 (negative control); (**C**), JZ666 carrying pAME63 (*Arabidopsis* PP2C); (**D**) JZ666 carrying pREP3-SpTBP (*S.pombe* TBP); (**E**) JZ666 carrying pREP3-ScTBP (*S.cerevisiae* TBP); (**F**) JZ666 carrying pAME66 (*Arabidopsis* TBP); and (**G**), JZ666 carrying pAME67 (*Arabidopsis* Dr1). (Right panel) Cells in each patch shown in the left panel were observed under the phase-contrast microscope. Sporulated cells were infrequently detected in (D), whereas practically no sporulated cells were seen in (B).

an *Xba*I cutting site and an *Eco*RI cutting site on it (data not shown; consistent with the DNA sequence), these results suggest that the gene cloned in pAME63 has no close homolog in the *Arabidopsis* genome. In contrast, the pAME67 probe, which carries no cutting site for *Xba*I, *Eco*RI and *Hin*dIII, gave a weak hybridization band in addition to a strong one in each kind of digest (Fig. 1B). This suggests that the gene carried by pAME67 may have its homolog on the *Arabidopsis* chromosomes, although a firm conclusion in this regard should await more detailed analysis.

pAME63 encodes putative protein phosphatase 2C and pAME67 encodes a protein similar to human Dr1

We determined the nucleotide sequences of the 1.4 kb insert in pAME63 and the 0.9 kb insert in pAME67 (DDBJ accession numbers D38109 and D38110, respectively). The coding potential of the gene carried on pAME63 was 399 amino acids, and the deduced gene product was 35% identical with two isoforms of rat PP2C, termed PP2C1 and PP2C2 (29,30), and 31% identical with *S.cerevisiae* PP2C encoded by the *PTC1* gene (31) (Fig. 2A). The coding potential of the gene carried on pAME67 was 159 amino acids. The deduced gene product was 41% identical with human Dr1 (Fig. 2B), which interacts with the TATA-binding protein (TBP) and blocks its ability to activate transcription (32). We tentatively call these gene products as *Arabidopsis* PP2C and *Arabidopsis* Dr1, respectively, in this paper.

pAME66 encodes TBP

Determination of the nucleotide sequence of the 1.1 kb insert in pAME66 suggested that the deduced gene product is identical with *Arabidopsis* TBP, reported as TFIID-1 previously (33). The nucleotide sequence we obtained matched perfectly with the published data, except that our clone was polyadenylated at position 1137 instead of 1190 as reported (33) (data not shown).

The ability of the isolated *Arabidopsis* clones and *S.cerevisiae* TBP to suppress the mating- and sporulation-deficiency of the *S.pombe pde1* mutant

JZ666 was transformed with each *Arabidopsis* clone described above, the vector pREP3, and plasmids carrying either *S.pombe* or *S.cerevisiae* TBP. The last two plasmids were prepared as described in Materials and Methods. Transformed cells were placed as a patch on agar medium containing only a limited amount of nitrogen (SSA), incubated at 30°C for 7 days, and stained with iodine vapor to estimate the degree of mating and sporulation. As shown in Fig. 3, cells expressing either *Arabidopsis* PP2C, *Arabidopsis* TBP, *Arabidopsis* Dr1, or *S.cerevisiae* TBP were clearly stained dark brown, although the degree of staining was lower than that of the sporogenic wild-type control. In contrast to the heterologous TBPs, overexpression of *S.pombe* TBP showed only very weak suppression (Fig. 3D). Consistent results were obtained by microscopic inspection of the transformed cells (Fig. 3). These JZ666 transformants produced larger asci compared with the wild-type control,

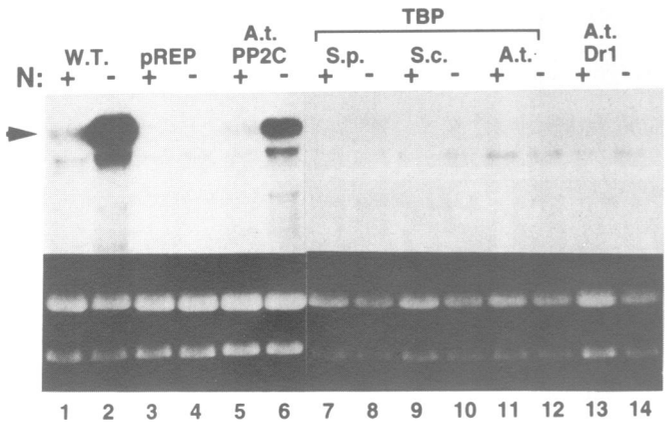

Figure 4. Expression of *ste11* in *S.pombe pde1* mutant cells transformed with *Arabidopsis* and *S.cerevisiae* clones. The same strains as examined in Fig. 3 were analyzed by Northern blotting. Lanes 1 and 2, JY450 carrying pREP1; 3 and 4, JZ666 carrying pREP3; 5 and 6, JZ666 carrying pAME63; 7 and 8, JZ666 carrying pREP3-SpTBP; 9 and 10, JZ666 carrying pREP3-ScTBP; 11 and 12, JZ666 carrying pAME66; and 13 and 14, JZ666 carrying pAME67. Odd-numbered lanes received RNA samples prepared from cells growing in the presence of rich nutrition, whereas even-numbered lanes received RNA samples prepared from cells starved for nitrogen. The arrowhead indicates the position of *ste11* mRNA. Approximately equal loadings of RNA were verified by ethidium bromide staining of ribosomal RNA, as shown at the bottom.

suggesting that the effect of a high cAMP level to induce cell elongation under nutritional starvation persisted in these cells.

Expression of *ste11* in *S.pombe* cells transformed by heterologous genes

The *S.pombe ste11* gene encodes an HMG-family transcription factor, which is a key regulator of the expression of genes essential for sexual development (34). Transcription of *ste11* is induced by nitrogen starvation and a concomitant decrease in the level of intracellular cAMP (34). The level of expression of this gene is a good measure of the ability of the host cell to carry out sexual development. We therefore examined expression of *ste11* in those *S.pombe* transformants described above. As shown in Fig. 4, JZ666 (*pde1*$^-$) cells clearly recovered the ability to express *ste11* when they were transformed with pAME63, which carries *Arabidopsis* PP2C. The results obtained with pAME66 (*Arabidopsis* TBP), pAME67 (*Arabidopsis* Dr1) and pREP3-ScTBP (*S.cerevisiae* TBP) were less clear. The expression of *ste11* in these transformants was much weaker compared to the pAME63 transformant, although it could possibly be slightly higher than the basal level seen in the JZ666 cells transformed with the vector.

DISCUSSION

Two new genes from *Arabidopsis thaliana*

Although the original aim of this study, that is, identification of genes that encode factors involved in the cAMP cascade in plant cells, was not accomplished, we fortuitously isolated two novel genes from *Arabidopsis*, which have the ability to suppress the mating- and sporulation-deficiency of the *S.pombe pde1* mutant. The two genes encode putative PP2C and a Dr1-like protein, respectively. We also demonstrated that expression of *Arabidopsis* TBP can suppress *pde1*.

Suppression of *S.pombe pde1* by *Arabidopsis* PP2C

The suppression of *pde1* by expression of *Arabidopsis* PP2C will be explained by supposing that *Arabidopsis* PP2C counteracts *S.pombe* PKA. Activation of PKA in *S.pombe* cells results in the repression of expression of the *ste11* gene, which encodes a key transcription factor required for sexual development, but a direct target of *S.pombe* PKA that regulates this gene expression is not yet identified (34). Induction of *ste11* transcription was resumed in *S.pombe pde1* cells by expressing *Arabidopsis* PP2C. It thus appears likely that *Arabidopsis* PP2C dephosphorylates the substrate(s) of *S.pombe* PKA that plays a critical role in the regulation of *ste11* expression.

The *S.pombe ptc1* gene encoding PP2C has recently been identified (35). Arabidopsis PP2C is 33% identical with this *S.pombe* PP2C (data not shown). Analysis of the *ptc1* gene has suggested that it is required for the heat-shock response but has no significant function in vegetative growth and sexual development (35). The functional relationship of the *ptc1* gene product and the cAMP cascade is not known. Although it certainly is required to investigate further this relationship, we suspect that Ptc1 may not be a direct counterpart of the *Arabidopsis* PP2C we identified, since Shiozaki *et al.* refer to the presence of more *S.pombe* genes encoding PP2C (35). It will be interesting to see whether a subtype of PP2C physiologically counteracts PKA to regulate sexual development in the fission yeast.

Suppression of *S.pombe pde1* by *Arabidopsis* Dr1 and TBP

Dr1 is a protein that can associate with TBP, which was originally detected in HeLa cell extract (32). The association of Dr1 with TBP causes repression of both basal and activated levels of transcription, by preventing the association of TBP with general transcription factors (32). To our knowledge, homologs of Dr1 in other organisms have not been previously described. The occurrence of a Dr1-like protein in plant genera, as demonstrated in this study, strongly argues for its ubiquity and for a conserved mechanism involving Dr1 that regulates transcription initiation.

How does the *Arabidopsis* Dr1 suppress *S.pombe pde1*, then? We speculate as follows. Human Dr1 has been shown to complex with *S.cerevisiae* TBP (32). By analogy it is likely that *Arabidopsis* Dr1 interacts with *S.pombe* TBP. This interaction may block expression of a certain gene(s) that negatively regulates sexual development, presumably more severely than expression of other genes. Artificial expression of *Arabidopsis* Dr1 did not recover induction of *ste11* transcription conspicuously in *pde1* mutant cells, suggesting that *Arabidopsis* Dr1 may affect the regulatory pathway for sexual development downstream of the step at which *ste11* exerts its function.

Concerning the suppression of *pde1* by *Arabidopsis* and *Saccharomyces* TBPs, two opposing interpretations are possible. One interpretation assumes that these heterologous TBPs are not active in promotion of transcription in *S.pombe*. Then these TBPs are likely to sequester *S.pombe* general transcription factors and block gene expression that is inhibitory for sexual development. This mechanism of suppression resembles the one we postulated for *Arabidopsis* Dr1. Overproduction of its own TBP suppressed *S.pombe pde1* very weakly. This weak suppression may also be thought to result from the reduction of certain gene expression due to unbalanced distribution of the general transcription factors.

The other interpretation, in contrast, postulates that the heterologous TBPs may actively promote gene expression in

S.pombe. Although it is uncertain to which extent TBPs are exchangeable among species, at least *S.pombe* TBP has been shown to function in *S.cerevisiae* (19). Thus, *Arabidopsis* and *S.cerevisiae* TBPs may activate specific gene expression in *S.pombe* cells that promotes sexual development, rather than repress gene expression that inhibits it.

Despite the above discussion, other explanations of the observed suppression have not been completely excluded. Tamura *et al.* pointed out that rat PP2C1 shows similarity to the catalytic domain of *S.cerevisiae* adenylyl cyclase (29). We confirmed that *Arabidopsis* PP2C is 23% identical with the catalytic domain of *S.pombe* adenylyl cyclase over 370 amino acid residues (data not shown). It is thus conceivable that overproduction of *Arabidopsis* PP2C may interfere with the proper function of *S.pombe* adenylyl cyclase in one way or another. Likewise, as Dr1 has been shown to be a phosphoprotein (32), *Arabidopsis* Dr1 may possibly inhibit the activity of *S.pombe* PKA, behaving as a pseudosubstrate. If this interpretation is correct, the observed suppression will have little physiological significance.

Our observations suggest that it is urgent to answer the following questions. Does *S.pombe* have its own Dr1? Does this protein interact with *S.pombe* TBP? Is it a substrate of PKA? Does *S.pombe* Dr1 play a role in regulation of sexual development? Answering these questions is undoubtedly important, not only to explain the mechanism of the observed heterologous suppression, but to elaborate our knowledge how sexual development is regulated at the level of gene expression.

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