Volume 301, number 1, 29–33 FEBS 10909 April 1992

A gene family homologous to the S-phase specific gene in higher plants is essential for cell proliferation in *Saccharomyces cerevisiae*

Masaki Ito[a], Akira Yasui[b] and Atsushi Komamine[a]

[a]*Biological Institute, Faculty of Science, Tohoku University, Sendai 980, Japan* and [b]*Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai 980, Japan*

Received 6 February 1992; revised version received 20 February 1992

Previously we reported the isolation and characterization of the gene, *cyc07*, which was specifically expressed in the S phase during the cell cycle in synchronous cell division cultures of the higher plant, *Catharanthus roseus*. We found that the yeast *Saccharomyces cerevisiae* contains two closely related genes which show a high degree of similarity (about 64% at the amino acid level) to *cyc07* of *C. roseus*. Site-directed disruption mutations demonstrated that the two yeast genes, homologous to *cyc07*, constitute an essential gene family for cell proliferation in yeast cells. Furthermore, the rate of cell proliferation varied with the gene copy number.

Cell cycle; Gene sequence; *cyc07*; *Catharanthus roseus*; *Saccharomyces cerevisiae*

1. INTRODUCTION

The molecular mechanisms of the cell cycle in eukaryotic cells is one of the most important subjects to elucidate in cell biology. Recently, significant effort has been directed towards the isolation and characterization of genes whose functions are essential for the progression of the cell cycle.

The higher plant, *Catharanthus roseus*, grown in suspension can be synchronized by the double phosphate-starvation method [1]. Initial attempts concentrated on the isolation of genes which were expressed differentially during the cell cycle. In our synchronous cell division system, alteration of gene expression was demonstrated at both the protein and mRNA levels [2,3]. Several cDNA clones have been isolated which showed differential expression in synchronous cultures [4]. In our previous report, we described the characterization of one of these genes, *cyc07*, which was expressed specifically in the S phase during the cell cycle [5]. The expression of this gene was closely associated with cell proliferation in both intact plants and cultured cells. The gene, *cyc07*, encodes a highly basic protein with a molecular weight of 35 kDa. A data base search revealed that the nucleotide sequence of *cyc07* has significant similarity (64%) to that of an uncharacterized ORF that is partly contained in the yeast genomic clone containing the entire *SIR3* gene [6,7]. The homologous region to *cyc07* was located downstream from the reported 3′ end of the *SIR3* mRNA [7]. Thus, our previous results suggest that *Saccharomyces cerevisiae* contains a gene homologous to *cyc07* [5]. In this communication, we describe the molecular cloning and functional analysis of the yeast homologue of *cyc07*.

Abbreviations: ORF, open reading frame; PCR, polymerase chain reaction; ts, temperature sensitive; YEPD, yeast extract-peptone-dextrose.

Correspondence address: M. Ito, Biological Institute, Faculty of Science, Tohoku University, Sendai 980, Japan. Fax: (81) (22) 262 5146.

2. MATERIALS AND METHODS

2.1. *Cloning and sequence analysis*

Yeast genomic libraries were constructed with the vectors M13mp18 and λgt11 for isolating *PLC1* and *PLC2*, respectively. Library screening was performed by plaque hybridization with ^{32}P-labeled DNA probes. For amplification of the *SIR3* downstream region, 21mer primers, CGCTTGCTCTGGAGACCGTCC (3532–3552) and TCAGAGCTATCAGAAAGGTTA (4435–4455) were synthesized according to the sequence data in the EMBL Data Library and were used for PCR. Amplified 1.1 kb DNA fragments were used as probes for isolating the *PLC1* gene. DNA inserts of recombinant phage DNA were sub-cloned into pBluescript KS (Stratagene). Deletion derivatives of the clone were generated using a double-stranded nested deletion kit (Pharmacia LKB Biotechnology). Nucleotide sequences were determined by the dideoxynucleotide chain-termination method [8] with single-stranded DNA templates [9].

2.2. *Strains, growth conditions and genetic methods*

Yeast haploid strain SP1 (MAT a, leu2, ura3, his3 trp1, ade8, can1) and DC124 (MAT α, leu2, ura3, his4, trp1, ade8) were obtained from T. Toda (Department of Biophysics, Faculty of Science, Kyoto University, Japan). A diploid strain, DS00, was constructed by crossing SP1 and DC124. One-step gene disruption was performed as described by Rothstein [10]. Yeast cells were transformed by the spheroplast method [11]. Tetrads were dissected on YEPD agar plates, and spores were germinated at 27°C.

2.3. *Plasmid construction*

A 4.5 kb *Eco*RI–*Xho*I genomic fragment containing the *PLC2* gene

was subcloned into the single copy vector, pRS414 (Invitrogen), containing *TRP1*.

3. RESULTS

3.1. *Molecular cloning of the yeast homologues of* cyc07

To clone the downstream region of the *SIR3* gene, DNA sequences in the reported *SIR3* genomic clone were amplified by PCR from the yeast genomic DNA and were used as hybridization probes to screen the genomic library. A 3.3 kb *Xho*I–*Hin*dIII restriction fragment, which contained a 1.9 kb *Eco*RI fragment, was obtained (Fig. 1A). Determination of the nucleotide sequence for the 1.9 kb *Eco*RI fragment indicated the presence of a complete ORF of 768 nucleotides which encodes a polypeptide of 255 amino acids, only 10 amino acids longer than that encoded by *cyc07* (Fig. 2). Comparison of predicted amino acid sequences revealed that about 64% of amino acids were identical among 234 amino acids in the N-terminal region without appearance of any gaps (Fig. 3). This high degree of similarity clearly shows that the DNA sequence located downstream from the *SIR3* gene is a yeast homologue of *cyc07*.

Yeast chromosomal DNA digested with *Eco*RI was probed with the labeled 1.9 kb *Eco*RI fragment which contained the coding sequence of the homologous gene of *cyc07*. Hybridization to two DNA fragments, 1.9 and 4.5 kb (data not shown), suggested the existence of an additional copy of the gene. The 4.5 kb *Eco*RI fragment was cloned from the genomic library and a 2.3 kb *Pst*I fragment, contained in the cloned fragment, was sequenced (Fig. 1B). An ORF of 768 bp was found, which encoded a polypeptide of 255 amino acids. The nucleotide sequence of the ORF in the 4.5 kb *Eco*RI fragment showed approximately 92% similarity to that of the ORF in the 1.9 kb *Eco*RI fragment downstream from the *SIR3* gene. When protein sequences were compared, only 7 amino acid changes were found in 255 amino acids, all of which were conservative (Fig. 4). Thus, yeast contains two closely related genes which are homologous to *cyc07*. The gene downstream from *SIR3* and the other copy of the gene were named as *PLC1* and *PLC2*, respectively (yeast homologue of *PL*ant *C*ell-cycle-specific gene).

3.2. *Phenotypes of the gene disruption mutant*

To determine whether *PLC1* and *PLC2* are essential genes, we have analyzed the phenotype of cells caused by the disruption of *PLC1*, *PLC2* or *PLC1* and *PLC2*. Each gene contains similarly positioned *Hpa*I restriction sites within the coding region. Insertion of the DNA into this site with the *LEU2* gene in the case of *PLC1*, and with the *URA3* gene in the case of *PLC2*, was established (Fig. 1). To determine the phenotype of the disruption mutants, diploids containing one intact and one disrupted copy of either of two genes were

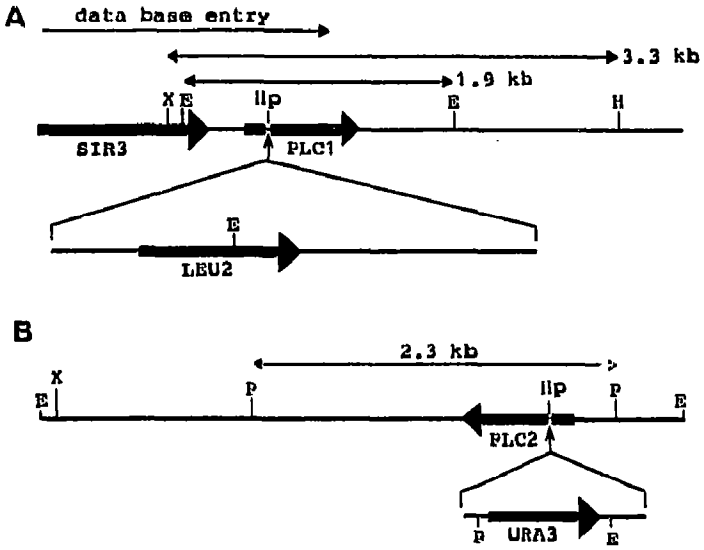

Fig. 1. Restriction maps of *PLC1*, *PLC2* genes and their insertional mutations. Location of the ORF of *PLC1* and *PLC2* are shown. Restriction enzyme recognition sites: X, *Xho*I; E, *Eco*RI; Hp, *Hpa*I; H, *Hin*dIII; P, *Pst*I.

sporulated, and the haploid progeny were analyzed by tetrad dissection (Fig. 5). Although spores carrying the disruption of either one of *PLC1* or *PLC2* were viable, they grew at a slower rate than the wild-type. In addition, cells carrying the mutation in *PLC2* (*PLC1*, *plc2*::*URA3*) grew slower than cells carrying the mutation in *PLC1*(*plc1*::*LEU2*, *PLC2*).

To examine whether the double *PLC1* and *PLC2* disruptants could grow or not, crosses were made between the single disruptants, *PLC1 plc2*::*URA3* × *plc1*::*LEU2 PLC2*. The resulting diploid cells (*PLC1*/*plc1*::*LEU2*, *PLC2*/*plc2*::*URA3*) were transformed with a single copy plasmid containing the wild-type *PLC2* gene and the *TRP1* gene, sporulated, and dissected. The spores showing Ura$^+$, Leu$^+$ and Trp$^+$ phenotypes were chosen. These cells carried mutations in both the chromosomal *PLC1* and the chromosomal *PLC2* genes and carried a wild-type *PLC2* gene on the plasmid. The loss of the plasmid was examined by a *TRP1* marker after the cells were grown under non-selective conditions. No plasmid loss was observed among more than 5,000 cells tested. However, many cells lost the plasmids if these cells, carrying the *TRP1 PLC2* plasmid, also carried a chromosomal *PLC1* or *PLC2* gene. These analyses demonstrate that cells must produce either the *PLC1* or *PLC2* protein for vegetative growth.

4. DISCUSSION

The isolation of the gene, namely *MFT1*, which is identical to *PLC2*, has recently been published by Garrett et al. [12]. They reported that this gene is involved in protein import into mitochondria. They showed that

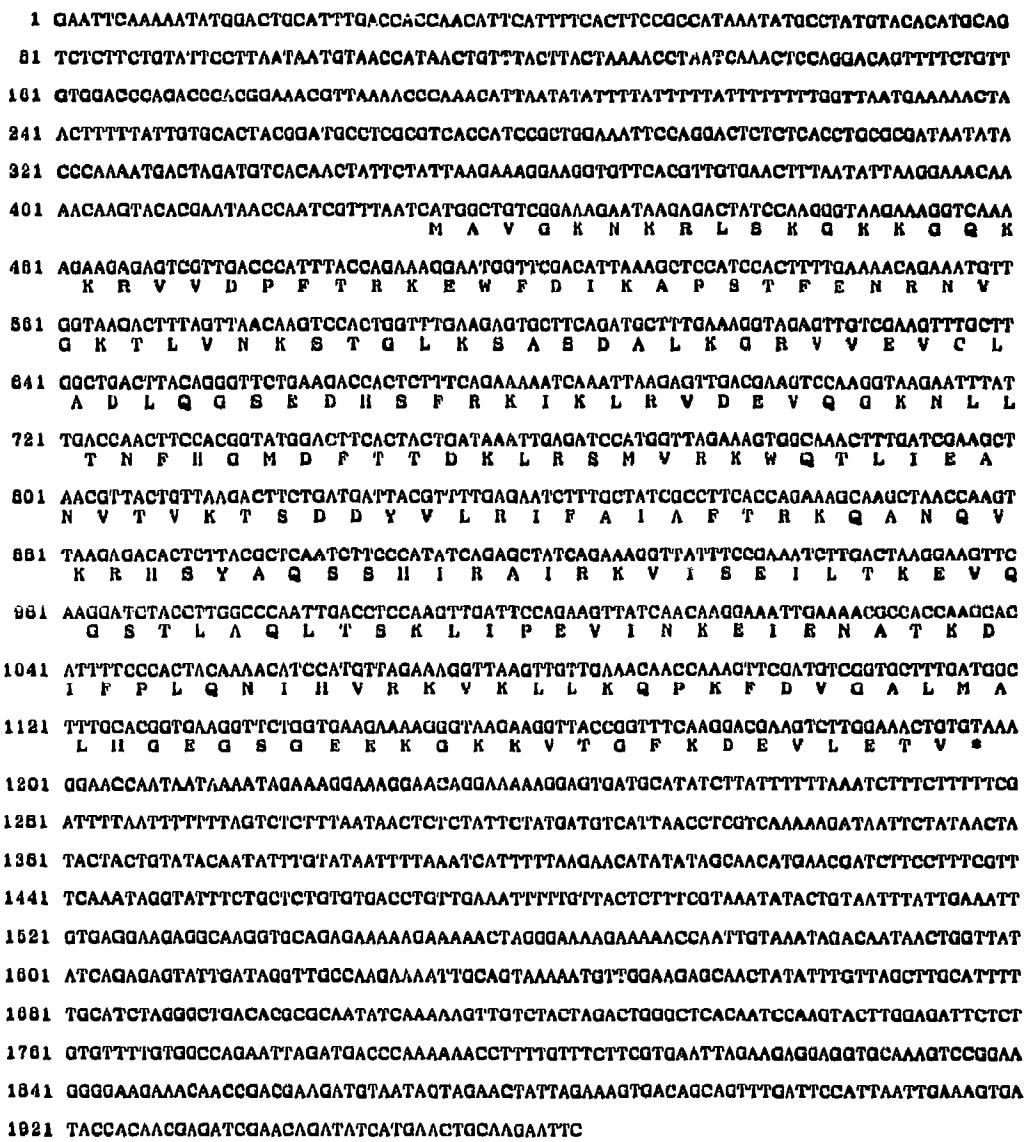

Fig. 2. Nucleotide sequence of the 1.9 kb *Eco*R1 fragment containing *PLC1* and deduced amino acid sequence.

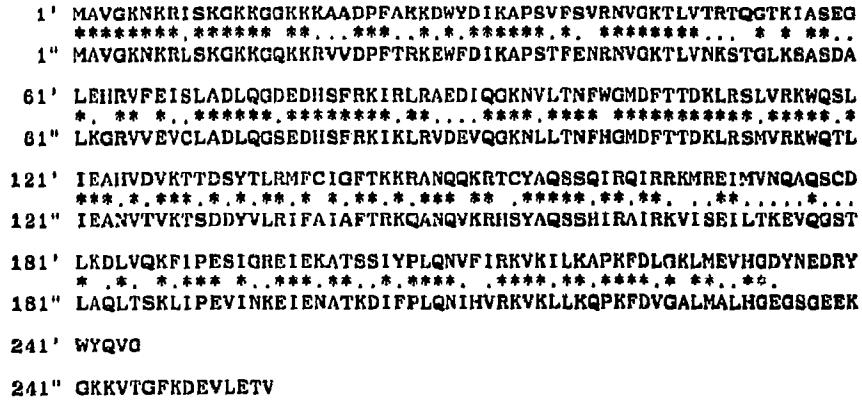

Fig. 3. Amino acid sequence comparison of proteins encoded by *cyc07* (upper lines) and *PLC1* (lower lines).

```
  1' MAVGKNKRLSKGKKGQKKRVVDPFTRKEWFDIKAPSTFENRNVGKTLVNKSTGLKSASDA
     ********** **** ** ************************************ ****
  1" MAVGKNKRLSRGKKGLKKKVVDPFTRKEWFDIKAPSTFENRNVGKTLVNKSTGLKNASDA

 61' LKGRVVEVCLADLQGSEDHSFRKIKLRVDEVQGKNLLTNFHGMDFTTDKLRSMVRKWQTL
     ************************ ************************************
 61" LKGRVVEVCLADLQGSEDHSFRKVKLRVDEVQGKNLLTNFHGMDFTTDKLRSMVRKWQTL

121' IEANVTVKTSDDYVLRIFAIAFTRKQANQVKRHSYAQSSHIRAIRKVISEILTKEVQGST
     ************************************************** *** **
121" IEANVTVKTSDDYVLRIFAIAFTRKQANQVKRHSYAQSSHIRAIRKVISEILTREVQNST

181' LAQLTSKLIPEVINKEIENATKDIFPLQNIHVRKVKLLKQPKFDVGALMALHGEGSGEEK
     ************************************************************
181" LAQLTSKLIPEVINKEIENATKDIFPLQNIHVRKVKLLKQPKFDVGALMALHGEGSGEEK

241' GKKVTGFKDEVLETV
     **** **********
241" GKKVSGFKDEVLETV
```

Fig. 4. Amino acid sequence comparison of proteins encoded by *PLC1* (upper lines) and *PLC2* (lower lines).

mitochondrial targeting of an Atp2–LacZ fusion protein results in disruption of the normal functioning of mitochondria and results in a respiration-deficient phenotype [13]. Mutants were isolated by reversion of the respiration-deficient phenotype resulting from the import of the fusion protein. One of such mutants, *mft1*, was also found to be temperature-sensitive (ts) for growth. The *MFT1* gene was isolated as a gene fragment which complemented the ts defect. Site-directed disruption of *MFT1* resulted in slower growth at 30°C and ts for growth at 37°C. From these observations, they concluded that *MFT1/PLC2* was a mutated gene in the *mft1* mutant [12]. However, in our experiments, disruption of *PLC2* did not cause the ts phenotype, i.e. the haploid carrying *plc2::URA3* could grow at 73°C. According to our observations, it is unlikely that *MFT1/PLC2* is a mutated gene in the *mft1* mutant. The experimental protocol used by Garrett et al. [12] differed from ours in that they deleted the *Pst*I 2.3 kb fragment which contains the *MFT1/PLC2* gene, while we inserted DNA into the coding region. Gene disruption produced by Garrett et al. can also mutate neighboring genes if they exist in the 2.3 kb *Pst*I fragment. Furthermore, we have found another ORF located downstream from

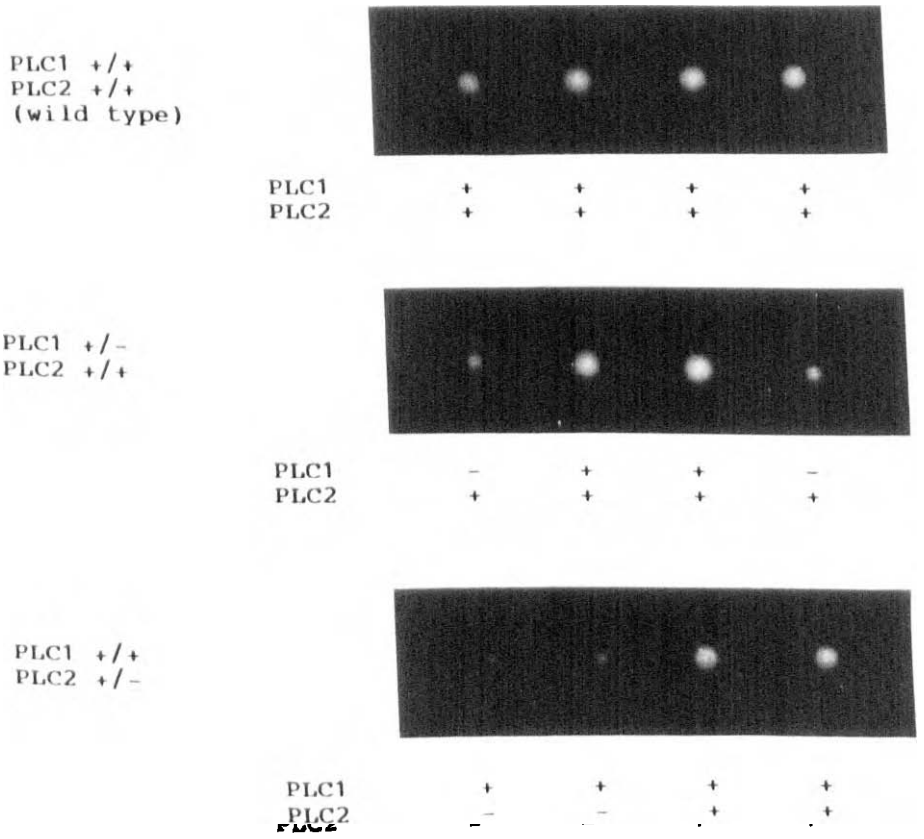

Fig. 5. Analysis of tetrads produced by cells heterozygous for either *PLC1* or *PLC2*.

MFT1/PLC2, which is partially contained in the *Pst1* 2.3 kb fragment. Here, we propose that the *PLC2* gene is involved in cell proliferation rather than protein import into mitochondria.

We have shown that *PLC* genes are essential for cell proliferation in yeast. From the observation that the gene copy number affects the rate of cell proliferation, we speculate that the amount of protein encoded by *PLC* genes is controlling the progression of the cell division cycle in yeast. Despite a vast evolutionary distance, the structures of *cyc07* from *C. roseus* and the two yeast genes are highly conserved, suggesting that these genes play the same role in biological systems, and possibly in a highly conserved aspect of cell proliferation. The expression pattern of *cyc07* in higher plant cells and gene function in yeast, together, suggest the importance of this gene for cell proliferation.

Acknowledgements: This research was supported by Grant-in Aid for Scientific Research (02454007) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

[1] Amino, S., Fujimura, T. and Komamine, A. (1983) Physiol. Plant. 59, 393–396.
[2] Kodama, H., Kawakami, N., Watanabe, A. and Komamine, A. (1989) Plant Physiol. 89, 910–917.
[3] Kodama, H., Ando, S. and Komamine, A. (1990) Physiol. Plant. 79, 319–326.
[4] Kodama, H., Ito, M., Hattori, T., Nakamura, K. and Komamine, A. (1991) Plant Physiol. 95, 406–411.
[5] Ito, M., Kodama, H. and Komamine, A. (1991) Plant J. 1, 141–148.
[6] Rine, J., Strathern, J.N., Hicks, J.B. and Herskowitz, I. (1979) Genetics 93, 877–901.
[7] Shore, D., Squire, M. and Nasmyth, K.A. (1984) EMBO J. 3, 2817–2823.
[8] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[9] Dente, L., Cesareni, G. and Cortese, R. (1983) Nucleic Acids Res. 11, 1645–1655.
[10] Rothstein, R.J. (1983) Methods Enzymol. 101, 202–210.
[11] Hinnen, A., Hicks, J.B. and Fink, G.R. (1978) Proc. Natl. Acad. Sci. USA 75, 1929–1933.
[12] Garrett, J.M., Singh, K.K., Vonder Haar, R.A. and Emr, S.D. (1991) Mol. Gen. Genet. 225, 483–491.
[13] Emr, S.D., Vassarotti, A., Garrett, J., Geller, B.L., Takeda, M. and Douglas, M.G. (1986) J. Cell Biol. 102, 523–533.