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# Precise mapping and molecular characterization of the *MFT1* gene involved in import of a fusion protein into mitochondria in *Saccharomyces cerevisiae*

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Garrett et al. [Mol. Gen. Genet. 225 (1991) 483–491] recently reported that an Atp2-lacZ fusion protein was transported into mitochondria in yeast, thus identifying the *MFT1* (mitochondrial fusion targeting) gene as a genomic fragment which complements a mutation (*mft1*) that failed in targeting a fusion protein into mitochondria [4]. They mapped this gene to the ORF, which we have independently identified as a gene homologous to the *cyc07* gene, which is expressed specifically in the S phase during the plant cell cycle. We have mapped the *MFT1* gene precisely and found that this gene should correspond to the neighboring ORF, rather than the ORF they identified.

Mitochondrial protein import; *MFT1*; *cyc07*; Gene sequence; *Saccharomyces cerevisiae*

## 1. INTRODUCTION

Recently, we have isolated the cDNA which corresponds to the S-phase specific gene, *cyc07*, from the synchronous cultures of plant cell, *Catharanthus roseus* [1]. The *cyc07* mRNA appeared only in the S phase during the cell cycle of plant cells examined [1,2]. In our previous work, the yeast, *Saccharomyces cerevisiae*, was found to have a gene family constituted by two closely related genes, namely *PLC1* and *PLC2*, which were homologous to *cyc07* from *C. roseus*. The lethal phenotype resulted from a double *PLC1* and *PLC2* gene disruption suggesting an important role for this gene in cell cycle progression [3]. The gene sequence that is identical to *PLC2* has recently been isolated from the yeast, *S. cerevisiae* by Garrett et al. [4]. The authors reported cloning the gene, named *MFT1*, that is involved in protein import into mitochondria.

The *ATP2* gene of yeast encodes for a cytoplasmically synthesized β-subunit protein of the mitochondrial $F_1$-ATPase. An *ATP2-lacZ* fusion gene was constructed in a single copy yeast/*E. coli* shuttle vector, which expressed hybrid protein comprising of the 380 N-terminal amino acids of Atp2 fused to *E. coli* β-galactosidase [5]. Mitochondrial targeting of the Atp2-lacZ fusion protein caused disruption of mitochondrial function and resulted in a respiration-defective phenotype ($Gly^-$ phenotype) [5]. Revertants with extragenic mutations which rendered the strain respiration competent were isolated. One of these mutants, *mft1*, also had a ts phenotype at 37°C, which co-segregated with the respiration-competent phenotype ($Gly^+$ phenotype) in the presence of the fusion protein. The *MFT1* genomic fragment was isolated by complementation of the ts defect [4]. In the experiments of Garrett et al. [4], a 4.2 kb *Eco*RI-*Hin*dIII fragment (Fig. 1A) was the smallest for complementation. They proposed the precise position of *MFT1* based on the observations that (1) a 2.3 kb *Pst*I fragment (Fig. 1A) hybridized to a 0.9 kb mRNA, but the *Pst*I-*Hin*dIII fragment (Fig. 1A) did not, (2) a hybridization analysis using strand-specific probes revealed that the orientation of the 0.9 kb mRNA was from *Eco*RI to *Hin*dIII, (3) a single 768 nucleotide ORF (ORF-1) was found within the *Pst*I 2.3 kb fragment, which had the expected size and orientation, and (4) the disruptant in which the *Pst*I 2.3 kb fragment was deleted by site-directed disruption mutation showed a ts phenotype at 37°C and $Gly^+$ phenotype under the expression of the *ATP2-lacZ* fusion gene. From these results, they attributed the *MFT1* gene to the 768-base ORF (ORF-1) within the *Pst*I fragment, which is identical to that of *PLC2* (Fig. 1A) [4].

However, as we reported previously, the *PLC2* gene disruptant created by insertional mutagenesis at the *Hpa*I site within ORF-1 (Fig. 1B) did not show the ts phenotype [3]. Furthermore, we found another ORF (ORF-2) located downstream from *PLC2* gene with an opposite orientation to *PLC2*, which was partially con-

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*Abbreviations:* ORF, open reading frame; ts, temperature sensitive; YEPD, yeast extract-peptone-dextrose.

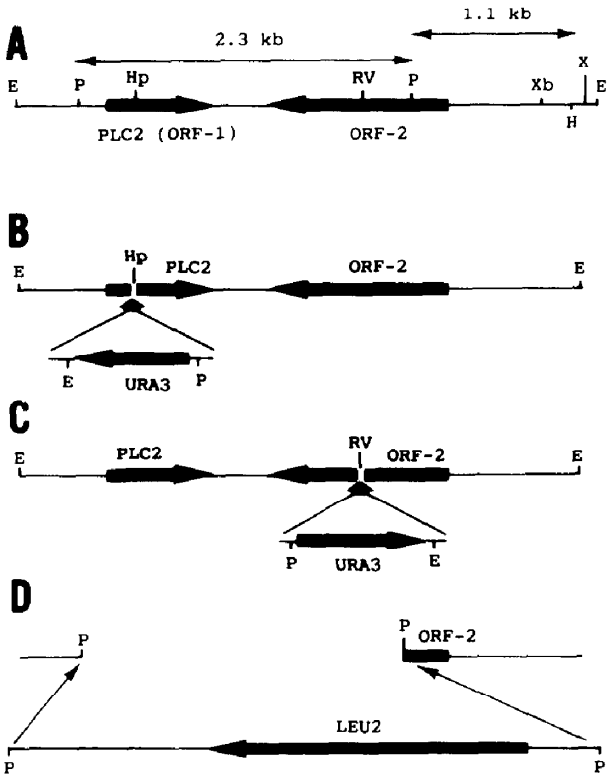


Fig. 1. Partial restriction maps of the 4.5-kb *Eco*RI fragment containing two ORFs (ORF-1 and ORF-2). A, wild type; B, insertional disrution in ORF-1; C, insertional disruption in ORF-2; D, deletion mutation of *Pst*I 2.3 kb fragment. Restriction enzyme recognition sites: E, *Eco*RI; P, *Pst*I; Hp, *Hpa*I; RV, *Eco*RV; Xb, *Xba*I; H, *Hin*dIII; X, *Xho*I.

tained in the *Pst*I 2.3 kb fragment (Fig. 1A). The gene disruption constructed by Garrett et al. [4] could mutate not only ORF-1 but also ORF-2. Thus, our previous results suggested that it was not the *PLC2* gene but ORF-2 that was mutated in the *mft1* mutant. Moreover, Garrett et al. [4] showed that the 2.3 kb *Pst*I fragment did not complement the *mft1* defect. Although they ascribed it to loss of the regulatory activity, it is not certain and they were never able to even shorten the complementing region from the 4.2 kb *Eco*RI-*Hin*dIII fragment. Here, we show the evidence that *PLC2* is different from the mutated gene in the *mft1* mutant, and describe the isolation and molecular characterization of the gene located downstream from *PLC2*, which is considered to be mutated in *mft1* mutant.

## 2. MATERIALS AND METHODS

### 2.1. *Strains and growth conditions*

*Saccharomyces cerevisiae* haploid strain SP1 (MATα, leu2, ura3, trp1, ade8, can1) and DC124 (MATα, leu2, ura3, his4, trp1, ade8) were crossed to produce the diploid strain DS00 [3]. For gene disruption mutations, we transformed DS00. One-step gene disruption was performed as described by Rothstein [6]. Yeast cells were transformed by the spheroplast method [7], and tetrads were dissected and germinated at 27°C or 37°C.

For analyzing vegetative growth at 27°C and 37°C, strains were grown in liquid YEPD medium with vigorous aeration at 27°C to mid-log phase, diluted to $5 \times 10^5$ cells/ml into YEPD medium and cultured at either 27°C or 37°C. Samples were taken at various times and counted with a hemacytometer.

### 2.2. *Plasmid constructions*

For insertional disruption of the gene downstream from *PLC2*, a *URA3*-containing 1.3 kb fragment was purified. This fragment was inserted at the *Eco*RV site, which is located within the ORF, on the plasmid carrying the 2.3 kb *Pst*I fragment, and subsequently digested with *Pvu*II. The resultant linearized 4.1 kb fragment was purified and introduced into yeast cells.

For deletion disruption of 2.3 kb *Pst*I fragment, a 4.2 kb *Eco*RI-*Hin*dIII fragment was sub-cloned into pUC19. On the plasmid, the 2.3 kb *Pst*I fragment was replaced with a 4.6 kb *Pst*I fragment of *LEU2* from pEMBL-Ye30 [8]. A linearized fragment used for transformation was prepared by digestion with *Pvu*II.

The yeast *ATP2* gene was presented by Dr. T. Endo (Department of Chemistry, Faculty of Science, Nagoya University), which was cloned in the *E. coli*/yeast shuttle vector, YEpM4. A 1.1 kb *Hin*dIII-*Bam*HI fragment was purified, which contained approxiamtely 0.7 kb of DNA upstream of the *ATP2* translation start site. It was cloned into the *Hin*dIII and *Bam*HI sites of plasmid pYT760 [9]. This generated a plasmid, pYT-ATP2, that contains an *ATP2-lacZ* gene fusion in which 380 amino-terminal amino acids of the Atp2 protein are fused in frame to *E coli* β-galactosidase

### 2.3. *Northern blot analysis*

Yeast cells were cultured at 27°C or 37°C in YEPD liquid medium, and were harvested when cultures reached mid-logarithmic phase. Yeast total RNA was prepared using glass beads as described by Carlson and Botstein [10]. Poly(A)-rich mRNA was purified from total RNA by chromatography on oligo(dT) cellulose (pharmacia). Northern blot analysis was performed as described in our previous report [2].

### 2.4. *Nucleotide sequencing*

Nucleotide sequences were determined by the dideoxynucleotide chain-termination method [11] with single-stranded templates [12].

## 3. RESULTS

### 3.1. *Gene disruption mutation in ORF downstream from PLC2*

Garrett et al. reported that the deletion of the 2.3 kb *Pst*I fragment, which contained both ORF-1 and ORF-2, resulted in a slower growth at 30°C and ts at 37°C [4]. However, in our work, an insertional mutation in *PLC2* (Fig. 1B) showed slower growth at 27°C, but did not show a ts phenotype (Fig. 2B). Therefore, we reasoned that the mutation in ORF-2 was responsible for the ts defect. The site-directed disruption mutation was produced by insertion of the yeast *URA3* gene into the *Eco*RV site within ORF-2 (Fig. 1C). The resulting heterozygous diploid cells were induced to sporulate, and tetrads were dissected. Four normal colonies were produced at 27°C, however, when spores were germinated at 37°C, the two $Ura^+$ spores grew much slower than normal (Fig. 2C). To analyze the growth rate more precisely, yeast strains were cultured in the YEPD liquid medium. The growth rate was measured by taking samples every two hours and counting cell numbers. Each strain was grown at 27°C, diluted in YEPD medium and cultured at 27°C or 37°C. The haploid cells carry-

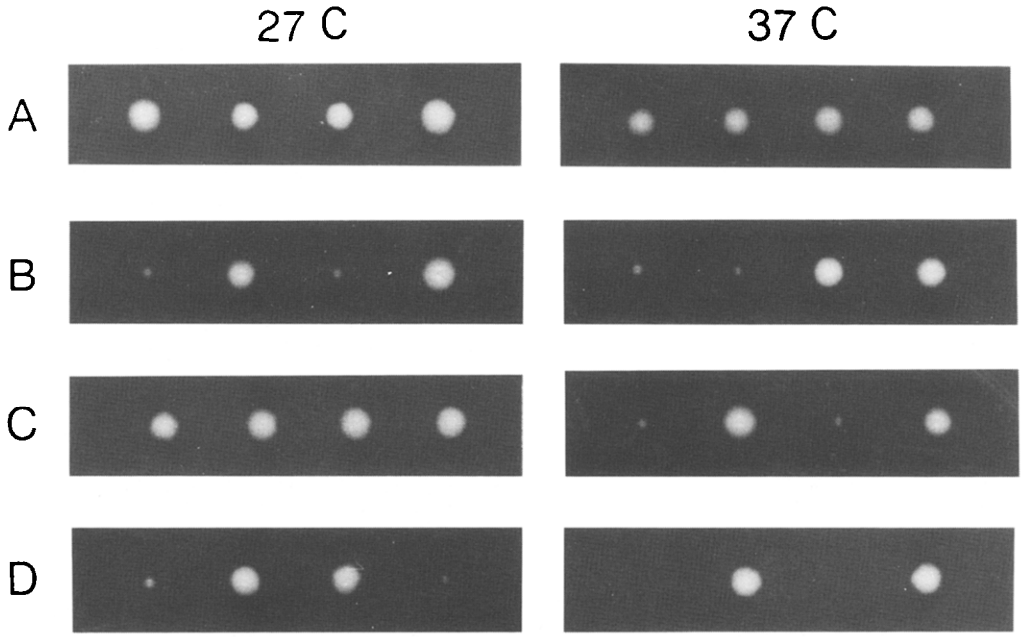


Fig. 2. Analysis of tetrads produced by wild-type cells or gene disruption transformants. A, wild-type cells; B, cells carrying the insertional disruption in ORF-1; C, cells carrying the insertional disruption in ORF-2; D, cells with the deletion disruption of 2.3-kb *Pst*I fragment. Four spores from a single ascus were germinated at 27°C or 37°C.

ing the disruption mutation in ORF2 showed the same growth rate as wild-type cells at 27°C, however, at 37°C, they grew substantially slower than wild-type cells (Fig. 3). In contrast, the cells carrying the disruption mutation in ORF-1 (*PLC2*) showed slower growth at both 27°C and 37°C (Fig. 3).

Experiments were undertaken to verify previous work done by Garrett et al. [4]. The *Pst*I 2.3 kb fragment was deleted and replaced by a 4.6 kb fragment containing the *LEU2* gene of yeast (Fig 1D). Heterozygous mutants transformed to Leu$^+$ were induced to sporulate and tetrads were analyzed for the ability to grow at 27°C or 37°C. The *LEU2* marker segregated 2:2 and all Leu$^+$ progeny grew slower at 27°C (Fig. 2D),

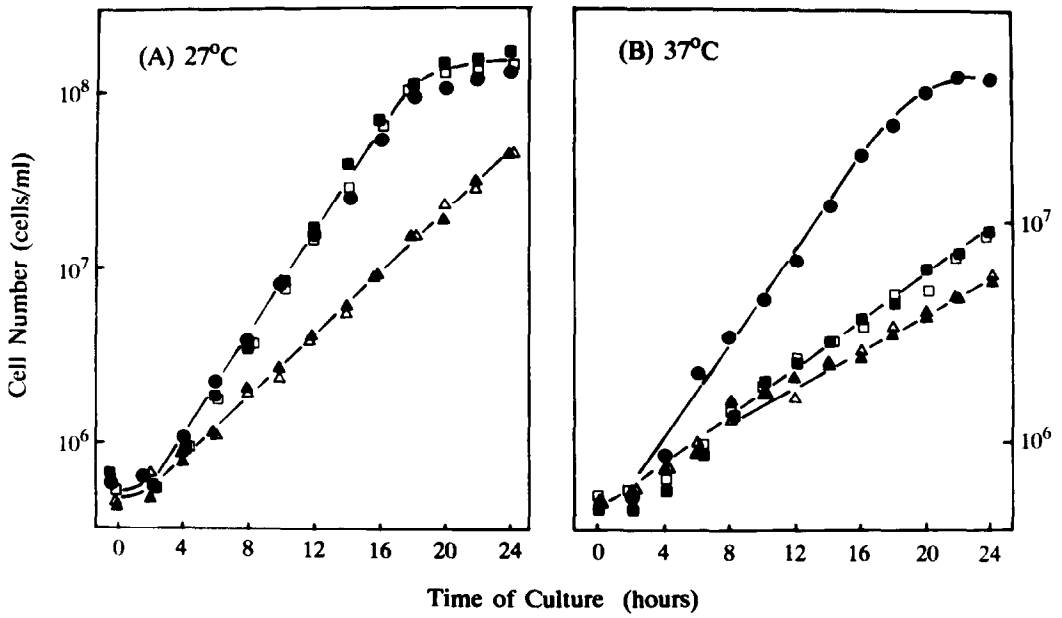


Fig. 3. Determination that mutation in orf-2 affects growth at 37°C but not 27°C. (A) Growth at 27°C. (B) Growth at 37°C. Cells were inoculated in YEPD and counted at indicated times. Two independent transformants were analyzed for each gene disruption mutatnt. (●), wild-type cells; (▲, △), cells carrying disruption mutation in ORF-1; (■, □), cells carrying disruption mutation in ORF-2.

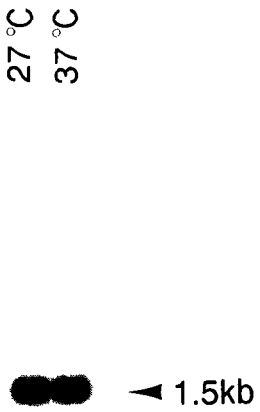


Fig. 4. Detection of transcripts corresponding to ORF-2. Poly(A)-rich mRNA was extracted from yeast cells grown either at 27°C or at 37°C, and was used for Northern blot analysis.

similar to *PLC2* insertional mutants (Fig. 2B). When spores were germinated at 37°C, only two spores gave rise to colonies in most tetrads (Fig. 2D). However, in some cases, three (two normal and one small) or four (two normal and two small) colonies could be formed (data not shown).

Gly$^+$ phenotype for *plc2::URA3* cells and *orf2::URA3* cells in the presence of *ATP2-lacZ* fusion gene was tested. Wild-type cells, *plc2::URA3* cells and *orf2::URA3* cells were transformed with pYT-ATP2, which expresses the Atp2 protein fused to $\beta$-galactosidase, just same as the report of Garrett et al. [4] These transformants formed blue colonies on X-Gal indicator plates, revealing the expression of the fusion protein. To determine if these transformants had respiration-competent phenotype, the cells were plated on minimal-glycerol plates. Wild-type cells and *plc2::URA3* cells formed much smaller colonies than *orf2::URA3* cells and wild-type cells transformed with the vector, pYT760, alone. Thus, presence of the *ATP2-lacZ* fusion gene results in a respiration-deficient phenotype for wild-type cells and *plc2::URA3* cells, but not for *orf2::URA3* cells.

### 3.2. *Expression of the gene downstream from PLC2*

Total RNA extracted from yeast cells was hybridized to a probe derived from the 2.3 kb *Pst*I fragment containing both ORF-1 and ORF-2. Although two RNA species of 1.0 kb and 0.9 kb were detected, which corresponded to *PLC1* and *PLC2*, respectively, no additional mRNA species were detected (data not shown) in agreement the report from Garrett et al. [4]. Poly(A)-rich mRNA was purified in order to increase sensitivity of mRNA detection. Northern blot hybridization with an *Eco*RV-*Pst*I 345-base fragment (Fig. 1A), which is within ORF-2, revealed the presence of a 1.5 kb mRNA corresponding to ORF-2 (Fig. 4). Since gene disruption in ORF-2 influenced the growth rate at 37°C, but not at 27°C, there was the possibility that the gene expression corresponding to ORF-2 is induced by heat. However, no significant change in the amount of the tran-

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   1 AGTAAAATACAACAACTGCCTGTAAAAAAGGAATCAAAGAACTAAAGCCA
  51 AAGGAGACTAACTCACAATGCCTCTGTCACAAAAACAAATAGACCAAGTT
                M  P  L  S  Q  K  Q  I  D  Q  V
 101 AGAACCAAAGTGCACTACAGTGAAGTAGATACTCCATTCAATAAATATTT
      R  T  K  V  H  Y  S  E  V  D  T  P  F  N  K  Y  L
 151 AGACATCTTAGGAAAAGTAACCAAGCTGACAGGAAGCATTATAAATGGCA
      D  I  L  G  K  V  T  K  L  T  G  S  I  I  N  G  T
 201 CATTATCCAATGATGATAGCAAGATTGAAAAACTTACTGAGCAAAATATC
      L  S  N  D  D  S  K  I  E  K  L  T  E  Q  N  I
 251 TCCCAACTAAAAGAAAGTGCTCATCTTCGATTTTTGGATCTGCAGTCATC
      S  Q  L  K  E  S  A  H  L  R  F  L  D  L  Q  S  S
 301 AATTGACACAAAGAAAGTAGCAGACGAAAATTGGGAAACATGCCAACAGG
      I  D  T  K  K  V  A  D  E  N  W  E  T  C  Q  Q  E
 351 AGACATTGGCCAAGCTGGAAAATCTCAAAGATAAATTGCCTGATATAAAG
      T  L  A  K  L  E  N  L  K  D  K  L  P  D  I  K
 401 AGCATCCATAGCAAGTTGCTTTTACGTATTGGAAAACTACAAGGTCTTTA
      S  I  H  S  K  L  L  L  R  I  G  K  L  Q  G  L  Y
 451 CGATTCTGTCCAAGTAATTAACAGAGAGGTGGAAGGCTTGTCAGAAGGCC
      D  S  V  Q  V  I  N  R  E  V  E  G  L  S  E  G  R
 501 GTACCAGCCTTGTGGTGACACGTGCGGAATGGGAGAAAGAACTTGGCACT
      T  S  L  V  V  T  R  A  E  W  E  K  E  L  G  T
 551 GATCTGGTCAAGTTTTTAATTGAAAAGAATTATCTCAAACTAGTTGATCC
      D  L  V  K  F  L  I  E  K  N  Y  L  K  L  V  D  P
 601 GGGCCTGAAAAAGGATAGTTCAGAGGAGAGATATCGTATTTATGACGATT
      G  L  K  K  D  S  S  E  E  R  Y  R  I  Y  D  D  F
 651 TCTCTAAGGGCCCAAAAGAGTTAGAAAGTATCAACGCCTCAATGAAATCG
      S  K  G  P  K  E  L  E  S  I  N  A  S  M  K  S
 701 GATATAGAAAACGTAAGGCAGGAGGTATCGTCTTACAAAGAGAAGTGGCT
      D  I  E  N  V  R  Q  E  V  S  S  Y  K  E  K  W  L
 751 AAGAGATGCAGAAATATTTGGCAAGATCACATCAATATTCAAAGAAGAAC
      R  D  A  E  I  F  G  K  I  T  S  I  F  K  E  E  L
 801 TTCTGAAGAGAGATGGCCTGCTCAATGAGGCAGAAGGAGATAACATTGAT
      L  K  R  D  G  L  L  N  E  A  E  G  D  N  I  D
 851 GAAGATTATGAATCGGATGAAGATGAGGAAAGAAAAGAGAGGTTTAAAAG
      E  D  Y  E  S  D  E  D  E  E  R  K  E  R  F  K  R
 901 GCAGAGATCAATGGTGGAAGTGAATACTATAGAAAATGTGGACGAAAAAG
      Q  R  S  M  V  E  V  N  T  I  E  N  V  D  E  K  E
 951 AGGAAAGCGATCATGAATATGACGATCAGGAGGATGAAGAAAATGAAGAG
      E  S  D  H  E  Y  D  D  Q  E  D  E  E  N  E  E
1001 GAAGATGATATGGAAGTAGACGTTGAGGATATAAAAGAGGATAATGAAGT
      E  D  D  M  E  V  D  V  E  D  I  K  E  D  N  E  V
1051 TGATGGGGAAAGCAGTCAACAAGAAGATAATAGTCGCCAGGGTAATAATG
      D  G  E  S  S  Q  Q  E  D  N  S  R  Q  G  N  N  E
1101 AGGAAACAGACAAAGAAACTGGAGTGATAGAAGAGCCAGATGCGGTTAAT
      E  T  D  K  E  T  G  V  I  E  E  P  D  A  V  N
1151 GACGCAGAGGAGGCAGATAGCGATCACTCAAGCAGGAAACTTGGAGGCAC
      D  A  E  E  A  D  S  D  H  S  S  R  K  L  G  G  T
1201 TACAAGCGATTTTAGTGCGTCTTCCTCTGTTGAAGAAGTAAAATGACCAC
      T  S  D  F  S  A  S  S  S  V  E  E  V  K  *
1251 GTATAATGCATAGCTCTTACTAAATAGAAAAGGCATATAGACACATAGGC
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Fig. 5. Nucleotide sequence of ORF-2 and deduced amino acid sequence

scripts was observed between cells grown at 37°C and those grown at 27°C (Fig. 4).

### 3.3. *Molecular cloning of the gene downstream from PLC2*

As ORF-2 had been only partially sequenced [4], it was necessary to determine the complete nucleotide sequence. In our previous work, a 4.5 kb *Eco*RI genomic fragment containing *PLC2* was cloned and the 2.3 kb *Pst*I fragment was sequenced (Fig. 1A) [3]. A *Pst*I-*Hin*dIII 1.1 kb fragment was sub-cloned and sequenced. A complete nucleotide sequence of ORF-2 and deduced protein sequence are shown in Fig. 5. A polypeptide of 392 amino acids was encoded by the ORF-2 comprising 1179 bases. The deduced protein was rich in acidic amino acids and leucine. No significant similarity to other known proteins was found in protein sequence databases.

## 4. DISCUSSION

The report of the *MFT1* gene sequence led us to consider that the *cyc07* gene from *C. roseus* may be a plant homolog of the yeast gene for protein import into mitochondria. Our work presented here demonstrates that the mutated gene in the *mft1* mutant is not *PLC2*. *MFT1* should be attributed to the gene downstream from *PLC2*, which corresponds to ORF-2, since disruption of ORF-2 alone resulted in the ts defect and respiration-competent phenotype in the presence of the fusion protein. Therefore, the precise function of the *cyc07* gene and its yeast homologs still remains to be determined.

Recently, the isolation of a mammalian gene homologous to *cyc07* and *PLC2* was reported by Kho and Zarbl [13]. Revertants were isolated from v-*fos*-transformed rat cells, which resulted from a mutagenic insertion of a transfected plasmid into a genome. The gene mutated in the revertant, named *fte-1* after 'v-*fos* transformation effector gene', was found to be homologous to *cyc07* and *PLC2*. Sequence similarity between *fte-1* and *MFT1* was found, and it was suggested that there was a possibility that *fte-1* regulates or plays a role in transport of mammalian protein into mitochondria [13]. However, according to our results, *fte-1* cannot be regarded as a gene involved in mitochondrial protein import, because the mutated gene in the *mft1* mutant was different from the reported *MFT1* gene sequence.

Although, at present, the functions of the genes, *cyc07*, its yeast homolog (*PLC1* and *PLC2*) and possible mammalian homolog *fte-1* are not clearly identified, as we suggested in our previous report, an essential function of the *PLC* genes in yeast cell proliferation, the cell-cycle specific expression of *cyc07* in higher plant, and highly conserved structure among evolutionally distant species, all together, make it likely that the *cyc07* and its homologs are involved in cell proliferation in eukaryotic cells.

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## REFERENCES

[1] Kodama, H., Ito, M., Hattori, T., Nakamura, K. and Komamine, A. (1991) Plant Physiol. 95, 406–411.
[2] Ito, M., Kodama, H. and Komamine, A. (1991) Plant J. 1, 141–148.
[3] Ito, M., Yasui, A. and Komamine, A. (1992) FEBS Lett. 301, 29–33.
[4] Garrett, J.M., Singh, K.K., Vonder Haar, R.A and Emr, S.D. (1991) Mol. Gen. Genet. 225, 483–491.
[5] Emr, S.D., Vassarotti, A., Garrett, J., Geller, B.L., Takeda, M. and Douglas, M.G. (1986) J. Cell Biol. 102, 523–533.
[6] Rothstein, R.J. (1983) Methods Enzymol. 101, 202–210.
[7] Hinnen, A., Hicks, J.B. and Fink, G.R. (1978) Proc. Natl. Acad. Sci. USA 75, 1929–1933.
[8] Baldari, C. and Cesareni, G. (1985) Gene 35, 27–32.
[9] Lolle, S., Skipper, N., Bussey, H. and Thomas, D.Y. (1984) EMBO J. 3, 1383–1387.
[10] Carlson, M. and Botstein, D. (1982) Cell 28, 145–154.
[11] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[12] Dente, L., Cesareni, G. and Cortese, R. (1983) Nucleic Acids Res. 11, 1645–1655.
[13] Kho, C.-J. and Zarbl, H. (1992) Proc. Natl. Acad. Sci. USA 89, 2200–2204.