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Isolation and characterization of mutations affecting expression of the Δ9- fatty acid desaturase gene, *OLE1*, in *Saccharomyces cerevisiae*

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Abstract **Expression of the Δ9- fatty acid desaturase gene, *OLE1*, of *Saccharomyces cerevisiae* is negatively regulated transcriptionally and post-transcriptionally by unsaturated fatty acids. In order to isolate mutants exhibiting irregulation of *OLE1* expression, we constructed an *OLE1p–PHO5* fusion gene as a reporter consisting of the *PHO5* gene encoding repressible acid phosphatase (rAPase) under the control of the *OLE1* promoter (*OLE1p*). By EMS mutagenesis, we isolated three classes of mutants, *pfo1*, *pfo2* and *pfo3* (positive regulatory factor for *OLE1*) mutants, which show decreased rAPase activity under derepression conditions (absence of oleic acid). Analysis of the transcription of *OLE1* in these *pfo* mutants revealed that *pfo1* and *pfo3* mutants have a defect in the regulation of *OLE1* expression at the transcriptional level while *pfo2* mutants were suggested to have a mutation affecting *OLE1* expression at a post-transcriptional step. In addition, four other classes of mutants, *nfo1*, *nfo2*, *nfo3* and *nfo4* (negative factor for *OLE1*) mutants that have mutations causing strong expression of the *OLE1p–PHO5* fusion gene under repression conditions (presence of oleic acid), were isolated. Results of Northern analysis of *OLE1* as well as *OLE1p-PHO5* transcripts in *nfo* mutants suggested that these mutations occurred in genes encoding global repressors. We also demonstrated that *TUP1* and *SSN6* gene products are required for full repression of *OLE1* gene expression, by showing that either *tup1* or *ssn6* mutations greatly increase the level of the *OLE1* transcript.**

Key words: Fatty acid desaturase; Mutation; *OLE1*; Regulation; (*Saccharomyces cerevisiae*)

1. Introduction

In vivo, the lipid contents of the biological membranes change to maintain membrane fluidity and permeability depending on the environmental conditions [1]. A relatively large number of genes in mammals, micro-organisms and higher plants involved in lipid biosynthesis have been identified [2–16]. For example, *Saccharomyces cerevisiae* genes encoding acetyl-CoA carboxylase (*ACC1*), fatty acid synthases (*FAS1*, *FAS2*), acyl-CoA-binding protein (*ACBP*), long-chain acyl-CoA synthetases (*FAA1*, *FAA2*, *FAA3*, *FAA4*) and fatty acid desaturase (*OLE1*) have been cloned [10–16]. The expression of these genes has been shown to be regulated by fatty acids [10–14]. Recently, *cis*-acting elements that regulate the expression of some of these genes have been identified [11,13,14]. However, the signal transduction pathway of saturated fatty acids (SFA) and unsaturated fatty acids (UFA) is still poorly understood.

In this study to identify components involved in the signal transduction pathway of fatty acids, we focused on the *OLE1* gene of *S. cerevisiae* encoding Δ9- fatty acid desaturase. Δ9-fatty acid desaturase catalyzes the introduction of the initial double bond between the 9th and 10th carbons of palmitoyl CoA and stearoyl CoA. Expression of the *OLE1* gene has been reported to be strictly repressed transcriptionally and post-transcriptionally by UFAs [17]. Recently, Choi et al. identified the fatty acid-regulated element (FAR) which is essential for activation and repression of *OLE1* by SFAs and UFAs [13]. *ACBP*, and *FAA1* and *FAA4* genes have been involved in activation and UFA-mediated repression of *OLE1* gene expression [13]. McHale et al. isolated the *frm2* mutant which is defective in UFA-mediated repression of *OLE1* gene expression and suggested that the FRM2 protein is involved in the fatty acid signal transduction pathway [18].

Tup1–Ssn6 complex was proposed to be a general repressor of transcription in yeasts and recruited to the promoter region of a wide variety of target genes by DNA-binding proteins [19]. *OLE1* expression was suggested to be regulated by Rox1 (Martin, personal communication in [20]), a DNA-binding protein, which was reported to function in conjunction with the Tup1–Ssn6 complex [21–24].

In this study, we isolated *pfo* (positive regulatory factor for *OLE1*) mutants exhibiting decreased expression of *OLE1* in medium containing no UFA and *nfo* (negative factor for *OLE1*) mutants displaying increased expression of *OLE1* in the presence of UFA by using a reporter gene driven by the *OLE1* promoter. Results of analysis revealed that these *pfo* mutants have an impaired *OLE1* expression regulatory mechanism while *nfo* mutants carry mutations of general repressors. We also found that either *tup1* or *ssn6* disruptants exhibited higher levels of the *OLE1* transcript than did the wild-type and *rox1*-disrupted strains.

2. Materials and methods

2.1. Microorganisms

The *S. cerevisiae* strains used in this work are listed in Table 1. *Escherichia coli* strain TG1 [25] was used as a host for the propagation and manipulation of plasmid DNA.

2.2. Plasmids

Plasmid p1093, which contains a 345 bp upstream of the *OLE1* gene which we termed ΔUAS-*OLE1p* connected to the structural region of *PHO5* encoding repressible acid phosphatase (rAPase; EC 3.1.3.2), was constructed as follows: a 345 bp fragment of *OLE1* (nt positions −345 to −1, taking the base A of ATG codon as +1 [26]) was amplified by the polymerase chain reaction (PCR) using

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chromosomal DNA of *S. cerevisiae* strain S288C [27] as a template and oligonucleotides, 5′-CGAAGCTTACACTCAACAAACCTTAT-3′ and 5′-GCGGATCCTTTGTTGTAATGTTTTAG-3′, corresponding to the nt positions −345 to −327 and −18 to −2 of the *OLE1* gene as forward and reverse primers, respectively. The PCR products were doubly digested with *Hin*dIII and *Bam*HI and cloned into the *Hin*dIII–*Bam*HI gap of pSH39 [28] to obtain plasmid p1093.

Plasmid p1166, which contains a 935 bp upstream of *OLE1* fused with the *PHO5* gene, was constructed by integration–eviction method [29]. Firstly, p1093 linearized at a unique *Bgl*II site present in the ΔUAS-*OLE1p* region was integrated at the *OLE1* locus of diploid strains, SH3975, and chromosomal DNA was prepared from cells of transformed strain, and digested with *Hin*dIII. Eight kbp *Hin*dIII fragments which were assumed to contain the 935 bp upstream of *OLE1* fused with the *PHO5* gene were purified and subjected to a self-ligation reaction with T4 DNA ligase and introduced into *E. coli* by selecting ampicillin resistance transformants. One of the plasmids (p1166) (Fig. 1A) which was extracted from the *E. coli* transformants was found to harbor the 935 bp upstream of *OLE1* connected to *PHO5* which was verified by nt sequencing. We termed this 935 bp upstream region of *OLE1 OLE1p*.

All plasmids bearing the *PHO5* reporter fused with promoters of various genes were constructed similarly to plasmid p1093. The promoter regions which were amplified by PCR correspond to a 619 bp fragment for *HIS5p* (nt positions −619 to −1) [30], a 762 bp fragment for *SUC2p* (nt positions −762 to −1) [31], a 777 bp fragment for *PGK1p* (nt positions −777 to −1) [32], a 990 bp fragment for *CYC1p* (nt positions −996 to −7) (our unpublished data), an 800 bp fragment for *MET3p* (nt positions −820 to −21) [33] and a 470 bp fragment for *STE6p* (nt positions −470 to −1) [34] whose open reading frames encode histidinol phosphate aminotransferase, invertase, phosphoglycerate kinase, iso-1-cytochrome *c*, ATP sulphurylase and **a**-specific P-glycoprotein which is necessary for the biosynthesis of **a**-factor, respectively.

2.3. Media

The nutrient (YPAD), minimal and sporulation media for the yeast [35] and the LB medium for *E. coli* [25] were described previously. Synthetic complete medium containing amino acids except for uracil (SC-URA) [35] and SC-URA supplemented with oleic acid (0.02% (v/v) oleic acid (Wako Chemicals, Osaka, Japan) and 1% (v/v) Triton X-100 (Nacalai Tesque, Kyoto, Japan)) were used to screen mutants. Ura$^-$ clones of *S. cerevisiae* were selected using 5-fluoroortic acid (5-FOA) as described [36].

2.4. Genetic and biochemical methods

Methods for the genetic manipulation of *S. cerevisiae* cells were performed as described [35]. *S. cerevisiae* and *E. coli* were transformed as described [37] and [25], respectively. *S. cerevisiae* mutants were screened after ethyl methanesulfonate (EMS) mutagenesis [38]. rAPase activity of yeast colonies was detected by a staining method based upon a diazo-coupling reaction [39] and rAPase activity in cell suspensions was assayed as described [40]. Yeast chromosomal DNA and RNA was prepared as described [41,42] and Northern blot analysis was carried out by the method of Ausubel et al. [42]. Bacterial plasmid DNA was isolated by the alkaline lysis method [25]. Nucleotide sequences were determined by the dideoxy-chain termination method [43].

3. Results and discussion

3.1. Performance of the OLE1p–PHO5 fusion gene as a reporter

When we began this study, only 345 bp of nt sequence upstream region of *OLE1* (ΔUAS–*OLE1p*) was available [26]. Plasmid p1093 which is an integration plasmid bearing a ΔUAS–*OLE1p–PHO5* fusion gene was constructed (see Section 2) and introduction into the *ura3* locus of *S. cerevisiae* SH2553. Colonies of the transformant strain displayed inefficient expression of the ΔUAS–*OLE1p–PHO5* reporter gene (pink phenotype on YPAD and YPAD containing oleic acid) as determined by rAPase staining [39]. Therefore, we cloned a region further upstream of *OLE1* using the integration–eviction method [29]. Through this procedure we obtained plasmid p1166 (Fig. 1A) which contains a 935 bp upstream of *OLE1* connected to the *PHO5* gene (*OLE1p–PHO5*) (see Section 2). The plasmid p1166 was integrated at the *ura3* locus of *S. cerevisiae* SH2676 by linearization at the *Stu*I site within the *URA3* gene. By rAPase staining, colonies of the transformant (SH4130) displayed a deep red phenotype on YPAD (derepression condition) and a pink phenotype on YPAD containing oleic acid (repression condition). The decrease in rAPase activity due to the addition of oleic acid (Fig. 1B) occurred as a result of decrease of the *OLE1p–PHO5* transcript (Fig. 1C). Therefore, we decided to use this *OLE1p–PHO5* reporter as a legitimate reporter for screening *OLE1* regulatory mutations.

3.2. Isolation and genetic characterization of pfo mutants

Cells of strain SH4130 were subjected to EMS mutagenesis. By rAPase staining assay, we identified nineteen mutants, from approximately 40 000 colonies formed on the SC-URA plate, showing decreased level of rAPase activity (a pink phenotype) compared with wild-type strain on the medium without oleic acid. These mutants were designated as *pfo* (*positive regulatory factor for OLE1*) mutants. When each of these 19 mutants was crossed with wild-type strain (SH4129), all of the resultant diploids exhibited wild-type level of rAPase activity (a deep red phenotype) by staining. Thus, mutations in all of these *pfo* mutants were recessive. The diploids were induced to sporulate and asci were dissected. Results showed 2:2 segregation for the deep red:pink phenotype, indicating that a

Table 1
S. cerevisiae strains used

Strain	Mating type	Genotype	Source
SH2553	**α**	*ura3-52 trp1 his3 Δ pho3-1 pho5-1*	our laboratory
SH2676	**a**	*ura3-52 leu2-3,112 trp1 his1-29 pho3-1 pho5-1*	our laboratory
SH3975	**a/α**	SH2553×SH2676	our laboratory
SH4129	**α**	*ura3-52*::[*URA3 OLE1p-PHO5*][b] *trp1 his3 Δ pho3-1 pho5-1*	this study
SH4130	**a**	*ura3-52*::[*URA3 OLE1p-PHO5*][b] *leu2-3,112 trp1 his1-29 pho3-1 pho5-1*	this study
TD4	**a**	*ura3-52 leu2-3,112 trp1 his4-519 can*^{r}	[51]
KY934	**a**	*rox1*::*LEU2* distruptant of TD4	Kirin Brewery Co., Ltd.
KY935	**a**	*tup1*::*LEU2* distruptant of TD4	Kirin Brewery Co., Ltd.
KY936	**a**	*ssn6*::*LEU2* distruptant of TD4	Kirin Brewery Co., Ltd.

[a]*ura3-52*::[*URA3* Δ UAS-*OLE1p-PHO5*] represents the insertion of the short upstream region, i.e. 350 bp upstream of the *OLE1* gene connected to the structural part of the *PHO5* gene, at the *ura3-52* locus.
[b]*ura3-52*::[*URA3 OLE1p-PHO5*] represents the insertion of the long upstream region, i.e. 935 bp upstream of the *OLE1* gene connected to the structural part of the *PHO5* gene, at the *ura3-52* locus.

single nuclear mutation conferred these phenotypes. By standard complementation test, three complementation groups, *pfo1* (*pfo1-1*), *pfo2* (*pfo2-1* to *pfo2-15*) and *pfo3* (*pfo3-1* to *pfo3-3*), were found among the 19 mutants (data not shown). Interestingly, we noted that the *pfo3* mutants showed the petite phenotype (Pet$^-$) which cosegregated in tetrad analysis with the pink phenotype, suggesting that the *PFO3* gene is involved in not only positive regulation of *OLE1* expression but also mitochondrial function or alternatively mitochondrial function is required for *OLE1* expression. Although disruption of *OLE1* is known to be lethal [46], none of the *pfo* mutations apparently influenced cell growth. This is probably because the supply of the *OLE1* gene product is still sufficient to support cell growth in the *pfo* mutants.

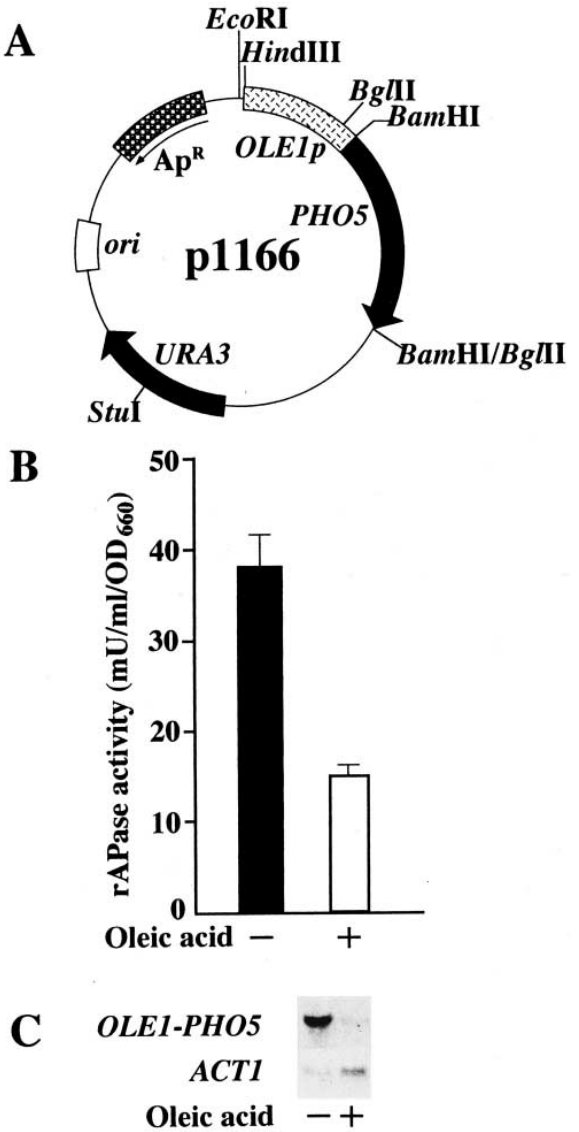

Fig. 1. Performance of the *OLE1p–PHO5* fusion gene as a reporter. A: Structure of plasmid p1166 harboring the *OLE1p–PHO5* reporter gene procedures for the construction are described in Section 2. B: rAPase activity in cells of SH4130 (wild-type) harboring the *OLE1p–PHO5* fusion gene cultivated in YPAD medium with (+) or without (−) oleic acid was determined as described [40]. *Error bars* in the figure represent standard deviations determined from a minimum of three independent measurements. C: Repression of *OLE1* expression occurs mainly at the level of transcription under the repression condition. Total RNA was isolated from cells of strain SH4130 which was cultivated in YPAD medium with (+) or without (−) oleic acid. Samples (10 μg) of RNA were electrophoresed in a 1.3% agarose gel in the presence of formaldehyde, transferred to a nylon membrane and hybridized with a ^{32}P-labelled 0.5 kbp *Bst*EII–*Eco*RV fragment containing a region of the *PHO5* ORF from plasmid pPHO5 [44] for detection of the transcript of *OLE1p–PHO5*. A 1.1-kbp *Xho*I–*Hin*dIII fragment encoding the *ACT1* gene prepared from plasmid pYA301 [45] was used as an internal control.

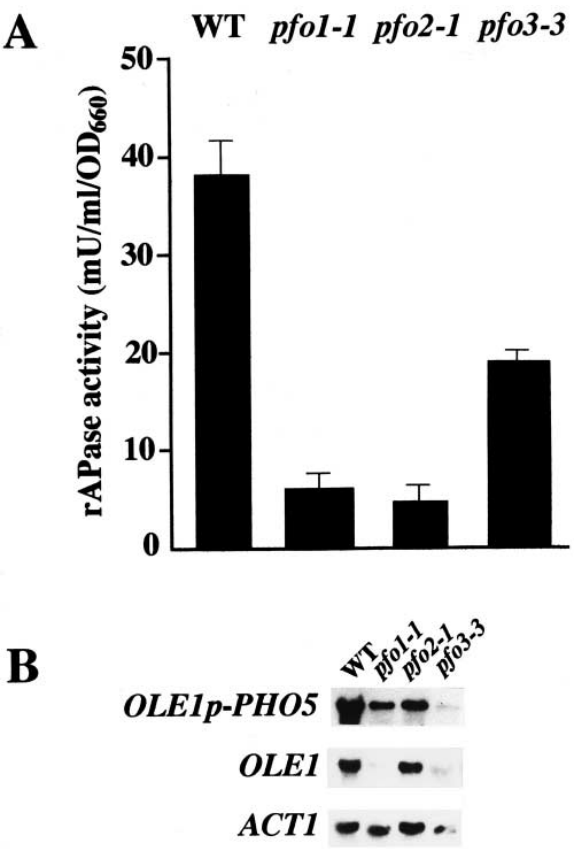

Fig. 2. rAPase activity of and Northern analysis of the *OLE1* and *OLE1p–PHO5* transcripts of the wild-type and *pfo* mutants. A: rAPase activity from the *OLE1p–PHO5* fusion gene in the wild-type (SH4130) and *pfo* mutants (*pfo1-1*, *pfo2-1* and *pfo3-3*) cultivated in YPAD medium was determined as described [40]. *Error bars* in the figure represent standard deviations determined from a minimum of three independent measurements. B: Total RNAs were prepared from cells of the wild-type and *pfo* mutants cultivated in YPAD medium. Northern blot analysis was performed as described in the legend of Fig. 1C. A ^{32}P-labelled 0.4-kbp *Eco*RI fragment containing a region of the *OLE1* ORF [46] was used as probe to detect endogenous *OLE1* mRNA. *PHO5* and *ACT1* probes were prepared and used as described in Fig. 1C legend.

Results from Northern hybridization (Fig. 2B) showed that the amounts of transcripts of *OLE1* and *OLE1p–PHO5* fusion gene in *pfo1-1* and *pfo3-3* mutants were significantly decreased while those in *pfo2-1* mutant was not although its rAPase activity was markedly decreased (Fig. 2A). These results suggest that expression of *OLE1* as well as of *OLE1p–PHO5* in *pfo1* and *pfo3* mutants were decreased at the transcriptional level while that in *pfo2* mutant appeared to occur at the post-transcriptional level.

To determine whether *pfo* mutations affect the expression of other genes, the *OLE1p–PHO5* fusion gene was eliminated from the *pfo* mutants by using 5-FOA [36], and Ura$^-$ clones thus obtained were transformed with plasmids bearing *PHO5*

fusion genes driven by either the *HIS5p*, *SUC2p*, *PGK1p*, *CYC1p*, *MET3p* or *STE6p* promoter. The rAPase activity of transformants harboring each of these *PHO5* fusion genes except for *CYC1p–PHO5* was at the same level as that of the wild-type strain. The rAPase activity from the *CYC1p–PHO5* fusion gene was slightly lower in all of the *pfo* mutants than in the wild-type strain (data not shown), suggesting that *pfo* mutations are specific to *OLE1* and possibly to *CYC1* expression. The expression of both *OLE1* (Martin, personal communication in [20]) and *CYC1* [20,47] has been suggested to be affected by molecular oxygen. Therefore, these genes are likely co-regulated by UFA and oxygen.

3.3. Mutation leading to increased expression of OLE1p–PHO5 in the presence of UFA

To isolate mutants which have a defect in UFA-mediated repression, cells of strain SH4130 were subjected to EMS mutagenesis and the mutants were screened on SC-URA plates supplemented with oleic acid. Seven of approximately 68 000 colonies exhibited deep red phenotype, as determined by the rAPase staining assay, on these plates. Thus, these mutants were designated *nfo* (negative factor for *OLE1*) mutants. It was found that each of these *nfo* mutants had a single nuclear mutation and these mutants could be assigned to four complementation groups, *nfo1* (*nfo1-1* to *nfo1-4*), *nfo2* (*nfo2-1*), *nfo3* (*nfo3-1*) and *nfo4* (*nfo4-1*) (detailed procedure not shown). The rAPase activities of these *nfo* mutants were 2–10-fold higher than that of the wild-type strain under the repression condition (Fig. 3A). However, the rAPase activities in the *nfo1-1*, *nfo2-1* and *nfo3-1* mutants were decreased in the presence of UFA while that in the *nfo4-1* mutant was constitutively high irrespective of the presence or absence of UFA. These results are consistent with the results of Northern analysis (Fig. 3B). However, when we investigated the endogenous *OLE1* transcription in *nfo* mutants, all of the *nfo* mutants showed a response to UFA similar to that of the wild-type although the level of *OLE1* transcription in *nfo3* was significantly higher than that in the wild-type strain (Fig. 3B). These results suggest that the effect of the *nfo* mutations are affected by the chromosomal position of the *OLE1* promoter and might not directly affect *OLE1* regulation but rather affect general repression systems such as those by nucleosomes and chromatin. In fact, we found that the *nfo1* and *nfo2* mutations were suppressed by *SIN4*, a gene proposed to be involved in the maintenance of correct chromatin structure [29,48]. Recently, Jiang et al. demonstrated that high dosage of *RGR1* could suppress *sin4* mutation [49]. However, none of the *nfo1* to *nfo4* mutations were suppressed by single or multicopies of the *RGR1* gene (our unpublished results). Neither *nfo3-1* nor *nfo4-1* mutations were suppressed by either *FRM2* [18], *ROX1*, *TUP1* or *SSN6* [21,22,50] gene (our unpublished results), suggesting that *NFO3* and *NFO4* are likely unidentified genes encoding global repressors.

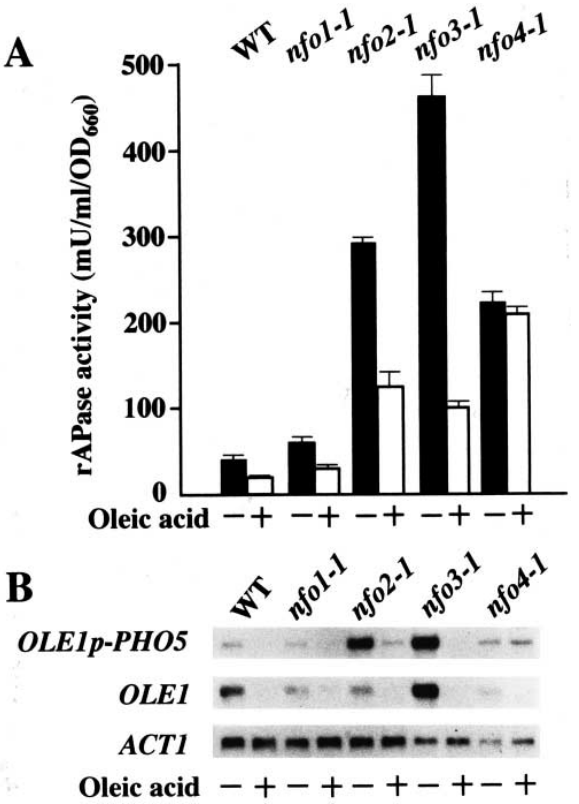

Fig. 3. rAPase activity of and Northern analysis of the *OLE1* and *OLE1p–PHO5* transcripts of the wild-type and *nfo* mutants. A: rAPase activity from the *OLE1p–PHO5* fusion gene in the wild-type (SH4130) and *nfo* mutants (*nfo1-1*, *nfo2-1*, *nfo3-1* and *nfo4-1*) cultivated in YPAD medium with (+) or without (−) oleic acid was determined as described [40]. *Error bars* in the figure represent standard deviations determined from a minimum of three independent experiments. B: Total RNAs were prepared from the wild-type and *nfo* mutants cultivated in YPAD medium with (+) or without (−) oleic acid. Northern blot analysis was performed as described in the legend of Fig. 1C.

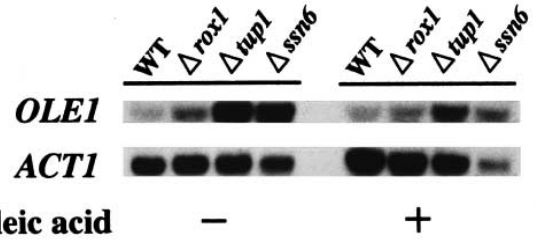

Fig. 4. Northern analysis of the *OLE1* transcripts in *rox1*, *tup1*, and *ssn6* disruptants. Total RNAs were prepared from cells of the wild-type (TD4) and *rox1* (KY934), *tup1* (KY935), and *ssn6* (KY936) disruptants cultivated in YPAD medium with (+) or without (−) oleic acid. Northern blot analysis was performed as described in the legend of Fig. 1C.

3.4. Tup1 and Ssn6 are required for repression of OLE1 gene expression

OLE1 gene expression is assumed to be regulated by two regulatory factors, a fatty acid [13] and oxygen [20,22]. Rox1 was suggested to be involved in the oxygen regulatory pathway of *OLE1* transcription (Martin, personal communication in [20]). We found putative Rox1-binding sites, 5′-YYYATTGTTCTC-3′ (where Y represents pyrimidine) [24], at nt positions −130 to −119, 5′-TTTATTGTTCTA-3′, −272 to −261, 5′-CCTATTGTTACG-3′, and −392 to −381, 5′-CTTAAGGTTCTC-3′ (the exact nucleotide matches are underlined) of the *OLE1* promoter. Since there is no experimental evidence indicating that the Tup1–Ssn6–Rox1 repression system operates in *OLE1* transcription, we analysed the *OLE1* transcripts of *tup1*, *ssn6* and *rox1* disruptants which were cultivated under aerobic condition by Northern hybridization. Results shown in Fig. 4 revealed that the amount of

OLE1 transcripts in either *tup1* or *ssn6* disruptants is markedly higher than that in the wild-type strain and that in the *rox1* disruptant is also slightly increased although the *OLE1* transcripts in all of the disruptants is still repressed by UFA. These results suggest that Tup1 and Ssn6 possibly in conjunction with Rox1 are involved in the transcriptional repression of *OLE1* expression independent of the fatty acid regulatory pathway and under aerobic conditions an unidentified DNA-binding protein distinct from Rox1 also functions in conjunction with Tup1 and Ssn6 to repress *OLE1* transcription.

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