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Promoter analysis of *Mucor rouxii* Δ^9-desaturase: Its implication for transcriptional regulation in *Saccharomyces cerevisiae*

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Abstract

Promoter study was performed to understand the transcriptional control of Δ^9-desaturase gene of *Mucor rouxii*. Several putative *cis*-elements involved in lipid metabolism were mapped by computational analysis. 5′ deletion analysis shows the presence of elements with repressing activity, especially in 122 bp located upstream of the transcription start site. Truncation of these repressor domains showed that the promoter of *M. rouxii* is functional in *Saccharomyces cerevisiae* without additional components and is insensitive to nutritional depletion. The promoter also drove effectively the expression of a *M. rouxii* Δ^{12}-desaturase gene, and the linoleic acid content increased with the age of the yeast culture in parallel with the promoter activity. This approach provides a genetic tool for programming heterologous protein production in the yeast.

Keywords: Δ^9-Desaturase; *Mucor rouxii*; Regulatory element; Promoter; *Saccharomyces cerevisiae*; Heterologous expression

Mucor rouxii is a dimorphic phycomycete that produces essential unsaturated fatty acids, particularly γ-linolenic acid [1]. Generally, unsaturated fatty acids, which are present in storage oils, are required in the membrane participating in the formation of a structure with the appropriate membrane physical state [2–4]. In addition, they have critical roles as precursors of some biological metabolites [5]. Unsaturated fatty acids are synthesized by desaturation of pre-formed fatty acyl chains using an enzyme complex, cytochrome b_5, cytochrome b_5 reductase, and desaturases. Δ^9-Desaturase is a membrane-bound enzyme that introduces the first double bond into saturated fatty acids to form mono-unsaturated fatty acids, palmitoleic acid (C16:1Δ^9), and oleic acid (C18:1Δ^9) [6]. The expression of the cloned Δ^9-desaturase genes of many organisms is strongly influenced by nutrients as well as by physical and environmental factors [7–9]. While Δ^9-desaturase genes share common transcriptional regulatory mechanisms, specific regulators and environmental conditions are operative in each organism. The fatty acid-regulated (FAR) element, which is responsible for unsaturated fatty acid-mediated repression of Δ^9-desaturase gene (*OLE1*) of *Saccharomyces cerevisiae*, has been identified [10]. Several regulatory elements have been mapped in the upstream promoter of the rat *SCD1* gene, encoding stearoyl-CoA desaturase [11,12]. It has been shown that a nuclear factor 1 is essential for transcriptional

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activation of *SCD1* gene during preadipocyte differentiation [13].

Transcriptional expression of the Δ^9-desaturase gene has been previously studied in *M. rouxii* [14]. As in many poikilothermic organisms, temperature change appears to be a key factor regulating Δ^9-desaturase gene expression. Moreover, the level of gene expression is associated with the vegetative growth of *M. rouxii*. To gain further insights into molecular mechanisms involved in the control of transcription of *M. rouxii* Δ^9-desaturase gene, the promoter was sequenced and studied by 5′ deletion analysis. We tested the ability of the Δ^9-desaturase promoter of *M. rouxii* to produce a heterologous protein in *S. cerevisiae*. This is the first example of the application of a *M. rouxii* promoter in constitutive fatty acid production in a heterologous yeast expressing a membrane-bound Δ^{12}-desaturase of *M. rouxii*, which is involved in the transformation of endogenous C16:1Δ^9 and C18:1Δ^9 to C16:2$\Delta^{9,12}$ and C18:2$\Delta^{9,12}$, respectively.

Materials and methods

Strains, growth conditions, and media. Saccharomyces cerevisiae DBY746 (α, *his3- Δ1*, *leu2–3*, *leu2–112*, *ura 3–52*, *trp 1–289*) was used as recipient strain and was grown at 30 °C in either complex (YPD) or synthetic minimal (SD) medium. Appropriate amino acids, 20 mg/ml L-tryptophan and L-histidine, and 30 mg/ml L-leucine, were added into the cultures when required. *Escherichia coli* strain DH5α was grown in Luria–Bertani medium at 37 °C [15]. When needed, 100 mg/liter of ampicillin was added.

Promoter sequence analysis of M. rouxii Δ⁹-desaturase. The nucleotide sequence of the 5′ upstream promoter of *M. rouxii* Δ^9-desaturase was determined by primer walking of the recombinant lambda clone containing the Δ^9-desaturase gene of *M. rouxii* [14]. The promoter sequence has been assigned the GenBank Accession No. AY995173. Based on comparative DNA analysis, we identified putative *cis*-regulatory promoter elements using two eukaryotic databases: the transcription factor database (TFD) [16] and the promoter database of *S. cerevisiae* (SCPD) [17]. The analytical methods, Tfsitescan and predefined consensus, were used to search the binding sites in TFD and SCPD, respectively.

Plasmid construction for 5′ deletion analysis of M. rouxii Δ⁹-desaturase promoter and yeast transformation. A shuttle vector, yEP356 [18], was used to construct a fusion between the Δ^9-desaturase promoter of *M. rouxii* and *lacZ* gene. Owing to lack of suitable restriction sites in the promoter sequence, a series of promoter fragments harboring the 5′ untranslated region and 61 bp of N-terminal coding sequence of *M. rouxii* Δ^9-desaturase gene were amplified by polymerase chain reaction (PCR). Forward oligonucleotide primers were derived from different regions of the 5′ flanking sequence of the promoter of *M. rouxii* Δ^9-desaturase gene to obtain 5′ deletion series. The sequences located between +141 and +159 relative to transcription start site were used to design a reverse primer for all PCR-generated constructs (Table 1). 5′ ends of forward and reverse primers were combined with *Pst*I and *Hin*dIII restriction sites, respectively, for in-frame fusion with the *E. coli lacZ* sequence and for further subcloning into YEp356. Subsequently, their nucleotide sequences were determined. These series of recombinant plasmids were designated as pDP as shown in Fig. 2. The pDP and YEp356 plasmids were transformed into *S. cerevisiae* using the lithium acetate method [19].

β-Galactosidase assay. The yeast cells containing the YEp356 and plasmids with Δ^9-desaturase promoter-*lacZ* fusion constructs were assayed for β-galactosidase activity in which *o*-nitrophenyl-β-D-galactoside (ONPG) was used as a substrate, as described previously [20]. The all yeast transformants were cultured in SD broth at 30 °C to logarithmic phase or to different phases of growth. Cell density was determined either by measuring the absorbance at A_{600} or by hemocytometer counts. Protein concentration in the extract was determined by the Bradford method [21] using the Bio-Rad assay kit and bovine serum albumin (BSA) as the standard.

Construction of expression vector containing the M. rouxii Δ⁹-desaturase promoter. To study the expression of Δ^9-desaturase promoter of *M. rouxii* in *S. cerevisiae*, the available gene encoding a membrane-bound Δ^{12}-desaturase enzyme of *M. rouxii* was fused downstream of the flanking sequences of Δ^9-desaturase promoter of *M. rouxii* by overlap extension PCR technique using *pfu* DNA polymerase (Promega, Madison, WI). The 281 bp fragment of Δ^9-desaturase promoter corresponding to nt −122 to +159 (+1 refers to the transcription start site) was synthesized by PCR using pDP122 plasmid as a template. The forward and reverse primers were: FP9-122, 5′-CGCGGATCCGGCTTGCACAACTTCCTA-3′ (the underlined letters indicate the *Bam*HI restriction site) and RP9-159, 5′-**CGTTTCTCTTGGTTGC**AGGCTTCATGGACTCCGT-3′ (the bold letters indicate the nucleotides complemented with the

Table 1
Oligonucleotide primers used in the plasmid construction for 5′ deletion analysis of *M. rouxii* Δ^9-desaturase promoter

Primer[a]	Sequence (5′–3′)[b]	Position[c]
FP18	AACTGCAGCCCAAGAGGAGATAATCA	−18 to −1
FP122	AACTGCAGGGCTTGCACAACTTCCTA	−122 to −105
FP154	AACTGCAGGTTATCATCGAAAAGGGA	−154 to −137
FP191	AACTGCAGAATTGACAATGATAGGAT	−191 to −174
FP222	AACTGCAGTTGAATCTGATTTGTTTA	−222 to −205
FP259	AACTGCAGTACTTGCTCGATAGTAGC	−259 to −242
FP311	AACTGCAGGGCATCTCGGGAACTGGA	−311 to −294
FP494	AACTGCAGCGACCAGCGTTTCAGTGT	−494 to −477
FP854	AACTGCAGCAGTATCTGATCGACATTGGTGC	−849 to −832
RPH	CCCAAGCTTGAGGCTTCATGGACTCCGT	+159 to +141

[a] FP and RP are forward and reverse primers, respectively.
[b] The underlined sequences represent the additional *Pst*I and *Hin*dIII restriction sites at the 5′ end of forward and reverse primers, respectively.
[c] The oligonucleotide positions are referred to the transcription start site of *M. rouxii* Δ^9-desaturase.