An investigation of subcellular localization of *Yl*Snf1p-EGFP in response to a fatty acid in *Yarrowia lipolytica*

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Abstract: *Yarrowia lipolytica* is a hydrophobic substrate-assimilating yeast, breaking down substrates such as fatty acids and *n*-alkanes as sole carbon sources. Nonetheless, the control of the mechanisms of hydrophobic substrate utilization in the yeast has not been completely understood. From our previous finding, the deletion of *YLSNF1* in *Y. lipolytica*, encoding a sucrose non-fermenting 1 protein kinase, caused defective growth when the cells were cultured in fatty acid medium. As a result, the aim of this study was to elucidate the response of *Yl*Snf1p to fatty acid utilization in *Y. lipolytica* by investigating the subcellular localization of *Yl*Snf1p-EGFP. To begin with, pSNF1-EGFP was successfully constructed to express *Yl*Snf1p-EGFP under the control of its own promoter in Δ*Ylsnf1::ADE1*. Next, *Yl*Snf1p-EGFP was expressed and intact inside the yeast cells, using western blot analysis. Moreover, *Yl*Snf1p-EGFP could restore growth of *Yl*Snf1p in Δ*Ylsnf1::ADE1*. The subcellular localization of *Yl*Snf1p-EGFP was then performed by observing the EGFP signal inside *Y. lipolytica* using fluorescence microscopic technique. The result showed that *Yl*Snf1p-EGFP was mainly located in the whole cell body when glycerol was used as a carbon source. After the shift of carbon source from glycerol to other hydrophobic substrates, *Yl*Snf1p-EGFP displayed distinct localization patterns, positioned in cytosol and nucleus for glucose and oleic acid media, respectively. These results suggested that the relocalization of *Yl*Snf1p-EGFP was associated with the control of fatty acid utilizations in *Y. lipolytica*.

Keywords: Snf1-protein kinase; protein relocalization; control of fatty acid utilization

1. Introduction

Yarrowia lipolytica, a hemiascomycetous yeast, can utilize a variety of hydrophobic substates as sole carbon sources such as fatty acids and *n*-alkanes [1]. When fatty acids are utilized as the carbon source, they are activated by fatty acyl-CoA synthetase I (ACSI) in cytosol or fatty acyl-CoA synthetase II (ACSII) in peroxisomes to acyl-CoA [2-4]. Then, acyl-CoA is metabolized through β-oxidation which has been intensively studied. First, acyl-CoA is oxidized to trans-2,3-dehydroacyl-CoA and subsequently to 3-ketoacyl-CoA by acyl-CoA oxidases (*POX1* – *POX6*) and the multifunctional enzyme 2 (*MFE2*), respectively. This is followed by the thiolytic cleavage of 3-ketoacyl-CoA yielding acetyl-CoA and acyl-CoA by 3-ketoacyl-thiolase (*POT1*) or that of 3-acetoacetyl-CoA to two acetyl-CoA by acetoacetyl-CoA thiolase (*PAT1*) [1, 4, 5].

However, the molecular mechanisms controlling the lipid metabolism in *Y. lipolytica* have not yet been entirely understood. Previously, Poopanitpan *et al*. demonstrated that Primary Oleate Regulator 1 gene (*POR1*), coding for a Zn_2Cys_6 transcription factor, regulated the transcription of *POX2*, *POT1*, and *PAT1* which involve in beta-oxidation pathways in *Y. lipolytica* [6]. In addition to the transcription factor control, protein phosphorylation via 5' adenosine monophosphate-activated protein kinase (AMPK) family was

reported to associate with the control of lipid metabolism in *Y. lipolytica*. The disruption of Sucrose non-fermenting 1 protein kinase (Snf1p), which is an AMPK found in *Y. lipolytica* (*Yl*Snf1p) and an ortholog of *Sc*Snf1p found in *Saccharomyces cerevisiae*, was formerly described as a negative regulator in a lipid accumulation process in *Y. lipolytica* [7]. Moreover, we found that the deletion of *YLSNF1* caused retarded growth in *Y. lipolytica* when fatty acids were used as a sole carbon source [8]. Furthermore, protein relocalization in yeast cells was observed in response to different carbon source utilization. For alkane utilization in *Y. lipolytica*, Yas3p which is the repressor of Yas1p-Yas2p-Yas3p system, relocated from the nuclear to the ER-like compartments to activate *ALK1* expression in cytoplasm and, in turn, alkane degradation [5, 9]. Thus, an understanding in the subcellular localization of *Yl*Snf1p could potentially be used to explain the control of fatty acid degradation, a target for metabolic engineering in biodiesel production. As a result, it is of our interest to explore the subcellular localization of *Yl*Snf1p that was previously reported to associate with lipid metabolism in *Y. lipolytica* during fatty acid utilization by employing EGFP-tagged *Yl*Snf1p for visualization.

2. Materials and Methods

2.1. Yeast strains and media

Y. lipolytica strain CXAU/AI (*ura3*, *ade1*::*ADEI*) was kindly provided by Prof. Akinori Ohta, (Laboratory of Cellular Genetics, The University of Tokyo). The deletion mutant of *YLSNF1* (*ura3*, a*de1*, Δ*snf1*::*ADE1*) (Δ*Yl*snf1) was generated as previously described [8]. Yeast Nitrogen Base (YNB) medium (0.17% YNB without amino acid and ammonium sulfate, supplemented with 0.5% ammonium sulfate) and 2% of glycerol, glucose and oleic acid were mixed to make SG, SD, and SO media, respectively, for *Y. lipolytica* cultivation (24 mg/L of Uracil was added in SG medium, if necessary). For solid media, 0.2% fatty acid was dispersed by adding 0.05% Triton X-100.

2.2. Construction of pSNF1-EGFP

To construct a plasmid carrying *YLSNF1* containing *EGFP* gene at 3′-terminal, a genomic DNA from *Y. lipolytica* was used as a DNA template for amplifying *YLSNF1* gene by PCR. The primers indicated in Table 1 were designed to generate two *YLSNF1* gene fragments (101G and 101T) that cover the whole *YLSNF1* gene sequence. The 101G fragment consists of promoter and coding regions and the 101T fragment consists of a terminator region of *YLSNF1* gene. Next, both 101G and 101T fragments were double-digested by *Hin*dIII and *Avr*II. Then, both fragments were cloned into pSUT5-nonAvrII, a low copy plasmid, at *Hin*dIII site. This plasmid was designated as p101GT. Next, an *EGFP* fragment was amplified by specific primers in Table 1. Then, the *EGFP* fragment was inserted into p101GT between *Avr*II and *Kas*I sites. This plasmid was designated as pSNF1-EGFP. The sequences of *YLSNF1* and *EGFP* genes were confirmed by DNA sequencing (Macrogen, Korea). The plasmid was introduced into yeast cells by electroporation [10].

Table 1. List of specific primers

Fragment	Primer name	Sequence
101G	YalD02101SH-5F	5′- CCC*AAGCTT*GACGGGTGAAGCGGGAAATCAAG-3′
	101-fusion-R	5′- AATCCCTAGTGTGCTCTACAAGTGCTTA*GGCGCC* *CCTAGG*CTTCTCACTCTCCTTCTGAGAACTCAC-3′
101T	101-fusion-F	5′- GTGAGTTCTCAGAAGGAGAGTGAGAAG*CCTAGGGG* *CGCC*TAAGCACTTGTAGAGCACACTAGGGATT-3′
	YalD02101H3′-R	5′- CCC*AAGCTT*CGAATTGCACCAGTCGTTCA-3′
EGFP	EGFP-AvrII-F	5′- GG*CCTAGG*ATGGTGAGCAAGGGCGAGGA-3′
	EGFP-Kas-R	5′- GG*GGCGCC*CTTGTACAGCTCGTCCATGCC-3′

* Underlines indicate the site of restriction enzymes

2.3. Yeast propagation in different carbon sources

Y. lipolytica at 0.05 OD/mL was grown into 150 mL of SG medium for a large-scale culture at 30 °C, shaken at 220 rpm for 18-19 hours. After that, cells were washed twice by YNB medium at room temperature. Fifteen OD of yeast cells were further incubated in YNB medium containing 2% glycerol, glucose, or oleic acid at 30 °C, shaken at 220 rpm, for 1 hour (cell fixation) or for 3 hours (western blot analysis). After incubation, 0.1% Triton X-100 was added, and cells were collected by centrifugation at 5,000 rpm for 10 minutes at 4 °C. Finally, cells were washed with 1 mL of Phosphate Buffer Saline (PBS) and kept at -80 °C prior to proceeding with experiments.

2.4. Western blot analysis of EGFP-tagged SNF1 protein

Yeast pellets were lysed by using glass beads and Micro Smash™ MS-100R (TOMY, Japan) at 4 °C, 2700 rpm for 3 minutes. The concentrations of protein extracts were measured by Bio-Rad protein assay (Bio-Rad, USA). Forty micrograms of each protein lysate sample were performed in 10% SDS-PAGE. Afterward, separated proteins from SDS-PAGE was electro-transferred onto PVDF membrane and EGFP protein were detected by immunoblotting with a standard method using Living Colors® A.v. monoclonal antibody (JL-8) (Clontech, JAPAN) and anti-mouse IgG, HRP-linked Antibody (Cell signaling, USA) as primary and secondary antibodies, respectively. The signals were visualized using an SuperSignal™ West Pico Substrate Working Solution (Thermo Scientific, USA).

2.6. Growth analysis on different carbon sources

The yeast strains were prepared using serial dilution at 10^{-1} to 10^{-5} starting from 1 OD_{600nm} and spotted on glycerol, glucose, and fatty acid solid media. The yeast cells were incubated for 2 days on glycerol and glucose media and for 3 days on fatty acid medium.

2.7. Cell fixation, DAPI-staining, and Fluorescence microscopy

The yeast strains were prepared from the previous method. Cells were then fixed with 70% ethanol, collected, and washed twice by PBS. Next, the fixed cells were mixed with 1 μg/mL DAPI and incubated at room temperature for 10 minutes. Finally, the stained cells were washed 3 times by PBS. Fluorescence microscopic technique was used to observe, and images were recorded using IX73 microscope (Olympus, Japan).

3. Results and Discussion

3.1. Design of YlSnf1p-EGFP

For the design of *Yl*Snf1p-EGFP that expresses under its own promotor, domain analysis and structural modelling were performed to find an appropriate *Yl*Snf1p terminus for attaching EGFP-tag. According to the Motif Scan result, there are four domains that were detected on N-terminus of *Yl*Snf1p including protein kinase ATP-binding region signature (amino acids 37 to 60), Protein kinase domain (amino acids 31 to 282), Serine/threonine protein kinase active-site signature (amino acids 149 to 161), and Ubiquitin associated domain (amino acids 302 to 345) (Figure 1a) [11]. The structure of *Yl*Snf1p was predicted by homology modelling method using SWISS-MODEL [12, 13] as shown in Figure 1b. The top-ranked template of *Yl*Snf1p was Human AMPK (5'-AMP-activated protein kinase catalytic subunit alpha-2) Chain C with the sequence identity value of 47.78% and with the QMEAN value of -3.06. The predicted structure demonstrated that the C-terminus of *Yl*Snf1p did not contain any important domains and was not buried in the core. Thus, C-terminus of *Yl*Snf1p was chosen as the attaching site for EGFP-tag.

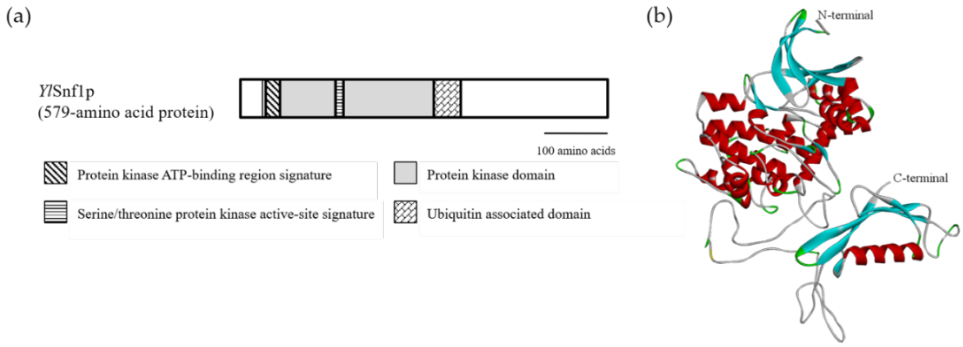

Figure 1. Structural domain and homology modeling of *Yl*Snf1p. **(a)** Schematic representation of functional domains in *Yl*Snf1p that were analyzed by Motif Scan. **(b)** The 3D structure of *Yl*Snf1p from homology modeling by SWISS-MODEL.

3.2. Generation and verification of YlSnf1p-EGFP

A schematic diagram representing the construction of the plasmid carrying *YLSNF1-EGFP* was shown in Figure 2a. Two *YLSNF1* fragments including 101G (3.0 kb) and 101T (1.0 kb) were successfully amplified by PCR as shown in Figure 2b. Then, both fragments, joining two fragments at *Avr*II site, were cloned into *Hin*dIII site of pSUT5-nonAvrII, yielding p101GT. The correct size of p101GT (9.8 kb), larger than pSUT5 (5.8 kb), was observed on agarose gel electrophoresis indicating the successful p101GT construct (Figure 2c) Next, an *EGFP* gene fragment was inserted between *Avr*II and *Kas*I sites of p101GT to obtain pSNF1-EGFP. The transformant plasmids were verified by colony PCR as shown in Figure 2d. In addition, DNA sequencing of the plasmid further confirmed that there was no mutation in pSNF1-EGFP (data not shown). Then, the expression of *Yl*Snf1p-EGFP in *Y. lipolytica* crude extract was detected by western blot analysis using Living Colors® A.v. monoclonal antibody (JL-8). The yeast cells were cultured in different carbon sources, including glycerol, glucose, and oleic acid. The result showed that *Yl*Snf1p-EGFP appeared intact in all three carbon sources (Figure 2e).

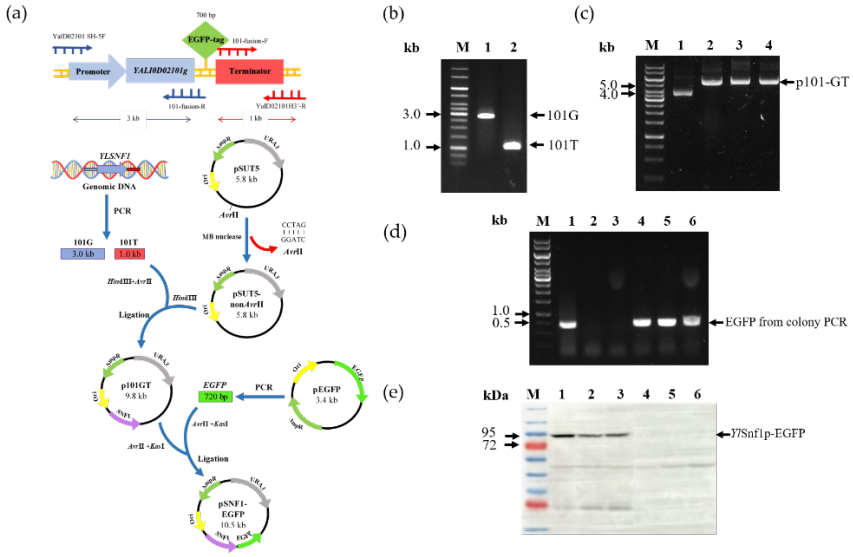

Figure 2. Generation and verification of *Yl*Snf1p-EGFP. **(a)** A plasmid construction diagram of pSNF1-EGFP. **(b)** PCR amplification products of 101G and 101T *SNF1* fragments on gel electrophoresis; lane M: 1 kb DNA ladder, lane1-2: PCR products of 101G and 101T, respectively. **(c)** Confirmation of p101GT on gel electrophoresis; lane M: 1 kb DNA ladder, lane1: pSUT5 nonAvrII control; lane 2-4: recombinant plasmids from clones 1-3. **(d)** Colony PCR of pSNF1-EGFP; lane M: 1

kb DNA ladder, lane1-6: PCR products from clones 1-6. **(e)** Western blot analysis of *Yl*Snf1p-EGFP in yeast cells; lane M: protein marker, lane 1-3: Δ*Ylsnf1*::*ADE1* carrying pSNF1-EGFP in glycerol, glucose and oleic acid media respectively, lane 4-6: CXAU/AI carrying pSUT5 in glycerol, glucose and oleic acid media respectively.

3.3. Growth analysis of ΔYlsnf1::ADE1 carrying pSNF1-EGFP on different carbon source

The growth of Δ*Ylsnf1*::*ADE1* carrying pSNF1-EGFP in various carbon sources was studied to confirm the function of *Yl*Snf1p-EGFP. In the experiment, the CXAU/AI carrying pSUT5 and Δ*Ylsnf1*::ADE1 carrying pSUT5 were used as a wildtype and a deletion mutant of *YLSNF1*, respectively. The results revealed that all strains grew normally on glycerol solid medium (Figure 3). The deletion of *YLSNF1* did not compromise the growth ability of the cells on glycerol. However, a decrease in growth rate of Δ*Ylsnf1*::*ADE1* carrying pSUT5 was observed in glucose solid medium, compared with wild type and Δ*Ylsnf1*::*ADE1* carrying pSNF1-EGFP. Similar to the cells on fatty acid media, Δ*Ylsnf1*::*ADE1* carrying pSUT5 exhibited growth retardation while the growth of wild type and Δ*Ylsnf1*::*ADE1* carrying pSNF1-EGFP was similar. This suggests that *Yl*Snf1p-EGFP was functional in the yeast cells and could restore the growth of Δ*Ylsnf1*::*ADE1* in various fatty acid media.

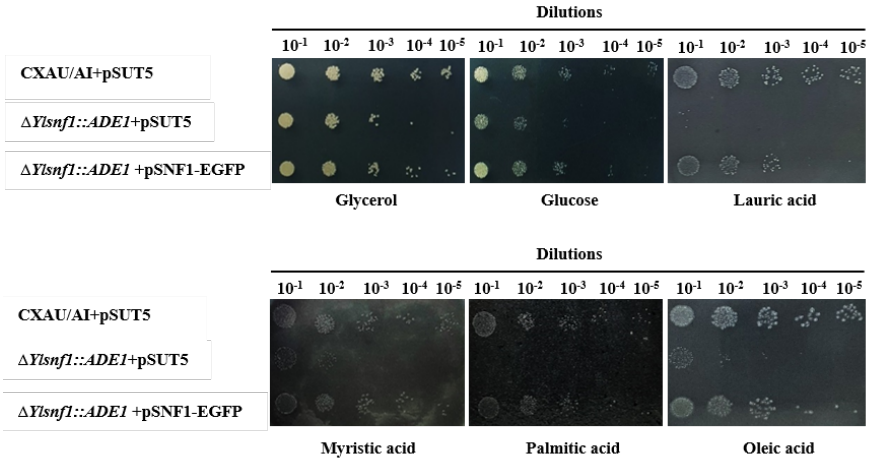

Figure 3. The growth of Δ*Ylsnf1*::*ADE1* carrying p101GT-EGFP on various carbon sources. The yeast strains were prepared by serial dilution from 10^{-1} to 10^{-5} and spotted on glycerol, glucose, and fatty acid media. The growth abilities of yeast in each condition were monitered for 2-3 days. CXAU/AI+pSUT5, Δ*Ylsnf1*::*ADE1*+pSUT5 and Δ*Ylsnf1*::*ADE1*+pSNF1-EGFP are representatives of wildtype, *YLSNF1* deletion mutant, and *YLSNF1* functional restoration strains, respectively.

3.4. Localization of YlSnf1p-EGFP in Y. lipolytica in different carbon sources

To investigate the subcellular localization of *Yl*Snf1p-EGFP, Δ*Ylsnf1*::*ADE1* carrying pSNF1-EGFP was grown to log-phase in glycerol medium. After washed with YNB, the yeast cells were shifted into media containing different carbon sources such as glycerol, glucose, and oleic acid, which was selected as a representative of fatty acids, and incubated for 1 hour. The cells were then fixed with 70% ethanol and stained with DAPI for nucleus detection. Fluorescence microscopic technique was performed to visualize the expression of *Yl*Snf1p-EGFP.

The subcellular localization of *Yl*Snf1p-EGFP in *Y. lipolytica* grown in different carbon sources was shown in Figure 4. In glycerol medium, *Yl*Snf1p-EGFP was observed in the whole cell body (cytoplasm and nucleus). For glucose medium, *Yl*Snf1p-EGFP displayed a cytosol localization pattern, in addition to the whole cell protein expression

pattern. The cytosol localization pattern accounted for 51% and about 45% of the total yeast cells were comprised of the whole cell localization pattern (n = 100). Interestingly, for oleic acid medium, some of the yeast cells exhibited distinct *Yl*Snf1p-EGFP expression in the nucleus, contributing to approximately 19% of total yeast population. As a result, these results indicated that the shift in carbon sources from glycerol to glucose and oleic acid determined the localization of *Yl*Snf1p-EGFP in *Y. lipolytica*. Similar to our result, in *S. cerevisiae* utilizing glucose, *Sc*Snf1p was detected in the cytosolic area and rapidly repositioned to the nucleus after switching carbon sources from glucose to glycerol [14, 15]. Furthermore, the β-subunits of SNF1/AMPK heterotrimer complexes such as Sip1, Sip2, and Gal83 showed subcellular localization in response to glucose limitation. The localization patterns of Gal83, Sip1, and Sip2 were observed in nucleus, vacuole, and/or cytoplasm, respectively after a shift of glucose to other carbon sources [16-18]. The localization of Snf1-Gal83 was proposed to affect several gene expressions, relying on the ability of transcription factors to move in and out of the nucleus [19]. However, the ortholog of β-subunits and γ-subunit of SNF1/AMPKs complex in *Y. lipolytica* are only identified as a part of negative regulator in lipid accumulation and the mechanism remains unknown [7].

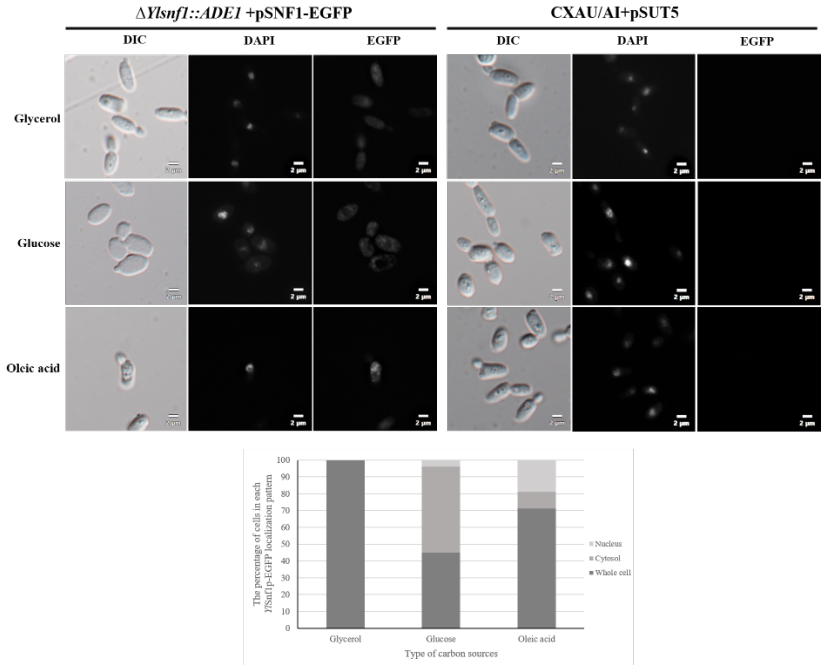

Figure 4. The subcellular localization of *Yl*Snf1p-EGFP. The yeast cells including Δ*Ylsnf1*::*ADE1*+pSNF1-EGFP and CXAU/AI+pSUT5 control were grown in glycerol for 18-19 hours and shifted into glucose, and oleic acid media for 1 hour. Images were obtained by fluorescence microscope IX73 using DIC and fluorescence modes. DAPI was used for nucleus staining (excitation at 358 nm and emission at 461 nm). Green fluorescence (excitation at 488 nm and emission at 509 nm) was used to detect the localization of *Yl*Snf1p-EGFP. The scale bars represent 2 μm. The percentage of cells in each *Yl*Snf1p-EGFP localization pattern were also calculated from different areas on the imaging slides (n = 100).

In *S. cerevisiae*, oleate induction is regulated by transcription factors, namely Oaf1p-Pip2p complex and Adr1p [20-24]. Adr1p was further known as one of the targets of *Sc*Snf1p [25]. For Oaf1p, it was also reported to be phosphorylated upon oleate induction but a protein kinase that phosphorylates it was still unclear [20]. Thus, protein phosphorylation of transcription factors affects the activation of genes associated with oleate induction. Unlike, *Y. lipolytica*, does not have such transcriptional control of fatty acid utilization [6]. Instead, *Y. lipolytica* possesses Por1p, an ortholog of FarA in *Aspergillus*

nidulans, acting as a positive regulator in beta oxidation pathway [6]. Additionally, Por1p was clearly positioned in the nucleus, regardless of carbon sources, according to our unpublished localization data. Moreover, we found that Por1p had a phosphorylation site for a protein kinase based on bioinformatic analysis. Therefore, Por1p might be a potential target for *Yl*Snf1p that relocated from the cytoplasm to the nucleus to activate genes involved in beta-oxidation. However, further studies are required to confirm that the phosphorylation of Por1p is a consequence of *Yl*Snf1p activity.

4. Conclusions

In this research, pSNF1-EGFP was successfully generated and introduced into Δ*Ylsnf1*::ADE1. The *Yl*Snf1p-EGFP appeared intact and functioned inside the yeast cells. In addition, the *Yl*Snf1p-EGFP could restore the function of fatty acid utilization in Δ*Ylsnf1*::ADE1. After the shift of the carbon sources from glycerol to glucose and oleic acid, *Yl*Snf1p-EGFP exhibited distinct subcellular relocalization patterns, largely in cytoplasm and nucleus, respectively. Altogether, our findings exhibited that the alternative carbon sources such as fatty acids affected the subcellular localization of *Yl*Snf1p, making it a promising target for controlling fatty acid utilization *Y. lipolytica.*

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Conflicts of Interest: "The authors declare no conflict of interest."

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