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MINI-REVIEW

Synthesis and production of unsaturated and polyunsaturated fatty acids in yeast: current state and perspectives

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Abstract Recently, many genes involved in the formation of unsaturated and polyunsaturated fatty acids (PUFAs) were isolated. In most cases, their activities were confirmed by expressing them in the well-studied model organism *Saccharomyces cerevisiae* because its fatty acid compositions are very simple and it does not contain PUFAs. Taking advantage of its genetic tractability and increasing wealth of accessible data, many groups are attempting to produce various useful fatty acids in the model yeasts, mainly in *S. cerevisiae*. This review describes typical such examples including a very recent study on the expression of a fatty acid hydroxylase gene in fission yeast *Schizosaccharomyces pombe*. Furthermore, the impact of the genetically engineered alteration of fatty acid composition on the stress tolerance is presented because unsaturated fatty acids have crucial roles in membrane fluidity and signaling processes. Lastly, recent attempts at increasing lipid content in *S. cerevisiae* are discussed.

Keywords *S. cerevisiae* · *S. pombe* · Polyunsaturated fatty acids · Fatty acid desaturase · Elongase · Fatty acid hydroxylase

Introduction

Unsaturated fatty acids are components of biological membranes and are essential for determining membrane structures and functions. They affect fluidity of phospholipid bilayer and regulate the mobility and function of embedded proteins (Sinensky 1974; Macartney et al. 1994; Vance and Vance 2002: Opekarova and Tanner 2003). They also serve as precursors for a number of biologically active molecules like eicosanoids. In mammals, eicosanoids like prostaglandins, leukotrienes, and thromboxanes mediate fever, inflammations, vasodilatation, blood pressure, clotting, pain, neurotransmission, and modulation of cholesterol metabolism (Funk 2001). Thus, unsaturated fatty acids especially polyunsaturated fatty acids (PUFAs) are required for the normal development and function of our body and are essential in maintaining human health. For instance, arachidonic acid (ARA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) are essential fatty acids found in brain tissues. They are also important dietary nutrients for neonatal babies owing to their involvement in the development of neural and retinal functions (Broun et al. 1999; Horrobin 2000; Mills et al. 2005; Napier and Sayanova 2005; Das 2006).

Since mammals including humans cannot synthesize linoleic acid (LA, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3), they are called essential fatty acids and many PUFAs including LA and ALA must be obtained from the diet. The findings that dietary supplementation of PUFAs, such as γ-linolenic acid (GLA, C18:3n-6), ARA, eicosapentaenoic acid (EPA, C20:5n-3), and DHA, significantly alleviates the symptoms of many chronic disease have attracted a great interest of general public and food manufacturers. But their natural sources are very limited. The principal sources for PUFAs (especially n-3 fatty acids) to date are fish oils. However, due to the increase of fish consumption and the expansion of marine pollution, fish hauls are decreasing. It is therefore highly desirable to produce PUFAs from alternative sources that are more economical, easier to handle, and sustainable through genetic engineering

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techniques. One option is to modify oil-seed crops to produce PUFAs through genetic engineering technique, and the other option is to produce them in well-studied model microorganisms such as *Saccharomyces cerevisiae*. Since *S. cerevisiae* has been serving as a model organism for the development of metabolic engineering strategies to produce certain metabolites (Ostergaard et al. 2000), the concept of obtaining PUFAs from *S. cerevisiae* in commercial and sustainable quantities is particularly attractive.

Therefore, this review focuses on the current typical studies on the production of various fatty acids in *S. cerevisiae*, together with one example in fission yeast *Schizosaccharomyces pombe*, another well-studied model yeast. Furthermore, the impact of the genetically engineered alteration of fatty acid composition on the stress response of *S. cerevisiae* is also presented, because unsaturated fatty acids have crucial roles in membrane biology and signaling processes, and future prospect of genetic engineering of fatty acid metabolism in *S. cerevisiae* is discussed.

Fatty acid composition in yeasts

The primary products of fatty acid biosynthesis in most organisms are 16- and 18-carbon fatty acids, and fatty acid desaturation is initiated by introducing a double bond at the Δ9 position of saturated fatty acids, palmitic (C16:0) and stearic (C18:0) acids. The relative ratio of chain lengths and the degree of unsaturation of these fatty acids vary widely depending on the microorganisms. *S. cerevisiae* primarily produces saturated and monounsaturated fatty acids of 16- and 18-carbon compounds, because it contains only one fatty acid desaturase, a Δ9-desaturase (*OLE1*), which is capable of producing monounsaturated palmitoleic (C16:1) and oleic (C18:1n-9) acids (Stukey et al. 1989).

Like *S. cerevisiae*, the fission yeast *S. pombe*, which is also well characterized and widely used in the field of basic research, is unable to synthesize LA (C18:2n-6), either (Ratledge and Evans 1989; Holic et al. 2012). In some organisms, however, oleic acid (C18:1n-9) is subsequently desaturated to LA by introducing the second double bond at the Δ12 position by a Δ12-fatty acid desaturase and then making ALA by a ω3-fatty acid desaturase (see Fig. 1). For instance, other budding yeasts such as *Saccharomyces kluyveri* and *Kluyveromyces lactis* can produce both the diunsaturated fatty acid LA (C18:2n-6) as well as the triunsaturated ALA (C18:3n-3) (Ratledge and Evans 1989; Kainou et al. 2006). In addition to *S. kluyveri* and *K. lactis*, *Candida albicans* and *Candida tropicalis* are able to produce up to ALA to a greater or lesser extent, but some other yeasts such as *Candida boidinii* and *Zygosaccharomyces rouxii* are able to produce only LA (Ratledge and Evans 1989). Among oleaginous yeasts, *Yarrowia lipolytica* (max lipid content 36 %) and *Trichosporon pullulans* (65 %) can produce around 1 % of ALA, but *Lipomyces starkeyi* (63 %), *Cryptococcus albidus* (65 %), and *Rhodotorula glutinis* (72 %) can produce only LA (Ratledge 1993). Fatty acid and lipid compositions in other yeasts are also summarized in the review by Ratledge and Evans (1989). For the fatty acid compositions in other lower eukaryotes such as fungi, algae, and protozoa, readers are referred to the review of Pereira et al. (2003) and the book of Ratledge and Wilkinson (1993).

Genetic manipulation of *S. cerevisiae* for the production of polyunsaturated fatty acids

PUFA synthesis in mammals proceeds predominantly by a Δ6-desaturation pathway, in which the first step is the Δ6-desaturation of dietary LA and ALA to yield GLA and stearidonic acid (STA, C18:4n-3), respectively. Then C20 fatty acids are synthesized by sequential desaturation and elongation (Broun et al. 1999; Napier et al. 1999; Huang et al. 2004; Ratledge 2004). Further fatty acid elongation and desaturation steps give rise to ARA (C20:4n-6) via dihomo-γ-linolenic acid (DGLA, C20:3n-6) in the n-6 pathway, and EPA and DHA via eicosatetraenoic acid (ETA, C20:4n-3) in the n-3 pathway (Fig. 1).

Desaturase enzymes are specific to the location, number, and stereochemistry of double bonds already present in fatty acids (Heinz 1993). Hence, various desaturases, together with elongases, which elongate carbon chain length of fatty acids, are required to introduce a series of desaturations and elongations into the fatty acyl to generate various PUFAs. Therefore, to make various fatty acids in *S. cerevisiae*, it is necessary to introduce appropriate desaturase and elongase genes.

Production of linoleic, α-linolenic, and γ-linolenic acids in *S. cerevisiae*

Animals in general are unable to produce LA (C18:2n-6) and ALA (C18:3:n-3) because of the lack of Δ12- and ω3-desaturases. Thus, these fatty acids are considered to be essential for us and must be obtained from the diet. In contrast, many plants and some fungi are able to synthesize these fatty acids. Hence, the genes encoding Δ12-desaturase (*FAD2*) and ω3-desaturase (*FAD3*) have mainly been reported from plants and fungi. For instance, Δ12-desaturases were isolated from *Arabidopsis* (Okuley et al. 1994), *Mortierella alpina* (Huang et al. 1999; Sakuradani et al. 1999), *Mucor rouxii* (Passorn et al. 1999), and *Aspergillus nidulans* (Calvo et al. 2001), and ω3-desaturases were isolated from *Arabidopsis* (Arondel et al. 1992; Yadav et al. 1993) and *Mortierella alpina* (Sakuradani et al. 2005; also see reviews by Tocher et al. 1998 and Pereira et al. 2003).

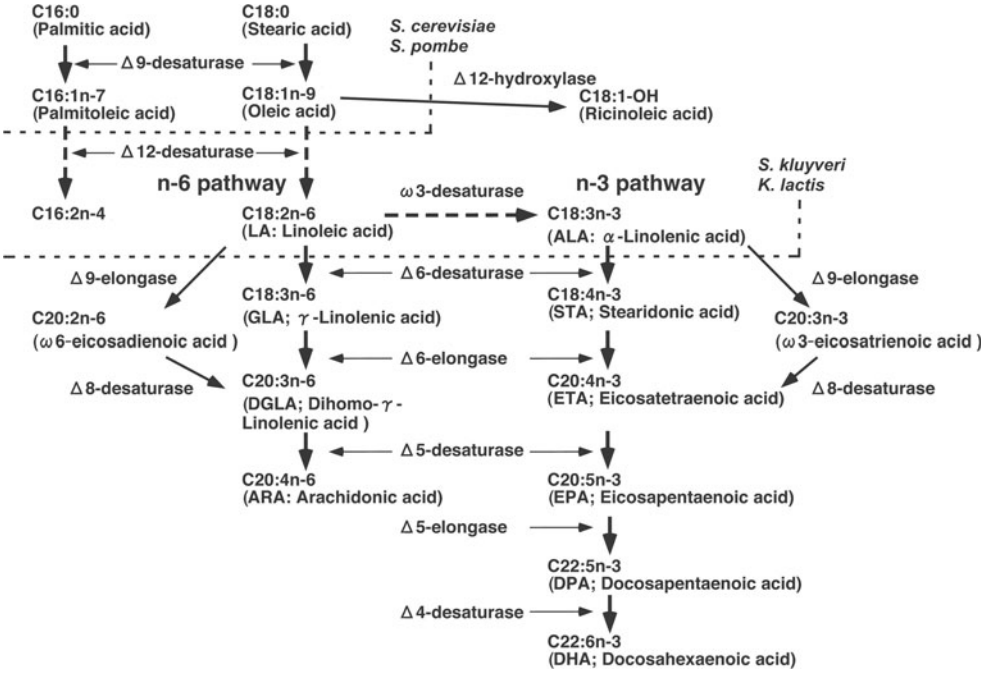

Fig. 1 Biosynthetic pathway for the production of long-chain PUFAs. Biosynthetic pathway for the production of long-chain PUFAs is indicated. Two steps indicated by *dotted arrows* (Δ12- and ω3-desaturases steps) do not exist in mammals and they need external supplementation of LA (C18:2) and ALA (C18:3n-3) (essential fatty acids). Ricinoleic acid is produced by introducing a hydroxyl group (−OH) to the 12th carbon of oleic acid by an oleate Δ12-hydroxylase (*FAH12*). *S. cerevisiae* and *S. pombe* are able to produce only up to oleic acid (C18:1n-9), but other yeasts such as *S. kluyveri* and *K. lactis* are able to produce up to ALA (C18:3n-3)

Reports on the isolation of Δ12- and ω3-desaturase genes from yeasts are fewer. First such desaturases reported were *FAD2* (Watanabe et al. 2004) and *FAD3* (Oura and Kajiwara 2004) from *S. kluyveri*. *K. lactis* can also produce LA and ALA (Ratledge and Evans 1989; Kainou et al. 2006). Based on the draft genome sequence of *K. lactis* (Dujon et al. 2004), two sequences (*KlFAD2* and *KlFAD3*), which encode proteins with the highest degree of homology to the *FAD2* and *FAD3* gene products of *S. kluyveri*, were identified. Co-expression of *KlFAD2* and *KlFAD3* in *S. cerevisiae* resulted in the endogenous production of both LA and ALA (Kainou et al. 2006). From fungus *M. alpina*, Δ6-desaturase cDNA was isolated in addition to Δ12-desaturase, and their co-expression in *S. cerevisiae* resulted in the endogenous production of GLA (Huang et al. 1999). The yield of GLA reached as high as 8 % to total fatty acids when the cells were grown at 15 °C for 2 days in 2 % galactose synthetic medium (Huang et al. 1999).

Production of long-chain PUFAs

To produce long-chain PUFAs in a heterologous host, genes encoding Δ4-, Δ5-, and Δ6-fatty acid desaturases are required. These genes have been cloned from a variety of organisms including higher plants, algae, mosses, fungi, nematodes, and mammals (reviewed by Pereira et al. 2003; Huang et al. 2004; Warude et al. 2006). In addition to desaturase genes, elongase genes are also required to produce fatty acids containing 20 carbons and more. *S. cerevisiae* also has three *ELO* genes, but they do not function to produce PUFAs described above, because Elo1p mainly elongates C14:0 to C16:0, and Elo2p and Elo3p are involved in the formation of very long-chain saturated fatty acid moiety of sphingolipids (Oh et al. 1997). The first elongase gene that can produce fatty acids containing 20 carbons was isolated from *M. alpina* cDNA library by directly measuring the elongation activity of GLA to DGLA in approximately 750 transformants of *S. cerevisiae* (Parker-Barnes et al. 2000). Subsequently, elongase genes were isolated from *Caenorhabditis elegans* (Beaudoin et al. 2000), moss *Physcomitrella patens* (Zank et al. 2002), rat (Inagaki et al. 2002), mouse (Leonard et al. 2002), and humans (Leonard et al. 2002; also see reviews by Leonard et al. 2004 and Warude et al. 2006).

To reconstitute PUFA synthesis pathways in *S. cerevisiae*, various combinations of multiple desaturase and elongase genes were attempted. Typical examples are shown below. Since Δ5- and Δ6-fatty acid desaturases can accept both n-3 and n-6 fatty acids as substrates as shown in Fig. 1, their transgenic *S. cerevisiae* strains produce either n-3 or n-6 fatty acids depending on the substrate fatty acid added in media. Beaudoin et al. (2000) produced ARA from exogenous LA and EPA from exogenous ALA in *S. cerevisiae* by using *C. elegans* elongase, *M. alpine* Δ5-desaturase, and *borage* Δ6-desaturase. By growing cells for 4 days at 25 °C in 2 % galactose synthetic medium with 0.5 mM of exogenous LA as a substrate, the composition of GLA, DGLA, and ARA to total fatty acids reached 6.8, 1.4, and 0.25 %, respectively. And with 0.5 mM of exogenous ALA as a substrate, 0.2 % of EPA was produced.

Domergue et al. (2002) produced ARA from exogenous LA and EPA from ALA in *S. cerevisiae* by using *Physcomitrella patens* Δ6-specific elongase, *Phaeodactylum tricornutum* Δ5- and Δ6-desaturases. The contents of DGLA and

ARA were 1.7 and 0.17 %, respectively, by growing cells for 4 days at 20 °C in 2 % galactose synthetic medium with 0.5 mM of exogenous LA. The content of EPA was 0.23 % when exogenous ALA was used as a substrate. Domergue et al. (2003) also produced ARA from exogenous LA by using algal Δ5- and Δ6-desaturases and moss Δ6-elongase and DGLA from exogenous LA by using human Δ6-desaturase and moss Δ6-elongase by growing cells for 2 days at 20 °C in 2 % galactose synthetic medium in the presence of 0.5 mM of the respective precursor fatty acids. Co-expressed of *M. alpina* elongase with *M. alpina* Δ5-desaturase cDNA in *S. cerevisiae* produced 0.5 % of ARA from GLA and 0.7 % of EPA from STA, respectively, by growing cells at 25 °C for 1 day in 2 % galactose synthetic medium in the presence of 0.025 mM of the respective precursors (Parker-Barnes et al. 2000).

A further long fatty acid, DHA, was also produced by introducing four genes into *S. cerevisiae*. Meyer et al. (2004) cloned alga *Ostreococcus tauri* elongase specific for the elongation of (Δ6-)C18-PUFAs, alga *Thalassiosira pseudonana* elongase specific for (Δ5-)C20-PUFAs, and fish *Oncorhynchus mykiss* bifunctional elongase (elongates both C18- and C20-PUFAs). Co-expression of Δ6-elongase (*T. pseudonana*) and Δ5-elongase (*O. tauri*) with the algal Δ5-desaturase (*Phaeodactylum tricornutum*) and Δ4-desaturase (*Euglena gracilis*), or three genes of the bifunctional elongase (*O. mykiss*) with the algal Δ5-desaturase (*Phaeodactylum tricornutum*) and Δ4-desaturase (*Euglena gracilis*), they observed around 0.5 % of DHA synthesis to total fatty acids by growing cells at 20 °C for 4 days in 2 % galactose synthetic medium in the presence of 0.25 to 0.5 mM STA as a precursor. In the two-gene co-expression of marine microalgae *Pavlova* elongase with *Isochrysis* Δ4-desaturase in *S. cerevisiae*, 3.1 % DPA and 3.8 % DHA were produced from EPA by growing cells at 20 °C for 2 days in 2 % galactose synthetic medium in the presence of 0.1 mM EPA (Pereira et al. 2004).

Li et al. (2011) isolated C18-Δ9-PUFAs-specific elongase from a DHA-rich microalga, *Isochrysis galbana* H29, and reconstituted in *S. cerevisiae* "Δ8 desaturation" pathway, an alternative pathway for the biosynthesis of C20-PUFAs in organisms that lack the Δ6-desaturase activity. Co-expression of this elongase with Δ8-desaturase from *Euglena gracilis* resulted in the production of DGLA (C20:3n-6) and ETA (C20:4n-3) from LA and ALA, respectively. The yields of DGLA and ETA were 7.2 and 2.0 % when the cells were grown in 2 % galactose synthetic medium at 22 °C for 2 days in the presence of 0.1 mM of LA and ALA precursors, respectively.

In all studies described in this section, substrate fatty acids were exogenously supplied in the media for the production of the desired fatty acids. In spite of the presence of a large excess of precursor fatty acids, their yields were generally low. One reason might be a difference of substrate specificity for desaturases and elongases. Elongation reaction adds two carbon units to the carboxyl end of the fatty acid chain and this reaction is initiated by the condensation of malonyl-CoA with a long-chain acyl-CoA (Leonard et al. 2004). In contrast to elongases that use fatty acid moiety of an acyl-CoA ester as substrates, fatty acid desaturases are classified into three types depending on their substrates: acyl-CoA, acyl-lipid, and acyl-acyl carrier protein (ACP) desaturases. The acyl-CoA desaturases are membrane-bound enzymes that desaturate fatty acids esterified to Co-enzyme A (CoA) and they are present in animals, yeasts, and fungi. The acyl-lipid desaturases are membrane-bound enzymes that introduce unsaturated bonds in lipid-bound fatty acids and they are found in plants, fungi, and cyanobacteria. The acyl-ACP desaturases desaturate fatty acids linked to an ACP, and they are found in plant plastids in a soluble form (Pereira et al. 2003; Uttaro 2006). Meyer et al. (2004) speculated that the low yield of DHA was largely attributed to the low activity of Δ5-desaturase because of the limited substrate availability. The Δ5-desaturase from *P. tricornutum* requires fatty acids acylated at the sn-2 position of phosphatidylcholine as substrates, whereas the elongase produces ETA, the substrate for Δ5-desaturase, as an acyl-CoA ester before being transferred to the various lipids, making the consecutive reaction of the elongase and the desaturase inefficient.

Construction of the complete pathway for the production of C20-PUFA from the endogenous oleic acid in *S. cerevisiae*

S. cerevisiae contains a Δ9-desaturase (*OLE1*) as the only one desaturase and cannot produce PUFAs. Thus, to construct a complete pathway to produce various PUFAs from the endogenous oleic acid, the first fatty acid desaturase to be introduced is a Δ12-desaturase gene that converts endogenous oleic acid to LA to produce n-6 fatty acids, and an additional ω3-desaturase gene to produce n-3 fatty acids.

To construct a complete pathway that allows DGLA biosynthesis without the need to supply exogenous fatty acids in the media, Yazawa et al. (2007a) introduced Δ12-desaturase from *K. lactis* (*KlFAD2*), rat Δ6-desaturase (*rFADS2*), and rat elongase (*rELO1*) genes in *S. cerevisiae*. DGLA is an encouraging target because it has unique biological activities (Stone et al. 1979; Horrobin and Huang 1987; Iversen et al. 1991, 1992; Rotondo et al. 1994; Williams et al. 1996; Vassilopoulos et al. 1997; Kahler and Du Plooy 1998; Dooper et al. 2003; Das 2006), but its natural sources are very limited. Media composition, cultivation temperature, and incubation time were examined to improve the yield of DGLA. Fatty acid content was increased by changing the medium from a standard synthetic dropout medium to a nitrogen limited minimal medium (NSD). Production of DGLA was higher in the cells grown

at 15 °C than those grown at 20 °C, and no DGLA production was observed in the cells grown at 30 °C. When the cells were grown in NSD for 7 days at 15 °C, the yield of DGLA reached 2.2 mg/mg of dry cell weight (DCW) and the composition of DGLA to total fatty acids was 2.7 % (Yazawa et al. 2007a), comparable to the values obtained in the systems of Beaudoin et al. (2000) and Domergue et al. (2002) as described above. This is the first case for *S. cerevisiae* of producing C20-PUFA without supplying the exogenous fatty acids. Construction of the complete metabolic pathway from the endogenous oleic acid of *S. cerevisiae* to the desired product would have a great advantage from the application point of view.

A similar entire biosynthetic pathway for ETA was demonstrated by using four genes. Tan et al. (2011) cloned a Δ6-desaturase cDNA (CoD6) and a Δ6-elongase cDNA (CoE6) from *Conidiobolus obscurus*, an entomopathogenic fungus able to infect aphids. Co-expression of CoD6 and CoE6 from *C. obscurus*, together with Δ12-desaturase (CpDes12) and ω3-desaturase (CpDesX) from *Claviceps purpurea* in *S. cerevisiae*, resulted in the endogenous production of the end product ETA, although its yield was very low (around 0.1 % to total fatty acids) when the cells were grown in 2 % galactose synthetic medium at 20 °C for 2 days.

Effect of cytochrome b5 on the activity of desaturases

In addition to desaturase and elongase genes that directly synthesize PUFAs, genes that facilitate their activities should also be considered. For instance, cytochrome b5 is required for the electron transport in desaturation reaction (Tamura et al. 1976; Certik and Shimizu 1999). While Ole1p and certain other desaturases contain their own cytochrome b5 domain (Mitchell and Martin 1995; Sperling et al. 1995), other desaturases, including Fad2p and Fad3p, do not (Mitchell and Martin 1997) and hence require an interacting cytochrome b5 (Mitchell and Martin 1995; Petrini et al. 2004).

DGLA production from endogenous oleic acid by using Δ12- and Δ6-desaturases and elongase demonstrated that its production was higher in the cells grown at 15 °C than those grown at 20 °C, and no DGLA production was observed in the cells grown at 30 °C (Yazawa et al. 2007a). The low temperature enhanced the activity of the Δ12-desaturase to produce LA from oleic acid. Since the double bond insertion into fatty acids is performed by a microsomal membrane-bound three-component enzyme system involving cytochrome b5, NADH-dependent cytochrome b5 reductase, and fatty acid desaturases, Yazawa et al. (2010) speculate that the increasing amount of cytochrome b5 would promote the complex formation between Δ12 fatty acid desaturase and cytochrome b5 and enhance the activity of fatty acid desaturase. Based on this idea, the effects of overexpression of *K. lactis* and *S. cerevisiae* cytochrome b5 (*CYB5*) genes on LA production by *KlFAD2* were examined in relation to incubation temperature. Without extra cytochrome b5, while LA synthesis was significant at 20 °C, it was marginal at 30 °C. Overexpression of *CYB5* at 20 °C did not affect the fatty acid synthesis much, but it significantly enhanced the synthesis of LA at 30 °C especially with *KlCYB5*. The higher activity of Δ12-desaturase with *KlCYB5* is presumably due to a better interaction between the desaturase and the cytochrome b5 of the same species, especially at 30 °C where unstable interaction was predicted between *K. lactis* desaturase and Cyb5p of *S. cerevisiae* (Yazawa et al. 2010). The enhancement of the desaturase activity at 30 °C is important to reduce the production cost in industrial application.

Production of ricinoleic acid in *S. cerevisiae*

In addition to PUFAs, which have biological functions, biological syntheses of fatty acid derivatives that can be petrochemical replacements are also drawing attention. Ricinoleic acid (12-hydroxy-octadeca-*cis*-9-enoic acid: C18:1-OH) is an important natural raw material with great values as a petrochemical replacement in a variety of industrial processes. Its derivatives have a considerable range of applications, especially in the production of lubricants, nylon, dyes, inks, soaps, adhesives, and plasticizers. The major source of ricinoleic acid is the seeds of castor-oil plant (*Ricinus communis*), in which ricinoleic acid constitutes approximately 90 % of the total fatty acids of the seed oil. However, castor bean is not an ideal source crop, because its cultivation is limited to tropical and sub-tropical regions, and their seeds must be harvested by hand. Furthermore, a highly poisonous protein (ricin) and strongly allergenic 2S albumins contained in castor seeds cause health problems for workers involved in planting, harvesting, and processing. Because of these problems, alternative sources that are more economical, easier to handle, and sustainable are highly desirable.

Ricinoleic acid biosynthesis is catalyzed by an oleate Δ12-hydroxylase (*FAH12*), which adds a hydroxyl group (−OH) to the 12th carbon of oleic acid moieties (Galliard and Stumpf 1966). The first hydroxylase gene was cloned from *R. communis* using EST sequencing/similarity searches (van de Loo et al. 1995), and soon afterward, a similar hydroxylase from *Lesquerella fendleri* involved in the first step of lesquerolic acid (14-hydroxyeicos-*cis*-11-enoic acid or 14-OH-C20:1-11c) biosynthesis was isolated by degenerate RT-PCR (Broun et al. 1998). Recently, by using degenerate RT-PCR targeted to conserved regions of fungal oleate desaturases, Meesapyodsuk and Qiu (2008) isolated the first non-plant Δ12-oleate hydroxylase from the sclerotium tissue of *C. purpurea* (*CpFAH12*), which has a high

sequence similarity to fungal desaturases, but a low similarity to plant fatty acid hydroxylases.

So far, several groups have attempted to produce ricinoleic acid in transgenic plants such as tobacco and *Arabidopsis* (van de Loo et al. 1995; Broun and Somerville 1997; Smith et al. 2003; Lu et al. 2006; Kumar et al. 2006; Meesapyodsuk and Qiu 2008). *S. cerevisiae* was also used to characterize the function of putative hydroxylase genes (Smith et al. 2003; Meesapyodsuk and Qiu 2008). Smith et al. (2003) expressed *Ricinus* and *Lesquerella* hydroxylase genes (*RcFAH12* and *LfFAH12*) under the control of *GAL10* promoter, but their activity was very low and only 0.8 and 1.6 % of ricinoleic acid to total fatty acids were produced, respectively, by growing cells at 30 °C for 5 days on 2 % galactose. Meanwhile, Meesapyodsuk and Qiu (2008) expressed *CpFAH12* under the control of *GAL1* promoter. They managed to boost ricinoleic acid production by adding 0.25 mM oleic acid, a substrate for hydroxylase, to media, and produced 19 % of ricinoleic acid to total fatty acids by growing cells at 20 °C for 2 days in the synthetic medium containing 2 % galactose.

Production of ricinoleic acid in *S. pombe*

In addition to *S. cerevisiae*, fission yeast *S. pombe* is also widely used as a model organism. Like *S. cerevisiae*, Δ9 fatty acid desaturase (*OLE1*) is the only desaturase in *S. pombe* (Wood et al. 2002); thus, it does not contain unsaturated fatty acids with two or more double bonds. However, the fatty acid compositions differ greatly between them. Palmitoleic acid (C16:1) is the major fatty acid in *S. cerevisiae*, but oleic acid (C18:1) is the major one (around 75 % of total fatty acid) in *S. pombe* (Ratledge and Evans 1989; Holic et al. 2012).

Since Fah12p converts oleic acid to ricinoleic acid, it was considered that *S. pombe*, in which around 75 % of total fatty acid is oleic acid, would accordingly be an ideal microorganism for high production of ricinoleic acid. Thus, Holic et al. (2012) introduced *C. purpurea* oleate Δ12-hydroxylase gene (*CpFAH12*) to *S. pombe*, putting it under the control of inducible *nmt1* promoter. Unfortunately, at the normal growth temperature 30 °C, *S. pombe* cells harboring *CpFAH12* grew poorly when the *CpFAH12* gene expression was induced, perhaps implicating ricinoleic acid as toxic in *S. pombe*. However, thermo-instability of Fah12p in *S. pombe* was discovered, and by contrast with 30 °C and lower temperatures, almost no growth inhibition that correlated with a very low level production of ricinoleic acid was observed at 37 °C. Accordingly, by taking advantage of the thermolabile characteristic of the hydroxylase, various optimization steps led to a regime with preliminary growth at 37 °C followed by 5-day incubation at 20 °C, and the level of ricinoleic acid reached 137.4 μg/ml of culture, that corresponded to 52.6 % of total fatty acids (Holic et al. 2012).

To demonstrate the advantage of *S. pombe*, Holic et al. (2012) also expressed *CpFAH12* in *S. cerevisiae*. The *CpFAH12* gene was expressed under the control of a strong glycolytic promoter *TDH3* (glyceraldehyde-3-phosphate dehydrogenase, isozyme 3), and 9.9 and 7.2 % of ricinoleic acid were obtained by growing cells at 20 and 30 °C for 5 days, respectively. These values were comparable to that of Meesapyodsuk and Qiu (2008) described in the previous section, because exogenous oleic acid was not provided in the media. Obviously, we cannot simply compare the values of *S. pombe* to those of *S. cerevisiae*, because many factors such as promoters and media were different, but these results clearly showed the advantage of *S. pombe*, in which more than 50 % of ricinoleic acid was produced in the absence of oleic acid in the media.

The higher hydroxylase activity of CpFah12p in *S. pombe* compared to *S. cerevisiae* makes this system a good candidate for use in metabolic engineering of ricinoleic acid, which has a specialized industrial use, in yeast. This is the first case of using *S. pombe* for the production of a useful fatty acid.

Effect of fatty acid compositions on stress response of *S. cerevisiae*

Unsaturated fatty acids have crucial roles in membrane fluidity and signaling processes, and fatty acid desaturases are functioning in most living organisms to help regulate the fluidity of membrane lipids. Thus, in addition to the production of useful fatty acids, manipulation of fatty acid composition and production of PUFAs in *S. cerevisiae* strains have a possibility to improve their stress response. Two such examples are presented.

Ethanol tolerance

S. cerevisiae is well known to produce a high concentration of ethanol and is commonly used for brewing and fuel ethanol production. Since ethanol is toxic to cells, the ethanol tolerance of *S. cerevisiae*, which is closely related to ethanol productivity (Jones 1989), is one of its most desirable characteristics. Despite many physiological, genetical, and the recent genome-wide gene expression studies (Attfield 1997; Casey and Ingledew 1986; Lloyd et al. 1993; Alexandre et al. 1994, 2001), the mechanism of ethanol tolerance still remains unclear. It has been known for a long time that the fatty acid composition of the membrane plays an important role in ethanol tolerance, although the correlation between ethanol tolerance and the increased degree of unsaturated fatty acyl residues in the membrane phospholipids is not yet completely understood (Thomas et al. 1978; Beavan et al. 1982; reviewed by Casey and Ingledew 1986; Mishra and Prasad 1989; Jones 1989). Some

researchers reported that the increase in any unsaturated fatty acids is sufficient for the acquisition of ethanol tolerance (Thomas et al. 1978; Beavan et al. 1982; reviewed by Casey and Ingledew 1986; Mishra and Prasad 1989). Rather than the first reports suggesting the importance of unsaturation but not of its specificity, the most recent reports point to oleic acid (C18:ln-9) but not palmitoleic acid (C16:1n-7) as important for ethanol tolerance (You et al. 2003; reviewed by Ma and Liu 2010). Genome-wide screening of ethanol tolerant mutants also suggested that the increase in oleic acid content was the factor in ethanol tolerance as well as the enhancement of the cell wall integrity (Yazawa et al. 2007b), and it was confirmed that the content of oleic acid increased as the initial concentration of ethanol in media increased (Yazawa et al. 2011).

To clarify the importance of oleic acid, and to create an alcohol stress tolerant *S. cerevisiae* by artificially altering the fatty acid composition of the cells instead of searching and isolating mutant strains, Yazawa et al. (2011) have attempted to make the content of oleic acid predominant even under the normal growth condition by introducing rat elongase genes that elongate C_{16} fatty acids to C_{18}. Rat has two elongase genes (*rELO1* and *rELO2*): *rELO1* catalyzes the elongation of mono- and polyunsaturated fatty acids of C_{16}-C_{20} size, while *rELO2* mainly catalyzes elongation of C16:0 to C18:0 (Inagaki et al. 2002). Introduction of *rELO1* produced vaccenic acid (C18:1n-7) instead of oleic acid (C18:1n-9) and did not affect ethanol tolerance. On the other hand, *rELO2* drastically increased oleic acid content without changing the unsaturation index and did contribute to the increase of ethanol tolerance, emphasizing the specific role of oleic acid. Furthermore, the transformant of *rELO2* also conferred tolerance to *n*-butanol, *n*-propanol, and 2-propanol.

Their result that oleic acid is a very efficacious unsaturated fatty acid in overcoming the toxic effects of ethanol agrees well with the observation by You et al. (2003) that showed introduction of extra desaturases increased the amount of oleic acid and enhanced the ethanol tolerance. The unique point of this approach was to have employed elongase genes instead of desaturase genes and demonstrated that the *rEOL2* expression was more effective for enhancing oleic acid accumulation: overexpression of *OLE1* from its own promoter and glycolytic *TDH3* promoter increased the content of oleic acid to 35.0 and 37.6 %, respectively, whereas the expression of *rELO2* from glycolytic *ADH1* promoter increased that to 44.0 %.

As for PUFA production, it has been reported as effective in conferring ethanol tolerance (Kajiwara et al. 1996), or not effective (Kim et al. 2011). The former group reported that the expression of *A. thaliana* Δ12-desaturase gene (*FAD2*) gave the strain a greater resistance to ethanol. In contrast, the latter group showed increase in LA and ALA by the overexpressed Δ12- and ω3-desarurases from *C. albicans* (*CaFAD2* and *CaFAD3*) did not enhance ethanol tolerance. Differences in growth conditions are doubtless important, as observed for *OLE1* effects on low temperature fermentations (Kajiwara et al. 2000). They could show better growth and ethanol productivity only in the medium containing 15 % glucose at 10 °C, but these differences were not observed under other culture conditions.

Alkaline growth tolerance

In order to understand the biological significance of Δ12- and ω3-fatty acid desaturation in yeast; furthermore, growth phenotypes of the *S. cerevisiae* strain co-expressing *K. lactis* Δ12- and ω3-desaturase genes (*KlFAD2* and *KlFAD3*) were examined. The strain producing LA and ALA showed an alkaline pH tolerant phenotype and could still grow under the condition of pH 8.2, while a control strain with empty vectors could not grow (Yazawa et al. 2009).

To identify the responsible genes on a genome-wide transcription basis, the effect of PUFA production was examined by DNA microarrays. DNA microarray analyses showed that the transcription of a set of genes whose expressions are under the repression of Rim101p were down-regulated in this strain, suggesting that Rim101p, a transcriptional repressor which governs the ion tolerance, was activated. In line with this activation, the strain also showed elevated resistance to Li+ and Na+ ions, and to zymolyase, a yeast lytic enzyme preparation containing mainly beta-1,3-glucanase, indicating that the cell wall integrity was also strengthened in this strain. These findings demonstrated a novel influence of PUFA production on transcriptional control that is likely to play an important role in the early stage of alkaline stress response (Yazawa et al. 2009). *K. lactis* strains were more tolerant to alkaline pH than *S. cerevisiae*, and since the alkaline tolerant phenotype of *K. lactis* has been transferred to *S. cerevisiae* by expressing its *FAD* genes in *S. cerevisiae*, the content of unsaturated fatty acids might be one of the factors involved in the alkali tolerance of yeast.

Rodríguez-Vargas et al. (2007) reported that the expression of sunflower Δ12-desaturase gene in *S. cerevisiae* increased its tolerance to freezing as well as NaCl accompanying with the increase in LA content and the unsaturation index. However, the molecular mechanism of the influence of fatty acid composition on the stress response is not well understood. If we could control the fatty acid composition as we wished, engineering of fatty acid composition might have a potential to become one of the tools to create *S. cerevisiae* strains with various stress tolerance.

Future perspectives

In recent years, more and more genes involved in the production of various fatty acids have been discovered. Taking

advantage of well-established host–vector systems of *S. cerevisiae*, many groups are attempting to produce various fatty acids in it. At the current moment, however, the disadvantage of the model yeasts is that their fatty acid contents are not sufficiently high to apply transgenic *S. cerevisiae* strains to industrial applications. *S. cerevisiae* does not accumulate storage lipids and usually only 5 to 7 % of lipids are produced per DCW, whereas some oleaginous yeasts and fungi have 30 to 50 % lipids. In order to produce useful fatty acids in sustainable quantities in transgenic *S. cerevisiae*, it is necessary to increase their lipid content per cell.

Attempts to increase lipid content in *S. cerevisiae*

The major storage lipids in yeasts are triacylglycerols (TAGs) and the limiting step in their biosynthesis is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) and phospholipid:diacyglycerol acyltransferase enzymes (reviewed by Yen et al. 2008). TAG metabolism is well characterized in *S. cerevisiae* (Sorger and Daum 2003; Czabany et al. 2007), and overexpression of DGAT genes has been reported to increase the lipid content in plants and yeasts (Hobbs et al. 1999; Bouvier-Nave et al. 2000; Jako et al. 2001).

Kamisaka et al. (2006) found that the disruption of *SNF2*, a gene encoding a transcription factor forming part of the SWI/SNF chromatin-remodeling complex, increased the lipid content in *S. cerevisiae*. Overexpression of *DGA1* (diacylglycerol acyltransferase) and *LEU2* genes in the *snf2* disruptant has achieved 29 % of total lipid content to DCW, high enough to use this strain as a model of oleaginous yeast (Kamisaka et al. 2007). When the rat Δ6-desaturase was expressed in this oleaginous *S. cerevisiae*, 14.6 μg of STA/mg of DCW was produced mainly in TAG by growing cells at 30 °C for 7 days in the presence of 0.7 g/l of ALA, whereas only 1.6 μg of STA/mg of DCW was produced in the control *SNF2*$^{+}$ strain (Kimura et al. 2009).

Key enzymes in oleaginous yeasts: malic enzyme and ATP: citrate lyase

Oil accumulation is only found in some yeasts, fungi, and algae. But since fatty acid biosynthetic pathways in most of such oleaginous microorganisms are the same as that in non-oleaginous yeast such as *S. cerevisiae*, their ability to accumulate large amount of oil must lie outside the main pathway of fatty acid biosynthesis. Studies in oleaginous yeasts and fungi have revealed that ATP:citrate lyase (ACL) and malic enzyme, which supply acetyl-CoA and NADPH, respectively, to the main pathway of fatty acid biosynthesis, are key enzymes that contribute to fatty acid synthesis and accumulation (Ratledge and Wynn 2002; Ratledge 2004; Zhang et al. 2007; Tang et al. 2009). In fact, overexpression of malic enzyme in the presence of malate in the culture medium resulted in a fourfold increase in intracellular lipids in *E. coli*, mimicking the lipid accumulation mechanism of oleaginous microorganisms (Meng et al. 2011). Thus, understanding of the underlying biochemistry and genetics of lipid accumulation in oleaginous microorganisms should lead to the manipulation of *S. cerevisiae* to increase its lipid content and to increase the content of specific PUFAs within the lipid.

Conclusion

Due to its genetic tractability and increasing wealth of accessible data, *S. cerevisiae* is an excellent model system for the study of the genetics, biochemistry, and cell biology of eukaryotic lipid metabolism. However, since the lipid content in *S. cerevisiae* is not high enough to use it for industrial application at the moment, further improvement of its potential for the production of useful fatty acids by metabolic engineering is crucial for its future applications.

TAG biosynthesis enzymes and modulators of lipid particle biogenesis play important roles in lipid accumulation (Murphy 2001; Sorger and Daum 2003; Czabany et al. 2007), and recent studies on lipid particles identified that Fld1p, a functional homologue of human seipin, regulates the size of lipid particles (Fei et al. 2008) and an increased level of cellular phosphatidic acid facilitates the formation of supersized lipid particles (Fei et al. 2011). Application of new technologies including metabolomics and lipidomics on *S. cerevisiae* are providing new information about regulation of lipid metabolism (Gaspar et al. 2007; Ejsing et al. 2009; Tian et al. 2010). Furthermore, genome sequencing of oleaginous yeast *Y. lipolytica* was completed recently and various information concerning its lipid metabolism and sequence data become available (Beopoulos et al. 2009a, b). Combination of these increasing knowledge on the regulation of lipid and fatty acid metabolism in *S. cerevisiae* itself and the application of the knowledge from the oleaginous yeasts will enable us to manipulate the model yeast to use it not only in the basic biology field, but also in the field of biotechnology of lipids and fatty acids.

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