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ORIGINAL RESEARCH PAPER

One-step integration of multiple genes into the oleaginous yeast *Yarrowia lipolytica*

Shuliang Gao · Linna Han · Li Zhu · Mei Ge · Sheng Yang · Yu Jiang · Daijie Chen

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Abstract *Yarrowia lipolytica* is an unconventional yeast, and is generally recognized as safe (GRAS). It provides a versatile fermentation platform that is used commercially to produce many added-value products. Here we report a multiple fragment assembly method that allows one-step integration of an entire β-carotene biosynthesis pathway (~11 kb, consisting of four genes) via in vivo homologous recombination into the rDNA locus of the *Y. lipolytica* chromosome. The highest efficiency was 21 %, and the highest production of β-carotene was 2.2 ± 0.3 mg per g dry cell weight. The total procedure was completed in less than one week, as compared to a previously reported sequential gene integration method that required n weeks for n genes. This time-saving method will facilitate synthetic biology, metabolic engineering and functional genomics studies of *Y. lipolytica*.

Keywords β-Carotene · DNA assembler · Integration · *Y. lipolytica*

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S. Gao · D. Chen
College of Bioengineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China
e-mail: slgao@cibt.ac.cn

L. Han
School of Pharmacology, Shandong University of Traditional Chinese Medicine, Jinan 250355, China

L. Zhu · M. Ge
Shanghai Laiyi Center for Biopharmaceutical R&D, 800 Dongchuan Road, Shanghai 200240, China

S. Yang (✉) · Y. Jiang
CAS Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China
e-mail: syang@sibs.ac.cn

S. Yang · Y. Jiang (✉)
Shanghai Research and Development Center of Industrial Biotechnology, 528 Ruiqing Road, Shanghai 201201, China
e-mail: yjiang01@sibs.ac.cn

S. Yang
Huzhou Research Center of Industrial Biotechnology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Huzhou 313000, China

S. Yang · D. Chen
Shanghai Collaborative Innovation Center for Biomanufacturing Technology, 130 Meilong Road, Shanghai 200237, China

D. Chen (✉)
Shanghai Institute of Pharmaceutical Industry, 1320 West Beijing Road, Shanghai 200040, China
e-mail: hccb001@163.com

Introduction

Yarrowia lipolytica is one of the most studied unconventional yeasts and is considered to be a generally recognized as safe (GRAS) organism, which has already been used for some biotechnological applications, including citric acid production and heterologous protein expression (Bankar et al. 2009; Beckerich et al.1998) and eicosapentaenoic acid production (Xue et al. 2013). *Y. lipolytica* can accumulate large amount of lipids either by *de novo* lipid synthesis or via the uptake of lipids and alkanes, and is considered to be a suitable host to produce valuable, lipid-derived compounds (Nicaud 2012). Among the oleaginous yeasts, *Y. lipolytica* is the only one for which genomic and efficient molecular genetic tools are currently available (Beopoulos et al. 2009). However, metabolic engineering of this strain is still time-consuming for sequential gene integrations (Celinska and Grajek 2013; Chuang et al. 2010; Xue et al. 2013). The assembly of large, recombinant DNAs encoding entire biochemical pathways represents a significant challenge. A DNA assembler, which allows the assembly of multiple fragments in a single step via in vivo homologous recombination, has been successfully applied in *Saccharomyces cerevisiae* (Gibson 2009; Shao et al. 2009), *Xanthophyllomyces dendrorhous* (Contreras et al. 2013) and *Kluyveromyces marxianus* (Heo et al. 2013). However, in vivo homologous recombination has not been used to assemble multiple-gene biochemical pathways in a one-step fashion in a chromosome of *Y. lipolytica*.

As a proof of concept, we report, for the first time, the use of a DNA assembler method to rapidly assemble β-carotene biosynthesis pathways, with sizes of approx. 11 kb, as well as their integration into a chromosome in *Y. lipolytica.*

Materials and methods

Strains, plasmids, reagents and medium

Strains and plasmids used in this study are listed in Supplementary Table 1. *Yarrowia lipolytica* ATCC 201249 was cultivated on yeast/peptone/dextrose (YPD) medium (10 g yeast extract/l, 20 g tryptone/l, and 20 g glucose/l). Synthetic complete minus uracil (SC-ura) medium (20 g glucose/l, 1.7 g yeast nitrogen base without amino acids/l, 5 g $(NH_4)_2SO_4$/l, supplemented with 50 mg leucine and 50 mg lysine/l) was used to select integrants containing the assembled biochemical pathways. For plates, agar (2 %) was added. Growth was at 30 °C.

DNA manipulation

One set of DNAs, comprising the *carB* and *carRP* genes from *Mucor circinelloides* (Velayos et al. 2000a,b), and the *GGS1* gene (Matthäus et al. 2013) and the selective marker *ura3* (accession number: AJ306421.1) from the *Y. lipolytica* NRRL Y-1095 genome, was synthesized *de novo* (Crolla and Kennedy 2001; Wang et al. 2013). The other set comprised the *crtE, crtI* and *crtYB* genes from *X. dendrorhous* ATCC24202 (Verwaal et al. 2007), and also the selective marker *ura3*. *Y. lipolytica* promoters (*TEF1p, EXP1p, FBAp* and *GPDp)*, terminators (*xpr2t*, *mig1t* and *lip2t*) and homologous fragments for integration into target regions (*rDNAu* and *rDNAd*) were obtained individually from the genomic DNA of *Y. lipolytica* strain NRRL Y-1095, and the terminator *cyc1t* was obtained from *S. cerevisiae* strain BY4742 (Blazeck et al. 2011). All primers used in this study are listed in Supplementary Table 3. The fragments were assembled by overlap extension PCR (OE-PCR) into the individual gene expression cassettes, and DNA mixtures of the individual cassettes were prepared for transformation using a previously described procedure (Shao et al. 2009). The PCRs were performed in 50 µl, as described in the instructions for the KOD-Plus-Neo kit (Toyobo, Japan).

Yeast transformation and genomic DNA extraction

Transformation of *Y. lipolytica* was performed using the Zymogen Frozen-EZ yeast transformation kit II (Zymo Research Corporation) (Blazeck et al. 2011). Genomic DNA (gDNA) was extracted from *Y. lipolytica* using the Axygen genomic DNA purification kit (Axygen, China).

Genotype confirmation

PCR analysis, restriction digestion of amplified fragments and Real–time qPCR were used to do genotype confirmation.

Confirmation of the correct DNA assembly was assessed by PCR amplification of each gene cassette from the genomic DNAs. The PCR products obtained were separated in agarose gels and purified. They were then subjected to digestion by the corresponding restriction endonucleases, and the correct restriction digest pattern was assessed via electrophoresis on 1 % agarose gels.

Real-time qPCR amplification and analysis were performed using a MyiQ2 Two Colour Real-Time PCR Detection System with iQ5 optical system software version 2.1 (Bio-Rad, USA). The host strain ATCC201249 was selected as the negative control. The *GGS1*, promoter *EXP1* and *GPD1* driven heterologous *carB* and *carRP*, and the residual *ura3* fragment in the genome were chosen as reference copy (primers listed in Supplementary Table 3).

Functional analysis of the assembled pathways

Colonies of yeast harboring correctly assembled DNAs were picked into 4 ml YPD liquid medium in 20 ml test tubes and incubated overnight at 30 °C, with shaking at 230 rpm. These were used to inoculate 20 ml fresh media in 150 ml flasks. Cells were grown at 30 °C, with shaking at 230 rpm, for 96 h. Cells that were harvested and dried at 80 °C for 16 h were used to calculate dry cell weight (DCW). Cells were harvested by centrifugation at 4,000 × *g* for 4 min, resuspended in 0.5 ml dimethyl sulfoxide, and incubated at 55 °C for 10 min and then at 45 °C for 15 min, after which an equal volume of acetone was added. Samples were then centrifuged at 13,000 × *g* for 5 min and the supernatants containing β-carotene were transferred to a new tube. Cell extracts were analyzed by HPLC, as described by Shao et al. (2009). A sample (5 µl) was loaded onto an Agilent Diamonsil SB-C18 column and analyzed at 472 nm after elution with acetonitrile (A) and tetrahydrofuran (B). The elution program is described in Supplementary Table 3.

Results and discussion

Design of the *Y. lipolytica* DNA assembler

As proof of concept, we designed and constructed two sets of multiple-genes: β-carotene biosynthetic pathways, named as MC or XD, as shown in Fig. 1a and Supplementary Fig. 1. The accumulation of β-carotene resulting from the successful assembly and integration of the biosynthetic pathways turns transformants orange or red, which enables their identification. An rDNA sequence was previously used as the target locus for integration in the chromosome of *Y. lipolytica* (Nicaud 2012), and there are more than 200 rDNA repeat units in various chromosomes of *Y. lipolytica*, as well as δ sites in *S. cerevisiae*, that are available for integration (Heo et al. 2013; Verbeke et al. 2013). Compared to *S. cerevisiae*, in which the efficiency of homologous integration of exogenous DNA is very high, even with short (50 bp) homologous DNA flanking sequences, homologous recombination in *Y. lipolytica* only occurs at acceptable rates (80 %) with 0.5–1 kb 5′- and 3′-flanking regions of the target sequence (Verbeke et al. 2013). An rDNA site was selected as the target locus for integration into the chromosome, and ∼0.6 kb 5′- and 3′-flanking regions were used as homologous flanking sequences. The two sets of β-carotene biosynthetic pathways (∼11 kb) were divided into four cassettes, namely *rDNAu-TEF1p-GGS1-xpr2t, EXP1p-carB-mig1t, GPDp-carRP-lip2t,* and *URA3-rDNAd*, MC pathway or *rDNAu-TEF1p-crtE-xpr2t, GPDp-crtI-lip2t, FBAp-crtYB-lip2t,* and *URA3-rDNAd*, XD pathway, with overlaps between two successive cassettes of ∼65 bp in MC or ∼40 bp in XD.

Confirmation of the assembled β-carotene biosynthetic pathways

Prepared DNA mixtures were transformed into *Y. lipolytica* strain ATCC 201249. Around 92 colonies or 240 colonies appeared after 60 h on SC-ura medium for the MC or XD pathways, respectively. One-third of the MC and one-sixth of the XD transformants were orange/red (Table 1). Ten red colonies and 10 white colonies were randomly picked for amplification using four set of primers outside or inside the assembled fragment using junction PCRs as indicated in Fig. 1a and Supplementary Table 3, and the resulting 4 bands have overlaps of the assembling overlap region. the correct assemblies exhibited all four expected bands obtained following PCR (Fig. 1c and Supplementary Fig. 1c), and these four fragments were further confirmed by *Pst*I/*Xba*I, *Hin*dIII/*Nde*I, *Hin*dIII/*Sac*I, *and Sma*I/*Sal*I/*Pst*I digestion, as indicated in Fig. 1d. Of the MC or XD transformants, 60 or 70 %, respectively, were correctly assembled, while none of the white

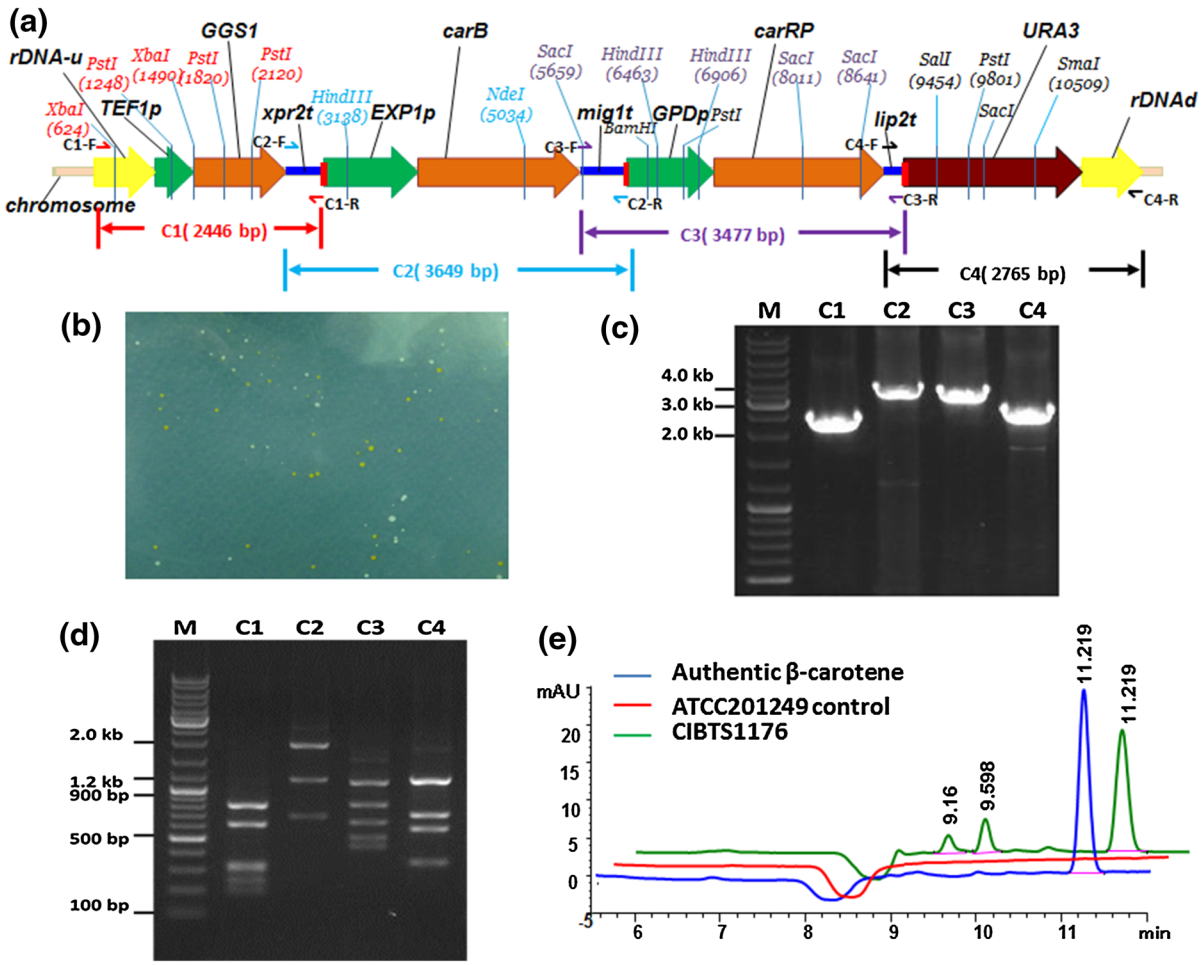


Fig. 1 Construction of a heterologous β-carotene biosynthetic pathway from *Mucor circinelloides* in *Y. lipolytica*, namely MC **a** The assembled β-carotene biosynthesis pathway with its corresponding verification primers **b** transformants on the selection plate **c** PCR analysis of the assembled, chromosomal β-carotene biosynthesis pathway using primers C1-F/C1-R, C2-F/C2-R, C3-F/C3-R, and C4-F/C4-Ralso demonstrated in Table S3 **d** Physical characterization of PCR analysis through restriction digestion. C1 was digested with *Pst*I and *Xba*I; the correct clones should exhibit six bands, with sizes of 195, 242, 300, 330, 624 and 767 bp. C2 was digested with *Hin*dIII/*Nde*I; the correct clones should exhibit three bands, with sizes of 660, 1093 and 1896 bp. C3 was digested with *Hin*dIII/*Sac*I; the correct clones should exhibit five bands, with sizes of 443, 500, 630, 837 and 1105 bp. C4 was digested with *Sma*I/*Sal*I/*Pst*I; the correct clones should exhibit four bands, with sizes of 347, 551, 708 and 1159 bp **e** HPLC analysis of the cell extracts from CIBTS1176 carrying the combined pathway. The host strain ATCC201249 was used as a negative control

colonies were correct (Table 1). The total efficiency of one-step assembly of these four-gene pathways was 20.9 % for MC and 11.7 % for XD (Table 1). Higher efficiency can be further improved by extending the length of the overlap region between two cassettes as previously reported in *S. cerevisiae* (Shao et al. 2009), or deletion of *KU70* in *Y. lipolytica* host (Verbeke et al. 2013). However, results of qPCR (supplementary information) indicate that CIBTS1176 assembled gene from MC which showed the deepest color underwent unexpected additional integrations of partial cassettes, including *GGS1*, *carRP*, and *ura3* into the genome (supplementary information). This was caused by the high rate of non-homologous end-joining (NHEJ) in *Y. lipolitica* (Kretzschmar et al. 2013).

From the HPLC results, a peak appeared at 11.2 min from the correctly assembled strains, consistent with the elution time of authentic β-carotene, whereas no such peak was observed for the cell extracts from the wild-type strain ATCC 201209 (Supplementary Figs. 1e, d).

Table 1 Probability of obtaining the correct colony after integration of multiple genes into *Y. lipolytica* using the DNA assembler

	DNA (μg) for transformation	Total colonies	Orange colonies	Genomic PCR				Cumulative probability (%)
				Orange colonies	Correct	White colonies	Correct	
MC	1.2*	92	32	10	6	10	0	20.9
XD	1.2*	240	40	10	7	10	0	11.7

%: The probability of obtaining a colony that produces β-carotene

* 300 ng DNA per cassette used for transformation

Among all the engineered strains, the highest producing strain, CIBTS1176, yielded 2.22 ± 0.34 mg of β-carotene per g DCW of MC, while all the XD transformants produced less than 1 mg of β-carotene/g DCW. As an example, strain CIBTS1187, shown in Supplementary Fig. 1d, produced 0.84 ± 0.05 mg β-carotene/g DCW. Additionally, to our knowledge, this was the first report of β-carotene production in engineered strains of *Y. lipolytica*, and the production can be further optimized with additional metabolic engineering tools or our DNA assembler.

While sequential gene integration in *Y. lipolytica* requires n weeks for n genes, as described previously (Celinska and Grajek 2013; Matthäus et al. 2013), our finding enables the integration of multiple genes (four genes of ~11 kb) within one week. Furthermore, the method demonstrated in this study was also used to transform multiple genes into other *Y. lipolytica* strains, including ATCC 76861 and ATCC MYA2613 (data not shown). Thus, it seems that simultaneous gene integration is an efficient method for multiple-gene transformations in *Y. lipolytica*. Thus, this work contributes to the development of genetic tools that allow efficient genomic modifications in *Yarrowia*.

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Supporting information Supplementary Table 1—Strains and plasmids used in this work

Supplementary Table 2—Primers used in this work

Supplementary Table 3—Procedure of elution for HPLC analysis

Supplementary Figure 1—Construction of engineering β-carotene biosynthesis pathway from *X. dendrorhous* in *Y. lipolytica*, namely XD

Supplementary Figure 2—A histogram representing the copy number of each cassette in mutant strain CIBTS 1176 by realtime qPCR

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