

Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–8, 2015

www.elsevier.com/locate/jbiosc

Transcription activator-like effector nucleases mediated metabolic engineering for enhanced fatty acids production in *Saccharomyces cerevisiae*

Mustapha Aouida, Lixin Li, Ali Mahjoub, Sahar Alshareef, Zahir Ali, Agnieszka Piatek, and Magdy M. Mahfouz*

Laboratory for Genome Engineering, Division of Biological Sciences, and Center for Desert Agriculture, King Abdullah University of Science and Technology, Thuwal 23955-6900, Saudi Arabia

Received 24 December 2014; accepted 24 February 2015
Available online xxx

Targeted engineering of microbial genomes holds much promise for diverse biotechnological applications. Transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats/Cas9 systems are capable of efficiently editing microbial genomes, including that of *Saccharomyces cerevisiae*. Here, we demonstrate the use of TALENs to edit the genome of *S. cerevisiae* with the aim of inducing the overproduction of fatty acids. Heterodimeric TALENs were designed to simultaneously edit the *FAA1* and *FAA4* genes encoding acyl-CoA synthetases in *S. cerevisiae*. Functional yeast double knockouts generated using these TALENs over-produce large amounts of free fatty acids into the cell. This study demonstrates the use of TALENs for targeted engineering of yeast and demonstrates that this technology can be used to stimulate the enhanced production of free fatty acids, which are potential substrates for biofuel production. This proof-of-principle study extends the utility of TALENs as excellent genome editing tools and highlights their potential use for metabolic engineering of yeast and other organisms, such as microalgae and plants, for biofuel production.

© 2015, The Society for Biotechnology, Japan. All rights reserved.

[**Key words:** Genome engineering; Metabolic engineering; Synthetic biology; Transcription activator-like effector nucleases; Biofuel production; Free fatty acids]

Owing to their potential use as dependable and renewable energy sources, biofuels derived from microbial fatty acids (FAs) have attracted much attention in recent years. Metabolic engineering and synthetic biology approaches have been used to produce alcohol, alkanes, and biodiesel (1–6). *Saccharomyces cerevisiae* has been used widely as the host of choice for numerous metabolic engineering and bioengineering applications (7). Hence, in recent years, there has been increasing interest in developing *S. cerevisiae* strains that act as cell factories for FA-derived biofuel production. For example, *S. cerevisiae* has been engineered to express heterologous lipase 2, which converts triacylglycerol or FAs to FA ethyl esters for biodiesel production (8). Overexpression of the active diacylglycerol acyltransferase, Dga1p, transforms *S. cerevisiae* into an oleaginous yeast that produces high levels of FAs (9). *S. cerevisiae* has also been engineered to produce FA-derived biofuels and chemicals from simple sugars (10), and a strain that produces short-chain FAs has been developed (11). Furthermore, the disruption of citrate turnover and overexpression of a heterologous ATP-citrate lyase leads to the accumulation of intracellular long chain FAs (12). Manipulation of glycerol-utilizing genes and FA biosynthesis in *S. cerevisiae* strains that use glycerol as a sole carbon source leads to the production of the FA-derived biofuel, FAEE (13). Notably, overproduction of FAs by *S. cerevisiae* is achieved by disrupting the beta-oxidation pathway, eliminating acyl-CoA synthetases, overexpressing thioesterases, and up-regulating the initial step of the FA synthesis pathway (14).

Targeted engineering of microbial genomes is an effective method of generating strains capable of overproducing select and valuable chemicals, and key gene targets that can be harnessed for metabolic engineering and synthetic biology applications have been identified. Site-specific nucleases can be used to generate functional knockouts, edit gene sequences, and introduce and stack multiple genes. In addition, genome engineering platforms using homing endonucleases, zinc finger nucleases, and transcription activator-like effector nucleases (TALENs) have also been developed (15–19). However, the production of homing endonucleases and zinc finger nucleases that are specific for a user-defined sequence is a time-consuming, labor-intensive, expensive, and difficult process (20); therefore, TALENs have been used to engineer the genomes of diverse eukaryotic species (20–28). TALENs are chimeric nucleases that are generated by fusing a TAL effector protein, which serves as DNA binding module, to the catalytic domain of the FokI endonuclease, which cleaves the target DNA in a site-specific manner (24,27). TAL effectors possess distinct structural features, including an N-terminal secretion domain, a central DNA-binding domain composed of repeats of 33–35 amino acids, and an acidic activation domain that mediates transcriptional regulation (22,23,25). The DNA binding specificity of TAL effectors is determined by residues 12 and 13 of the central

* Corresponding author. Tel.: +966 544700010; fax: +966 128020103.
E-mail address: magdy.mahfouz@kaust.edu.sa (M.M. Mahfouz).

Abbreviations: CRISPR, *clustered regularly interspaced short palindromic repeats*; EGFP, enhanced green fluorescent protein; FA, fatty acid; HRMA, high-resolution melting analysis; T7EI, T7 endonuclease I; TALEN, transcription activator-like effector nucleases; RVD, repeat-variable di-residue.

1389-1723/$ – see front matter © 2015, The Society for Biotechnology, Japan. All rights reserved.
http://dx.doi.org/10.1016/j.jbiosc.2015.02.017

repeats; these residues differ between proteins and are referred to as the repeat-variable di-residue (RVD) (23,28). The DNA binding abilities of specific RVDs have been deciphered for TAL effectors of *Xanthomonas* and *Ralstonia* sp. (29,30). The RVD sequences HD and ND bind specifically to C nucleotides; NH and NK bind specifically to G nucleotides; NI and NG bind specifically to A and T nucleotides, respectively; NN and HN bind specifically to A and G nucleotides; and NS binds to any nucleotide (29–32). The degeneracy of binding of RVD sequences can be utilized to design TAL effectors capable of binding to more than one genomic target, thereby expanding their overall utility and applications in genome engineering (21). Altering the sequence, number, and order of RVDs enables the production of TAL effectors and TALEN synthetic proteins with user-defined sequence specificity. In fact, several repeat assembly approaches have been developed, and the production of TALENs that bind and modify user-defined target sequences is an efficient and reproducible process (19,24).

The *S. cerevisiae* acyl-CoA synthetases Faa1 and Faa4 are involved in the recycling of FAs, and mutants that are deficient in these enzymes exhibit a transient FA secretion phenotype and significant accumulation of FAs in the culture medium (33). Here, to generate *S. cerevisiae* strains that overproduce FAs, we designed and produced synthetic heterodimeric TALENs for simultaneous targeted engineering of the *FAA1* and *FAA4* genes. We present a comprehensive workflow and establish the proof-of-concept that TALENs can be used as tools to generate *S. cerevisiae faa1Δfaa4Δ* double knockout mutants that displayed an increased FA accumulation in the cell. Overall, the results presented here suggest that TALENs are powerful tools for metabolic engineering and synthetic biology applications in yeast and other diverse eukaryotic species.

MATERIALS AND METHODS

Strains and growth media The BY4741 (*MATa his3Δ leu2Δ met15Δ ura3Δ*) *S. cerevisiae* strain was kindly provided by Dr. Ramotar (University of Montreal). The yeast cells were grown at 30°C in either YPD (1% yeast extract, 2% peptone, and 2% dextrose) or selective synthetic complete medium (0.67% yeast nitrogen base without amino acids, 2% dextrose, pH 5.7) supplemented with the appropriate dropout mix (Clontech, Mountain View, CA, USA) (34,35). As a control for gene expression under the *GAL* promoter, dextrose was substituted by raffinose and/or galactose at a final concentration of 2%. Yeast cells were transformed using the lithium acetate method (36).

Construction of the dTALENs heterodimer constructs Heterodimeric TALENs were assembled as described previously (37). Briefly, fragments containing repeat units were digested using BsmAI and simultaneously ligated into the dHax3 backbone to generate dTALENs with a particular sequence of RVDs (Fig. 3).

Yeast gateway cloning To enable the expression of the dTALEN heterodimers in yeast, LR reactions were performed using the pENTR211/dTALENS entry clones and the Gateway-compatible pAG423-GAL, pAG424-GAL, pAG425-GAL, and pAG426-GAL yeast expression vectors (all from Addgene) to generate pAG423-GAL-TALEN-FAA1-F, pAG424-GAL-TALEN-FAA1-R, pAG425-GAL-TALEN-FAA4-F, and pAG426-GAL-TALEN-FAA4-R, respectively (Fig. 2). Alternatively, to generate EGFP-tagged TALENs, LR reactions were performed using the pENTR211/dTALENs entry clones and the Gateway-compatible pAG523-EGFP-GAL, pAG524-EGFP-GAL, pAG425-EGFP-GAL, and pAG426-EGFP-GAL yeast expression vectors to generate pAG423-GAL-TALEN-FAA1-F-EGFP, pAG424-GAL-TALEN-FAA1-R-EGFP, pAG425-GAL-TALEN-FAA4-F-EGFP, and pAG426-GAL-TALEN-FAA4-R-EGFP, respectively. After transformation of *S. cerevisiae* with the expression plasmids, positive colonies were selected and the recombinant plasmids were purified and verified by restriction digestion with HindIII. The digestion produced six fragments of 1016, 870, 648, 408, 334, and 198 bp. Sanger sequencing using the Seqconf.F and Rep.Seqconf.R primers (Table 1) was also used to confirm the positive clones.

RT-PCR Overnight cultures were grown for 16 h in selective minimal medium supplemented with 2% galactose. Total RNA was then prepared from 5 ml of culture using the RiboPure-Yeast extraction kit (Ambion, Grand Island, NY, USA). M-MLV reverse transcriptase (Invitrogen, Grand Island, NY, USA) was used to synthesize cDNA from 2 μg of RNA. The PCR primers used to verify the expression level of TALEN-FAA1-F are listed in Table 1 and generated an amplicon of 793 bp. As a control, primers were designed to amplify a 375 bp fragment of the mRNA encoding actin (*ACT1*) (Table 1). The PCR program was as follows: 2 min at 95°C, followed by 25 cycles of 1 min at 94°C (denaturation), 2 min at 58°C (TALEN-FAA1-F) or 54°C (*ACT1*) (annealing), and 4 min at 72°C (extension), with a final extension for 7 min at 72°C. The PCR products were analyzed on a 1% agarose gel.

Confocal and electronic microscopy The cells were analyzed by confocal and electron microscopy as described previously (21).

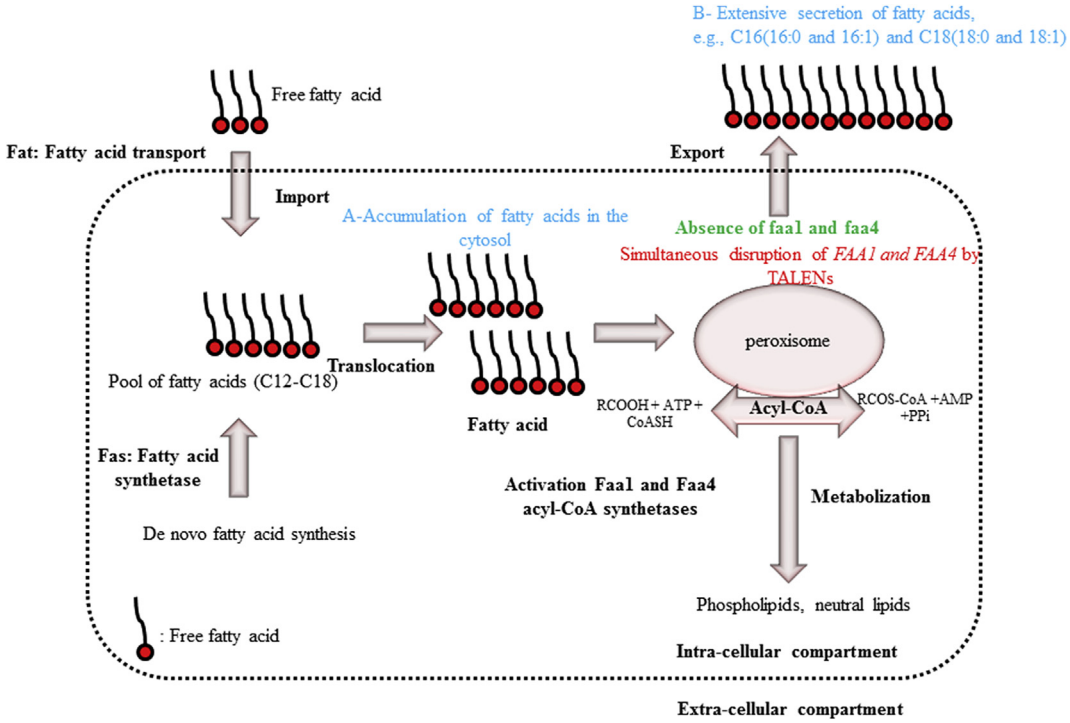


FIG. 1. Overview of fatty acid transport, synthesis, and activation in *Saccharomyces cerevisiae*. Engineered pathways for the production of fatty acids by simultaneous alteration of the *FAA1* and *FAA4* genes using specific TALENs. (A) Fatty acids accumulate in the cytosol at the early stage of growth. (B) Large amounts of fatty acids are secreted into the extracellular compartment at the late stage of growth.

Please cite this article in press as: Aouida, M., et al., Transcription activator-like effector nucleases mediated metabolic engineering for enhanced fatty acids production in *Saccharomyces cerevisiae*, J. Biosci. Bioeng., (2015), http://dx.doi.org/10.1016/j.jbiosc.2015.02.017

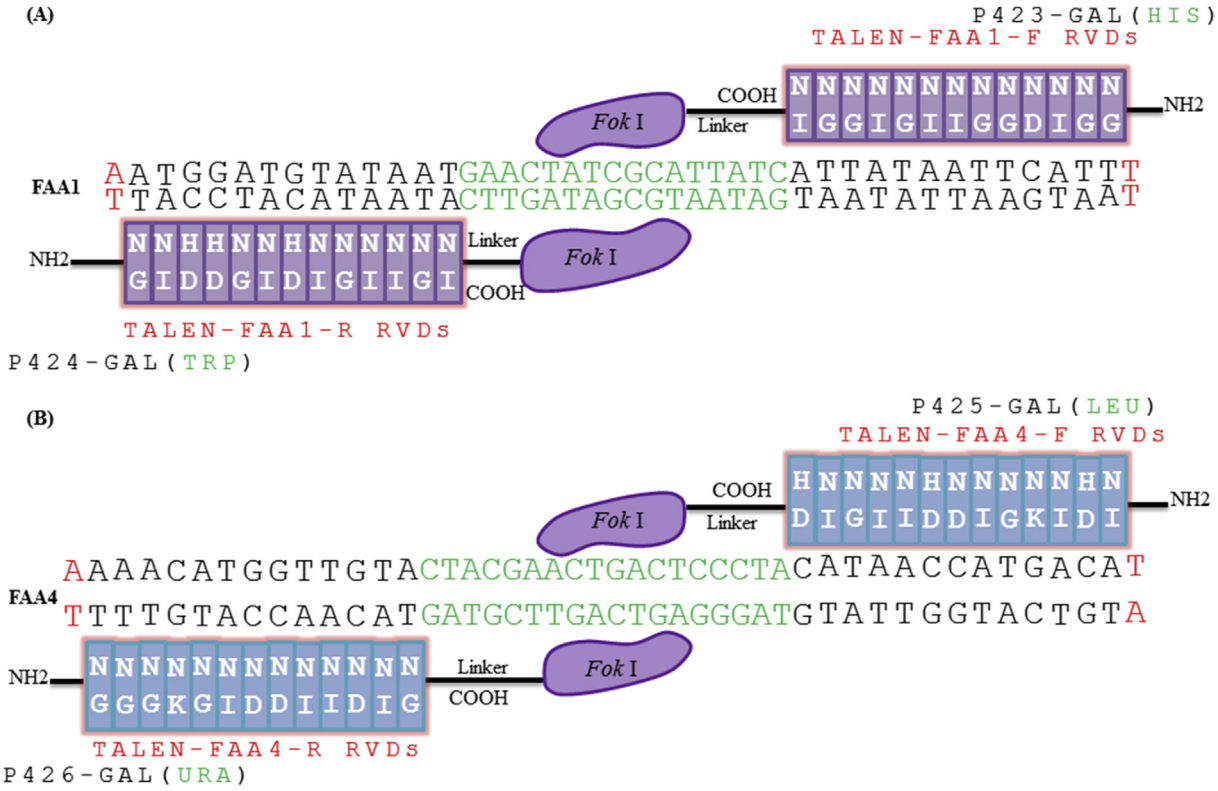


FIG. 2. Binding of the TALEN heterodimers to their target *FAA1* and *FAA4* genes. (A) To target the *FAA1* gene: TALEN-FAA1-F and TALEN-FAA1-R were cloned into pAG423-GAL and pAG424-GAL, respectively. (B) To target the *FAA4* gene: TALEN-FAA4-F and TALEN-FAA4-R were cloned into pAG425-GAL and pAG426-GAL, respectively. The plasmids were simultaneously transformed into the BY4741 yeast strain and colonies carrying the four heterodimers were isolated by culture in SD selective medium lacking His/Trp/Leu/Ura for 48 h at 30°C. The binding specificities of the repeat-variable di-residues (RVDs) are as follows: NI to A, HD and ND to C, NK to G, and NG to T.

High resolution melting analysis HRMA, an extremely sensitive method of detecting mutations in a mixed population of wild type and mutant template genomes (21,38), was used to detect mutations in wild type BY4741 cells carrying the four plasmids encoding TALEN-FAA1-F, TALEN-FAA1-R, TALEN-FAA4-F and TALEN-FAA4-R, as described previously (21).

Yeast FACS analysis for fatty acids detection The cell cycle distribution of yeast cells carrying the plasmids encoding TALEN-FAA1-F, TALEN-FAA1-R, TALEN-FAA4-F, and TALEN-FAA4-R was analyzed by flow cytometry after 16 h induction with 2% galactose. Flow cytometry was performed as described by Hutter and Eipel (39) using a Coulter Epics Profile flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Briefly, suspensions of 5×10^7 cells were collected by centrifugation, fixed in 70% ethanol for 10 min at 4°C, washed with 0.9 ml of cold S1 buffer (50 mM Tris–HCl, pH 8.0, and 20 mM EDTA), and then incubated with 1 mg/ml DNase-free RNase for 1 h at 37°C. After centrifugation, the cells were incubated with 5 mg/ml pepsin for 30 min at 23°C. The samples were then supplemented with 0.2 mg/ml propidium iodide, incubated in the dark at room temperature for 15 min, collected by centrifugation, resuspended in 0.9 ml of S1 buffer, sonicated for 3–5 s, and then analyzed by flow cytometry.

GC–MS analysis for lipid content in the cells For lipid content in the cells, yeast strains carrying the four empty vectors and the two pairs of TALENs (FAA1 and FAA4) were pre-cultured in SD selective medium lacking His/Trp/Leu/Ura at 30°C. Then cells were centrifuged and the pellet was washed three times with sterile water and cells were sub-cultured in 10 ml SD selective medium lacking His/Trp/Leu/Ura with a 2% galactose starting with $O.D._{600}$ ~0.2 and incubated for 16 h at 30°C. At the end of the incubation cells were collected by centrifugation and washed three times with sterile water and the pellet was dried using a vacuum freezer dryer. The Lipid fractions were eluted with chloroform and methylesterified by the addition of an equal volume of 10% methanolic hydrochloride then heating at 60°C for 3 h and finally were analyzed on a gas–liquid chromatography (Agilent Technologies).

RESULTS

Design and construction of TALENs targeting the *FAA1* and *FAA4* genes We hypothesized that using a pair of TALENs that specifically and simultaneously target the *FAA1* and *FAA4* genes (Figs. S1 and S2, respectively) would generate a mutant capable of enhanced cytosolic FA accumulation at the early stage of growth, and increased secretion of FAs into the culture medium at later stages of growth (Fig. 1). To generate specific TALENs, TAL effectors containing RVDs that conferred specificity to the sequences of the *FAA1* and *FAA4* genes were generated using a TALEN repeat assembly and engineering method, as described previously (37). Two pairs of heterodimeric TALENs that were designed to bind to target sequences in the *FAA1* (TALEN-FAA1-F and TALEN-FAA1-R) and *FAA4* (TALEN-FAA4-F and TALEN-FAA4-R) genes were assembled (Fig. 2). Sanger sequencing using primer pairs flanking the repeat domain confirmed the authenticity of the engineered TALENs. The DNA and protein sequences of each TALEN are listed in Additional information. The designer TALEN (dTALEN) backbone vectors were Gateway-compatible; therefore, the Gateway LR cloning method was used to transfer each TALEN into a yeast destination vector, namely pAG423, pAG424, pAG425, and pAG426 for TALEN-FAA1-F, TALEN-FAA1-R, TALEN-FAA4-F, and TALEN-FAA4-R, respectively (Fig. 3). The galactose-inducible expression clones were then transformed into the BY4741 yeast strain and transformants carrying the four plasmids were isolated in selective SD medium lacking His/Trp/Leu/Ura (Fig. 3).

Validation and optimization of TALENs RT-PCR analyses revealed that expression of TALEN-FAA1-F was induced by galactose in a concentration-dependent manner (Fig. 4A). Furthermore, supplementation of the culture medium with 2% glucose inhibited the expression of the four TALENs, whereas their expression levels were increased markedly by the addition of 2% galactose (Fig. 4A). To validate their in-frame fusion construction and proper nuclear localization, each TALEN was

Please cite this article in press as: Aouida, M., et al., Transcription activator-like effector nucleases mediated metabolic engineering for enhanced fatty acids production in *Saccharomyces cerevisiae*, J. Biosci. Bioeng., (2015), http://dx.doi.org/10.1016/j.jbiosc.2015.02.017

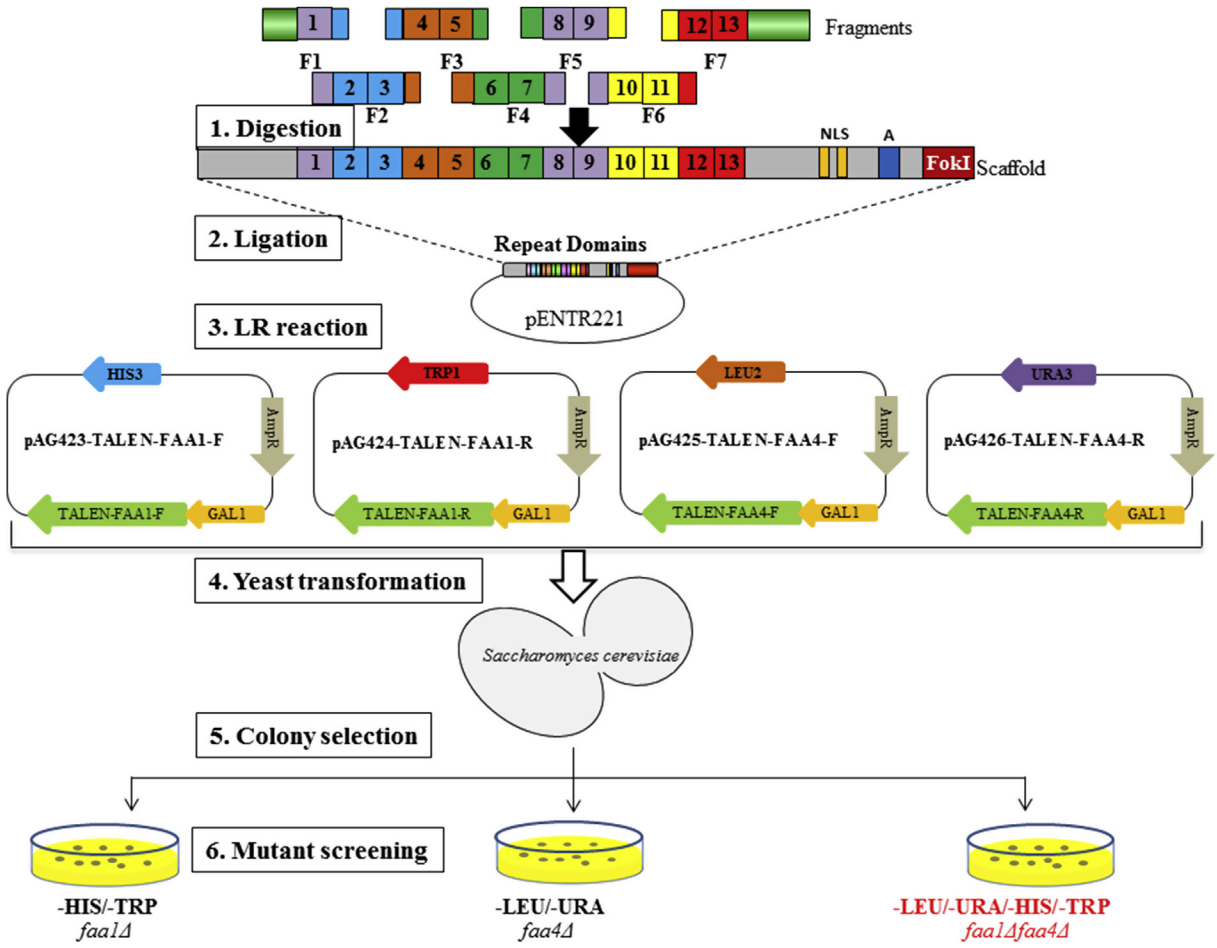


FIG. 3. Generation of *S. cerevisiae* clones expressing TALENs designed to simultaneously knockout the *FAA1* and *FAA4* genes. An overview of the Gateway cloning strategy used to generate yeast clones expressing TALEN-FAA1-F, TALEN-FAA1-R, TALEN-FAA4-F, and TALEN-FAA4-R. The positive clones carrying all four TALENs were isolated in SD selective medium lacking His/Trp/Leu/Ura. F refers to fragments that contained the specific RVDs, and pAG423, pAG424, pAG425, and pAG426 were used as the yeast destination plasmids.

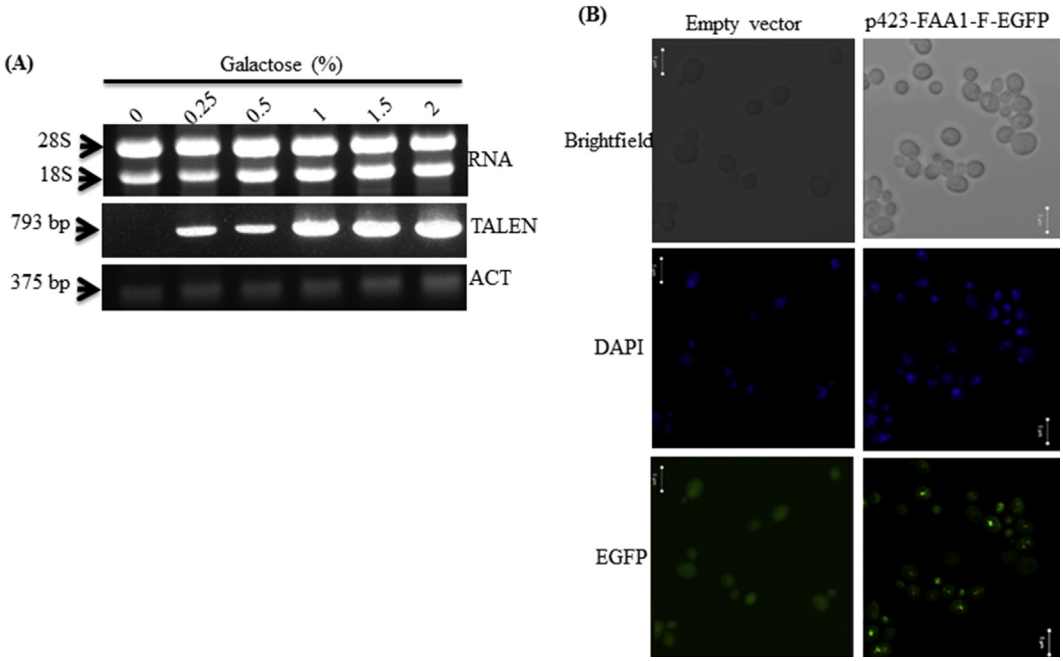


FIG. 4. Galactose-induced expression of TALEN-FAA1-F in yeast. (A) RT-PCR analysis of TALEN-FAA1-F expression in BY4741 cells cultured with the indicated concentrations of galactose for 16 h at 30°C. (B) Analysis of the nuclear localization of EGFP-tagged TALEN-FAA-1 by confocal microscopy (magnification 40×). *S. cerevisiae* BY4741 cells carrying the pAG423-GAL-EGFP (empty vector) or pAG423-GAL-TALEN-FAA1-F-EGFP were cultured in selective SD medium containing 2% galactose. Images of cells in the early log phase of growth are shown.

Please cite this article in press as: Aouida, M., et al., Transcription activator-like effector nucleases mediated metabolic engineering for enhanced fatty acids production in *Saccharomyces cerevisiae*, J. Biosci. Bioeng., (2015), http://dx.doi.org/10.1016/j.jbiosc.2015.02.017

TABLE 1. The primers used in this study.

Primer name	Sequence (5′–3′)
FAA1-F (HRMA assay)	GGCAAGGAGACTTCAGTGGA
FAA1-R (HRMA assay)	CCTATTTTCACCAACCCACG
FAA4-F (HRMA assay)	CAAGAGGGTGGATGGTAAGG
FAA4-R (HRMA assay)	CCAATCTTTATCAGCCCACG-
SPT10-F (HRMA assay)	TCGTGGAGGAAGAAAACTCAA
SPT10-R (HRMA assay)	TCTCGTTCATCACGGTTGAC
FAA1-F (T7EI assay)	CCGTTCCAGTTGGGAAAGCCGC
FAA1-R (T7EI assay)	ATGGCCTTAGACCCCGTTTGCACC
FAA4-F (T7EI assay)	ATATTCCGTTGCAGTTGGCGAAGCCGC
FAA4-F (T7EI assay)	AGTGTCGTACGCGGTGACCACAG
RT-PCR-TALEN-F	CGAATGGGACGAAGTCCAA AGTGGTTTAAGGGCAGC
RT-PCR-TALEN-R	TGCAATGGCAACGACTTGTTCGGGAGTTAACCCATGC
RT-PCR-ACT1-F	GAGTGTGCAAGAGCTGAAGAAGAGAGCTGCAGGGAA
RT-PCR-ACT1-R	ACTACACGCGATCCCAAAGCTAACGGTAATAAGCGC
Seqconf.F1	GGCGCAAGAGCATTAGAGGCGCTTCTCACCGTA
Rep.Seqconf.R2	TTTCTTAACGGCATCAAGTGCTGGTCGTCCTCCTAAACATGC

fused to enhanced green fluorescent protein (EGFP) in a yeast expression plasmid harboring a galactose-inducible promoter. The cells carrying pAG423-TALEN-FAA1-F-EGFP were cultured in selective media without His, induced with 2% galactose for 16 h, and then analyzed using confocal microscopy. The observed nuclear localization of TALEN-FAA1-F-EGFP confirmed the proper in-frame fusion and functionality of the nuclear localization signal in yeast cells (Fig. 4B). The same result was also obtained for EGFP fusions of TALEN-FAA1-R, TALEN-FAA4-F, and TALEN-FAA4-R (data not shown).

TALEN-induced cleavage of the *FAA1* and *FAA4* target genes Next, we determined the abilities of the TALEN heterodimer pairs to cleave their genomic target sequences. Several mutation detection assays, such as SURVEYOR and T7 endonuclease I (T7EI) assays, have been developed to assess the presence of mutations at target sequences; these assays are based on the identification and nuclease-mediated cleavage of a heteroduplex bulge formed by the hybridization of mutant and wild type sequences (40–42). These heteroduplexes can also be examined by high-resolution melting analysis (HRMA), particularly when the frequency of genome editing is expected to be less than 5% (21,38,43). To determine the activities of the *FAA1*-and *FAA4*-specific TALENs, HRMA was performed as described previously (21). In these experiments, specific primers were designed to generate short amplicons of 122 bp and 135 bp for *FAA1* and *FAA4*, respectively (Table 1 and Fig. S3). The cleavage efficiency of the TALENs towards the positive (control plasmids G and C) controls was approximately 50% (Fig. 5A). Yeast cells carrying the four TALENs exhibited site-specific nuclease activity at the target loci (Fig. 5B and C); however, no nuclease activity was detected at the non-target *SPT10* gene (Fig. 5D). The cleavage efficiencies of the TALENs towards *FAA1* and *FAA4* were approximately 2% and 5%, respectively. T7EI assays performed using the specific primers listed in Table 1 confirmed the targeted genomic modification of *FAA1* and *FAA4* by the specific TALENs (Fig. S3).

Yeast cells expressing TALENs targeting *FAA1* and *FAA4* exhibit a FA accumulation phenotype Several previous reports have demonstrated that mutants lacking both Faa1 and Faa4

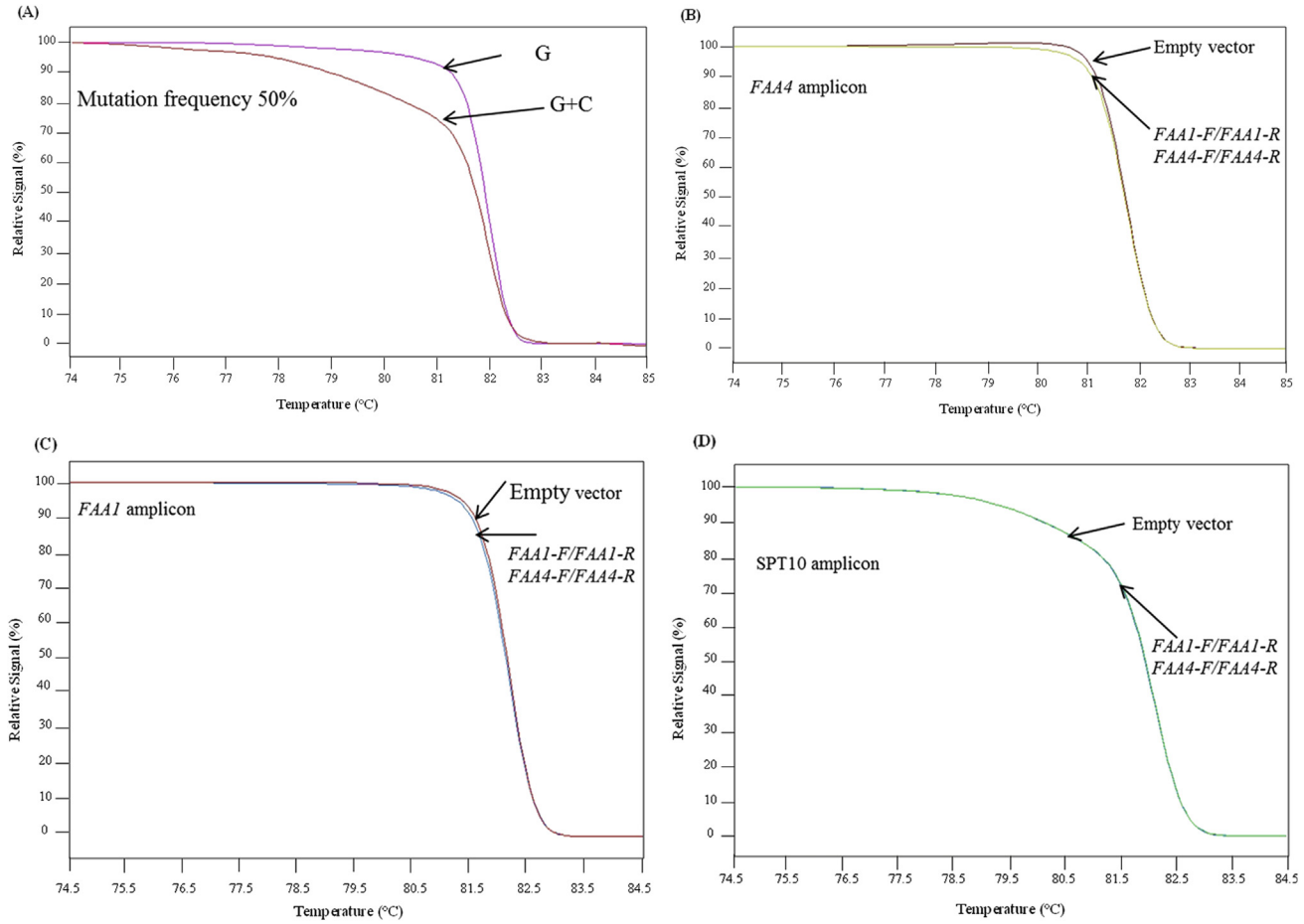


FIG. 5. HRMA of yeast cells expressing the four *FAA1*-and *FAA4*-specific heterodimeric TALENs. HRMA detects the presence of mutant alleles in mixed populations of wild type and mutant genomes. (A) HRMA of the control (Control G and Control C) plasmids provided in the SURVEYOR Mutation Detection Kit. These two control plasmids differ at a single base pair (C versus G). (B, C) HRMAs of the *FAA4* and *FAA1* genes in yeast cells expressing the four heterodimeric TALENs. (D) HRMA of the negative control (*SPT10*) gene.

Please cite this article in press as: Aouida, M., et al., Transcription activator-like effector nucleases mediated metabolic engineering for enhanced fatty acids production in *Saccharomyces cerevisiae*, J. Biosci. Bioeng., (2015), http://dx.doi.org/10.1016/j.jbiosc.2015.02.017

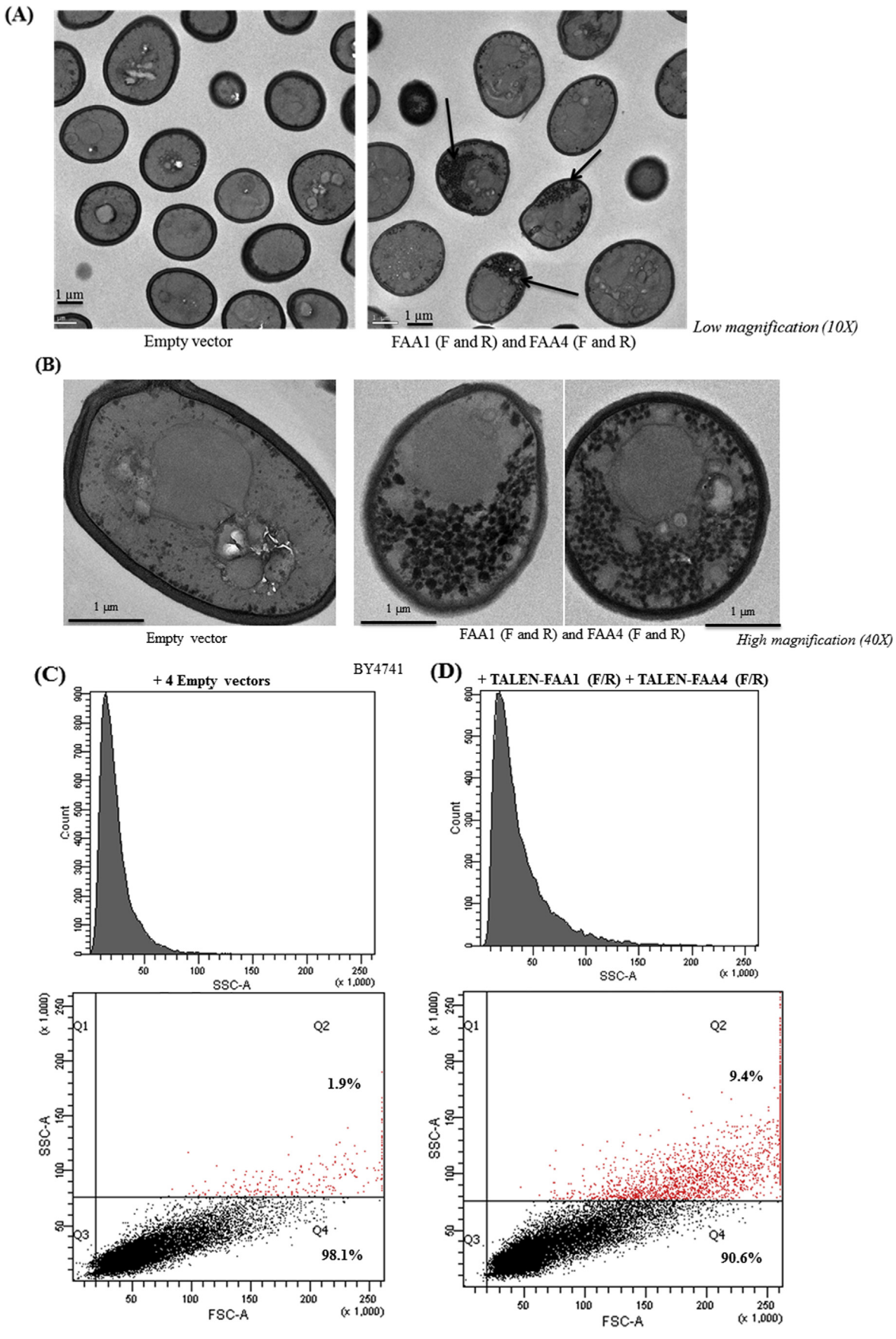


FIG. 6. Fatty acid accumulation in the cytosol of yeast cells expressing *FAA1*-and *FAA4*-specific TALENs. (A, B) Electronic microscopy analyses of yeast cells (log stage of growth) expressing TALEN-FAA1-F, TALEN-FAA1-R, TALEN-FAA4-F, and TALEN-FAA4-R, or the corresponding empty vectors. (C, D) Flow cytometric detection of lipid vesicles (droplets) in BY4741 cells expressing TALEN-FAA1-F, TALEN-FAA1-R, TALEN-FAA4-F, and TALEN-FAA4-R (C), or the corresponding empty vectors (D) at 16 h post-induction with 2% galactose. The lower panels show dot plots of side scatter (SSC; x-axis) versus forward scatter (FSC; y-axis). FSC represents the cell size, whereas SSC represents the cytoplasmic granular intensity.

Please cite this article in press as: Aouida, M., et al., Transcription activator-like effector nucleases mediated metabolic engineering for enhanced fatty acids production in *Saccharomyces cerevisiae*, J. Biosci. Bioeng., (2015), http://dx.doi.org/10.1016/j.jbiosc.2015.02.017

accumulate and then secrete a large amount of FAs into the culture medium (14,33,44,45). Molecular analyses (using HRMA and T7EI assays) of yeast cells expressing the engineered TALENs indicated the presence of mutations at the *FAA1* and *FAA4* loci (Fig. 5B and C, and Fig. S4); therefore, we expected that these cells would accumulate free FAs in the cytosol during log phase (16 h post-induction with galactose). FAs accumulation in yeast can be detected by electron microscopy as previously showed by Feron et al. (46). Electron microscopy analyses revealed that approximately 5% of the yeast cells expressing the *FAA1*-and *FAA4*-specific TALENs accumulated lipid vesicles (droplets) 16 h after induction with 2% galactose (Fig. 6A and B). By contrast, no lipid vesicles were detected in yeast cells carrying the four corresponding empty vectors, or in cells expressing only one pair of TALENs (TALEN-FAA1-F and TALEN-FAA1-R, or TALEN-FAA4-F and TALEN-FAA4-R; Fig. 6A and B).

FA accumulation in the cytosol of human fat cells can be monitored by fluorescence-activated cell sorting (47); therefore, we adapted this procedure to analyze the transformed yeast cells. Whereas 9.4% of the yeast cells expressing the *FAA1*-and *FAA4*-specific TALENs exhibited enhanced FA accumulation, only 1.9% of the control cells carrying the empty vectors displayed this phenotype (Fig. 6C and D). Then, we used gas chromatography (GC–MS) analysis to determine the amount of the total intracellular FAs in yeast cells carrying only empty vectors and yeast cells expressing FAA1-and FAA4-specific TALENs. The data showed an increase amount of FAs accumulation in cells expressing the *FAA1*-and *FAA4*-specific TALENs (10.28 ± 0.4 μg/10^8 cells) compared to the control cells that carrying only the empty vectors 9.45 ± 0.2 μg/10^8 cells). Taken together, these results confirm that the engineered TALENs disrupted the *FAA1* and *FAA4* genes, indicating that deleting both of these genes is necessary and sufficient for FA accumulation (14,33,44,45).

DISCUSSION

Targeted engineering of genomes is an effective means of harnessing the power of genetics for biotechnological applications and functional analyses of genes. Technologies that permit precise and site-specific manipulation of the genome are advancing with unprecedented pace. TALENs and *clustered regularly interspaced short palindromic repeats* (CRISPR)/Cas9 systems are powerful and efficient platforms for genome engineering (48,49). In both of these systems, proteins function like molecular scissors to introduce a targeted DNA double-strand break, which is repaired by one of two major mechanisms: (i) imprecise non-homologous end-joining, which leads to gene disruption by inducing mutations at the break site; or (ii) precise homologous recombination, which facilitates gene insertion, stacking, or replacement using exogenous DNA templates with ends that are homologous to the targeted sequence (48,49). Recently, a limited number of reports have demonstrated the use of genome editing tools for biotechnological applications (50). TALEN-based engineering platforms allow multiplex genome editing and the accelerated evolution of microbial genomes that enable the production of select and highly valuable chemical compounds. For example, yeast oligo-mediated engineering, which harnesses the yeast homologous DNA recombination machinery using template variants, has been used for multiplex genome editing (51). Technologies such as these will help to define the molecular basis of microbial phenotypes and will enable the engineering of specific microbes for industrial applications.

Here, we describe a comprehensive method of editing the *S. cerevisiae* genome using TALENs to simultaneously knockout the *FAA1* and *FAA4* genes, which are involved in the lipid metabolism of *S. cerevisiae*. Although a recent study used the CRISPR system to demonstrate the disruption of multiple genes in *S. cerevisiae* (52), to the best of our knowledge, the present study is the first to describe the generation of a yeast double knockout mutant using TALENs for a biotechnological application. The results presented here demonstrate the feasibility of using TALEN-mediated yeast engineering to induce the overproduction and enhanced secretion of free FAs by *S. cerevisiae*. Notably, these FAs can be used as precursors for generating advanced biofuels via trans-esterification reactions, as well as high-value oleo-chemicals.

The facile engineering of microbial and eukaryotic genomes using TALENs and the CRISPR/Cas9 system will advance synthetic biology and bioengineering applications aimed at overproducing bio-based chemicals and fuels. This proof-of-principle study extends the utility of TALENs as excellent genome engineering tools and highlights their potential use for targeted engineering of other organisms, including microalgae and plants. Because of their high growth rates and high photosynthetic efficiencies when grown in specific environments, microalgae (including cyanobacteria) are a potential feedstock for the production of bioenergy and biofuels.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2015.02.017.

ACKNOWLEDGMENTS

We thank the Bioscience Core Facility and Optical Imaging Core Laboratory at King Abdullah University of Science and Technology (KAUST) for technical assistance. We also thank the members of the genome engineering group at KAUST for their helpful discussions and technical assistance throughout the preparation of the manuscript. This research was funded King Abdullah University of Science and Technology (KAUST).

MA and MM designed research, analyzed data and wrote the manuscript, MA, LL AM, AZ and AP performed the experiments.

References

1. **Choi, Y. J. and Lee, S. Y.:** Microbial production of short-chain alkanes, Nature, **502**, 571–574 (2013).
2. **Howard, T. P., Middelhaufe, S., Moore, K., Edner, C., Kolak, D. M., Taylor, G. N., Parker, D. A., Lee, R., Smirnoff, N., Aves, S. J., and Love, J.:** Synthesis of customized petroleum-replica fuel molecules by targeted modification of free fatty acid pools in *Escherichia coli*, Proc. Natl. Acad. Sci. USA, **110**, 7636–7641 (2013).
3. **Lu, X., Vora, H., and Khosla, C.:** Overproduction of free fatty acids in *E. coli*: implications for biodiesel production, Metab. Eng., **10**, 333–339 (2008).
4. **Schirmer, A., Rude, M. A., Li, X., Popova, E., and del Cardayre, S. B.:** Microbial biosynthesis of alkanes, Science, **329**, 559–562 (2010).
5. **Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S. B., and Keasling, J. D.:** Microbial production of fatty-acid-derived fuels and chemicals from plant biomass, Nature, **463**, 559–562 (2010).
6. **Zheng, Y. N., Li, L. L., Liu, Q., Yang, J. M., Wang, X. W., Liu, W., Xu, X., Liu, H., Zhao, G., and Xian, M.:** Optimization of fatty alcohol biosynthesis pathway for selectively enhanced production of C12/14 and C16/18 fatty alcohols in engineered *Escherichia coli*, Microb. Cell Fact., **11**, 65 (2012).
7. **Ostergaard, S., Olsson, L., and Nielsen, J.:** Metabolic engineering of *Saccharomyces cerevisiae*, Microbiol. Mol. Biol. Rev., **64**, 34–50 (2000).
8. **Liu, J.-F., Nie, K.-L., Fan, L.-H., Wang, F., Tan, T.-W., and Deng, L.:** Increased production of FAEEs for biodiesel with lipase enhanced *Saccharomyces cerevisiae*, Process Biochem., **48**, 1212–1215 (2013).
9. **Kamisaka, Y., Kimura, K., Uemura, H., and Yamaoka, M.:** Overexpression of the active diacylglycerol acyltransferase variant transforms *Saccharomyces cerevisiae* into an oleaginous yeast, Appl. Microbiol. Biotechnol., **97**, 7345–7355 (2013).
10. **Runguphan, W. and Keasling, J. D.:** Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived biofuels and chemicals, Metab. Eng., **21**, 103–113 (2014).
11. **Leber, C. and Da Silva, N. A.:** Engineering of *Saccharomyces cerevisiae* for the synthesis of short chain fatty acids, Biotechnol. Bioeng., **111**, 347–358 (2014).
12. **Tang, X., Feng, H., and Chen, W. N.:** Metabolic engineering for enhanced fatty acids synthesis in *Saccharomyces cerevisiae*, Metab. Eng., **16**, 95–102 (2013).
13. **Yu, K. O., Jung, J., Kim, S. W., Park, C. H., and Han, S. O.:** Synthesis of FAEEs from glycerol in engineered *Saccharomyces cerevisiae* using endogenously

produced ethanol by heterologous expression of an unspecific bacterial acyltransferase, Biotechnol. Bioeng., **109**, 110–115 (2012).
14. **Li, X., Guo, D., Cheng, Y., Zhu, F., Deng, Z., and Liu, T.:** Overproduction of fatty acids in engineered *Saccharomyces cerevisiae*, Biotechnol. Bioeng., **111**, 1841–1852 (2014).
15. **Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A. J., and Voytas, D. F.:** Targeting DNA double-strand breaks with TAL effector nucleases, Genetics, **186**, 757–761 (2010).
16. **Deng, D., Yin, P., Yan, C., Pan, X., Gong, X., Qi, S., Xie, T., Mahfouz, M., Zhu, J. K., Yan, N., and Shi, Y.:** Recognition of methylated DNA by TAL effectors, Cell. Res., **22**, 1502–1504 (2012).
17. **Mahfouz, M. M. and Li, L.:** TALE nucleases and next generation GM crops, GM Crops, **2**, 99–103 (2011).
18. **Mahfouz, M. M., Li, L., Shamimuzzaman, M., Wibowo, A., Fang, X., and Zhu, J. K.:** De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks, Proc. Natl. Acad. Sci. USA, **108**, 2623–2628 (2011).
19. **Ramirez, C. L., Foley, J. E., Wright, D. A., Muller-Lerch, F., Rahman, S. H., Cornu, T. I., Winfrey, R. J., Sander, J. D., Fu, F., Townsend, J. A., and other 3 authors:** Unexpected failure rates for modular assembly of engineered zinc fingers, Nat. Methods, **5**, 374–375 (2008).
20. **Mahfouz, M. M., Piatek, A., and Stewart, C. N., Jr.:** Genome engineering via TALENs and CRISPR/Cas9 systems: challenges and perspectives, Plant Biotechnol. J., **12**, 1006–1014 (2014).
21. **Aouida, M., Piatek, M. J., Bangarusamy, D. K., and Mahfouz, M. M.:** Activities and specificities of homodimeric TALENs in *Saccharomyces cerevisiae*, Curr. Genet., **60**, 61–74 (2014).
22. **Boch, J. and Bonas, U.:** Xanthomonas AvrBs3 family-type III effectors: discovery and function, Annu Rev. Phytopathol., **48**, 419–436 (2010).
23. **Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., and Bonas, U.:** Breaking the code of DNA binding specificity of TAL-type III effectors, Science, **326**, 1509–1512 (2009).
24. **Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J. A., Somia, N. V., Bogdanove, A. J., and Voytas, D. F.:** Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting, Nucleic Acids Res., **39**, e82 (2011).
25. **Kay, S., Boch, J., and Bonas, U.:** Characterization of AvrBs3-like effectors from a Brassicaceae pathogen reveals virulence and avirulence activities and a protein with a novel repeat architecture. Molecular plant-microbe interactions, Mol. Plant Microbe Interact., **18**, 838–848 (2005).
26. **Kim, Y., Kweon, J., Kim, A., Chon, J. K., Yoo, J. Y., Kim, H. J., Kim, S., Lee, C., Jeong, E., Chung, E., and other 9 authors:** A library of TAL effector nucleases spanning the human genome, Nat. Biotechnol., **31**, 251–258 (2013).
27. **Li, T., Liu, B., Spalding, M. H., Weeks, D. P., and Yang, B.:** High-efficiency TALEN-based gene editing produces disease-resistant rice, Nat. Biotechnol., **30**, 390–392 (2012).
28. **Moscou, M. J. and Bogdanove, A. J.:** A simple cipher governs DNA recognition by TAL effectors, Science, **326**, 1501 (2009).
29. **Li, L., Atef, A., Piatek, A., Ali, Z., Piatek, M., Aouida, M., Sharakuu, A., Mahjoub, A., Wang, G., Khan, S., and other 3 authors:** Characterization and DNA-binding specificities of Ralstonia TAL-like effectors, Mol. Plant, **6**, 1318–1330 (2013).
30. **Morbitzer, R., Romer, P., Boch, J., and Lahaye, T.:** Regulation of selected genome loci using de novo-engineered transcription activator-like effector (TALE)-type transcription factors, Proc. Natl. Acad. Sci. USA, **107**, 21617–21622 (2010).
31. **Cong, L., Zhou, R., Kuo, Y. C., Cunniff, M., and Zhang, F.:** Comprehensive interrogation of natural TALE DNA-binding modules and transcriptional repressor domains, Nat. Biotechnol., **3**, 968 (2012).
32. **Huang, P., Xiao, A., Zhou, M., Zhu, Z., Lin, S., and Zhang, B.:** Heritable gene targeting in zebrafish using customized TALENs, Nat. Biotechnol., **29**, 699–700 (2011).
33. **Scharnewski, M., Pongdontri, P., Mora, G., Hoppert, M., and Fulda, M.:** Mutants of *Saccharomyces cerevisiae* deficient in acyl-CoA synthetases secrete fatty acids due to interrupted fatty acid recycling, FEBS J., **275**, 2765–2778 (2008).
34. **Sherman, F.:** Getting started with yeast, Methods Enzymol., **194**, 3–21 (1991).
35. **Sherman, F., Fink, G. R., and Hicks, J. B.:** Laboratory course manual for methods in yeast genetics. Cold Spring Harbor Laboratory, Plainview, NY (1983).
36. **Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H.:** Improved method for high efficiency transformation of intact yeast cells, Nucleic Acids Res., **20**, 1425 (1992).
37. **Li, L., Piatek, M. J., Atef, A., Piatek, A., Wibowo, A., Fang, X., Sabir, J. S., Zhu, J. K., and Mahfouz, M. M.:** Rapid and highly efficient construction of TALE-based transcriptional regulators and nucleases for genome modification, Plant Mol. Biol., **78**, 407–416 (2012).
38. **Dahlem, T. J., Hoshijima, K., Jurynec, M. J., Gunther, D., Starker, C. G., Locke, A. S., Weis, A. M., Voytas, D. F., and Grunwald, D. J.:** Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome, PLoS Genet., **8**, e1002861 (2012).
39. **Hutter, K. J. and Eipel, H. E.:** Microbial determinations by flow cytometry, J. Gen. Microbiol., **113**, 369–375 (1979).
40. **Cho, S. W., Kim, S., Kim, J. M., and Kim, J. S.:** Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease, Nat. Biotechnol., **31**, 230–232 (2013).
41. **Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., and Zhang, F.:** Multiplex genome engineering using CRISPR/Cas systems, Science, **339**, 819–823 (2013).
42. **Kim, H. J., Lee, H. J., Kim, H., Cho, S. W., and Kim, J. S.:** Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly, Genome Res., **19**, 1279–1288 (2009).
43. **Ota, S., Hisano, Y., Muraki, M., Hoshijima, K., Dahlem, T. J., Grunwald, D. J., Okada, Y., and Kawahara, A.:** Efficient identification of TALEN-mediated genome modifications using heteroduplex mobility assays, Genes Cells, **18**, 450–458 (2013).
44. **Chen, L., Zhang, J., Lee, J., and Chen, W. N.:** Enhancement of free fatty acid production in *Saccharomyces cerevisiae* by control of fatty acyl-CoA metabolism, Appl. Microbiol. Biotechnol., **98**, 6739–6750 (2014).
45. **Michinaka, Y., Shimauchi, T., Aki, T., Nakajima, T., Kawamoto, S., Shigeta, S., Suzuki, O., and Ono, K.:** Extracellular secretion of free fatty acids by disruption of a fatty acyl-CoA synthetase gene in *Saccharomyces cerevisiae*, J. Biosci. Bioeng., **95**, 435–440 (2003).
46. **Feron, G., Dufosse, L., Mauvais, G., Bonnarme, P., and Spinnler, H. E.:** Fatty acid accumulation in the yeast Sporidiobolus salmonicolor during batch production of gamma-decalactone, FEMS Microbiol. Lett., **149**, 17–24 (1997).
47. **Lee, Y. H., Chen, S. Y., Wiesner, R. J., and Huang, Y. F.:** Simple flow cytometric method used to assess lipid accumulation in fat cells, J. Lipid Res., **45**, 1162–1167 (2004).
48. **Gaj, T., Gersbach, C. A., and Barbas, C. F., 3rd:** ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering, Trends Biotechnol., **31**, 397–405 (2013).
49. **Silva, G., Poirot, L., Galetto, R., Smith, J., Montoya, G., Duchateau, P., and Paques, F.:** Meganucleases and other tools for targeted genome engineering: perspectives and challenges for gene therapy, Curr. Gene Ther., **11**, 11–27 (2011).
50. **Daboussi, F., Leduc, S., Marechal, A., Dubois, G., Guyot, V., Perez-Michaut, C., Amato, A., Falciatore, A., Juillerat, A., Beurdeley, M., and other 3 authors:** Genome engineering empowers the diato Phaeodactylum tricornutum for biotechnology, Nat. Commun., **5**, 3831 (2014).
51. **DiCarlo, J. E., Conley, A. J., Penttila, M., Jantti, J., Wang, H. H., and Church, G. M.:** Yeast oligo-mediated genome engineering (YOGE), ACS Synth. Biol., **2**, 741–749 (2013).
52. **Bao, Z., Xiao, H., Liang, J., Zhang, L., Xiong, X., Sun, N., Si, T., and Zhao, H.:** Homology-integrated CRISPR-Cas (HI-CRISPR) system for one-step Multigene disruption in Saccharomyces cerevisiae, ACS Synth. Biol., http://dx.doi.org/10.1021/sb500255k (published online 10 September 2014).

Please cite this article in press as: Aouida, M., et al., Transcription activator-like effector nucleases mediated metabolic engineering for enhanced fatty acids production in *Saccharomyces cerevisiae*, J. Biosci. Bioeng., (2015), http://dx.doi.org/10.1016/j.jbiosc.2015.02.017