Pathway grafting for polyunsaturated fatty acids production in *Ashbya gossypii* through Golden-Gate rapid assembly

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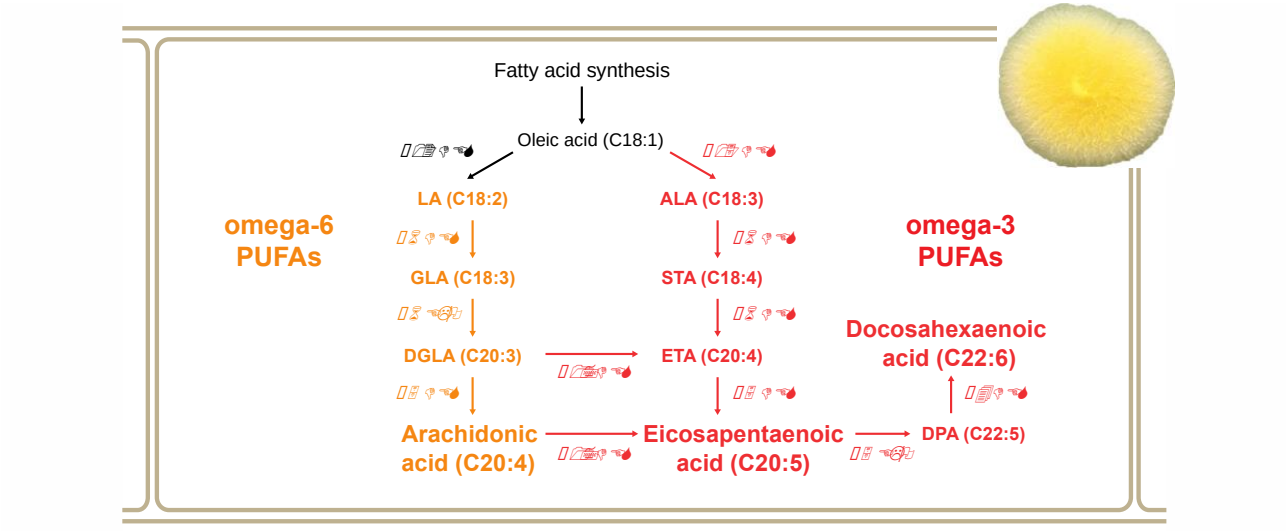
Abstract

Here we present a Golden-Gate assembly system adapted for the rapid genomic engineering of the industrial fungus *A. gossypii*. This biocatalyst is an excellent biotechnological chassis for synthetic biology applications and is currently used for the industrial production of riboflavin. Other bioprocesses such as the production of folic acid, nucleosides, amino acids and biolipids have been recently reported in *A. gossypii*. In this work, an efficient assembly system for the expression of heterologous complex pathways has been designed. The expression platform comprises interchangeable DNA modules, which provides flexibility for the use of different loci for integration, selection markers and regulatory sequences. The functionality of the system has been applied to engineer strains able to synthesize polyunsaturated fatty acids (up to 35% of total fatty acids). The production of the industrially relevant arachidonic, eicosapentanoic and docosahexanoic acids remarks the ~~high~~ potential of *A. gossypii* to produce these functional lipids.

Keywords: *Ashbya gosyypii*, Golden-Gate, omega polyunsaturated fatty acids, synthetic biology



Introduction

Microbial lipids are a sustainable source of fuels and chemicals and therefore, their efficient production has been extensively studied in the recent years. These oils can potentially replace fossil fuels for the production of polymers, plastics, pharmaceuticals, lubricants, surfactants or nutraceuticals [1]. One of these high-value nutraceuticals are the long-chain polyunsaturated fatty acids (≥C_{20}) (LC-PUFAs), and specifically omega-3 (ω3) fatty acids, which are essential components of the diet for humans and animals [2]. The commercial importance of these PUFAs prompted to the elucidation of several pathways leading to their production [3]. Usually, the single overexpression of the genes of the pathway lead to the production of very reduced amounts of PUFAs, typically under 4% in *Saccharomyces cerevisiae* [4-5]. Extensive manipulation was required in *Yarrowia lipolytica* to achieve a high production of PUFAs per cell, which reached 56% of the omega-3 eicosapentanoic acid (EPA; C20:5) using a multigene-multicopy approach comprising 30 copies of 9 different genes [6]. Anyhow, even small amounts of these PUFAs can have a ~~huge~~ commercial potential specially if they are produced in combination with other nutraceuticals such as proteins or vitamins and used for feeding. Besides, the production of these PUFAs from waste industrial by-products using the appropriate biocatalysts also constitute an important challenge for the biotechnological industry.

A. gossypii is a filamentous fungus that is currently used for the bio-based industrial production of riboflavin or vitamin B_2 [7-11]. One of the most important applications of the vitamin produced by *A. gossypii* is animal feeding. In addition, *A. gossypii* has been engineered to be a suitable biotechnological chassis for the implementation of bioprocesses such as the production of microbial oils [12-16], nucleosides [17-19] and other vitamins [20]. In this regard, a large number of biotechnological tools available for *A. gossypii* [21-23], together with the ability to grow using low-cost substrates, reinforce the versatility of *A. gossypii* as an ideal cell factory with broad-range capacities for microbial fermentations and potential nutraceutical applications.

A. gossypii is a genetically-tractable microorganism, however episomic plasmids containing *S. cerevisiae* ARS elements are not stable in *A. gossypii*. Therefore, the genetic manipulations of *A. gossypii* for metabolic engineering applications are mainly based on genomic integrations [21].

The manipulation of complex metabolic pathways in *A. gossypii* required many rounds of integration events using single-gene expression cassettes. Previous works in *A. gossypii* described the production of biolipids using single-gene heterologous expression of *ACL* genes from *Y. lipolytica*, the *pta* gene from *Bacillus subtilis* or the *xpkA* gene from *Aspergillus nidulans* [12, 14]. These single-gene approaches may present an important drawback, since the fitness and the sporulation ability can be affected after many events of genomic integrations.

Different approaches have been applied to enhance the production of biolipids in *A. gossypii*. The overexpression of *ACL* genes from *Y. lipolytica* together with the abolition of the FA beta-oxidation pathway resulted in engineered strains with high levels of lipid accumulation (up to 70% of total C.D.W.) [14]. Also, the production of biolipids from xylose in *A. gossypii* has been demonstrated by both the overexpression of the endogenous pathway for xylose utilization and the heterologous overexpression of a phosphoketolase pathway [12]. In addition, we have previously described the malleability of the *A. gossypii* fatty acid profile by engineering both the elongation and desaturation systems [15]. In that work, a strain overexpressing *AgDes589*, which encodes a Δ12 desaturase, was shown to accumulate high levels of linoleic acid (LA; C18:2), which is the immediate precursor of both omega-3 and omega-6 PUFAs (Figure 1) [15, 24]. However, *A. gossypii* lacks the enzymatic machinery required for the successive elongation and desaturation steps leading to the synthesis of omega-3/6 PUFAs from LA (Figure 1) [24]. The omega-6 arachidonic acid (ARA; C20:4) can be synthesized from LA through the so-called conventional Δ6 desaturase pathway, sequentially catalyzed by a Δ6 desaturase, a Δ6 C18/20 elongase and a Δ5 desaturase (Figure 1) [4]. On the other hand, the synthesis of the omega-3 EPA (C20:5) and docosahexaenoic acid (DHA; C22:6) requires, in addition to the Δ6 desaturase pathway, the activity of a Δ17 desaturase, a Δ5 C20/22 elongase and a Δ4 desaturase (Figure 1) [4].

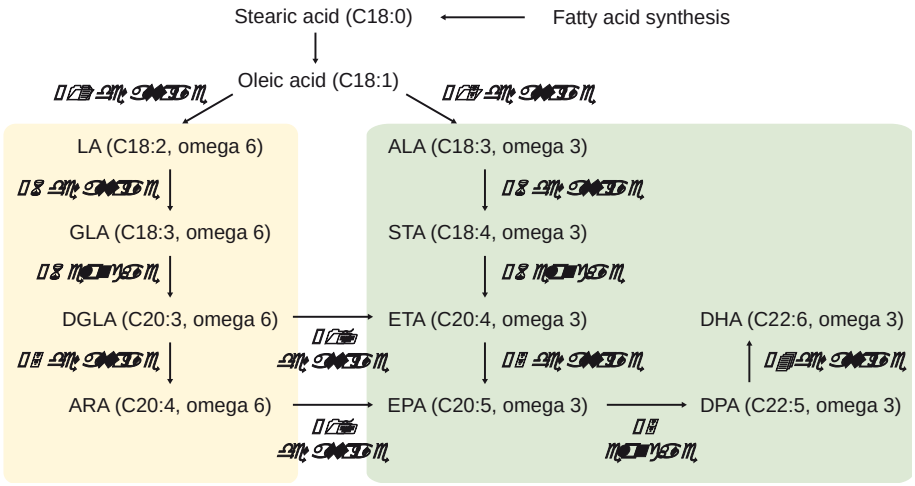


Figure 1. Schematic pathway of the omega PUFAs biosynthesis. The enzymatic activities that have been modified in this work are indicated in bold. SA, stearic acid; OA, oleic acid; LA, linoleic acid; GLA, gamma-linolenic acid; DGLA, di-homo-gamma-linolenic acid; ARA, arachidonic acid; ALA, alpha-linolenic acid; STA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

In this work, we have adapted the Golden Gate cloning system in *A. gossypii* and used it to express a multigene complex heterologous pathway for the production of omega-3/6 PUFAs. The utilisation of the GG system strongly accelerated the engineering process required to express a set of 7 heterologous genes. Moreover, the modularity of the systems allowed us to quickly test different promoter combinations in the expression cassettes. Interestingly, the engineered strains were able to produce between 20-35% of the total lipids as omega fatty acids.

Results and Discussion

As mentioned above, the overexpression of heterologous genes in *A. gossypii*, instead of using episomic expression plasmids, is mainly achieved using genomic integrations of overexpression cassettes that comprise integration, selection and expression modules (Figure 2A). The modularity of the system provides flexibility for the use of diverse genomic loci for integration, different selection markers and tunability of transcriptional activity by using different promoter sequences (Figure 2A). Therefore, the introduction of complex pathways, which involve a high number of heterologous genes, requires the development of timesaving approaches for a rapid assembly of efficient overexpression cassettes in *A. gossypii*. In this work, cassettes for the heterologous overexpression of different TUs have been constructed following a GG modular cloning system, which is based on the use of type IIs restriction enzymes (Figure 2B-C and Figure S1).

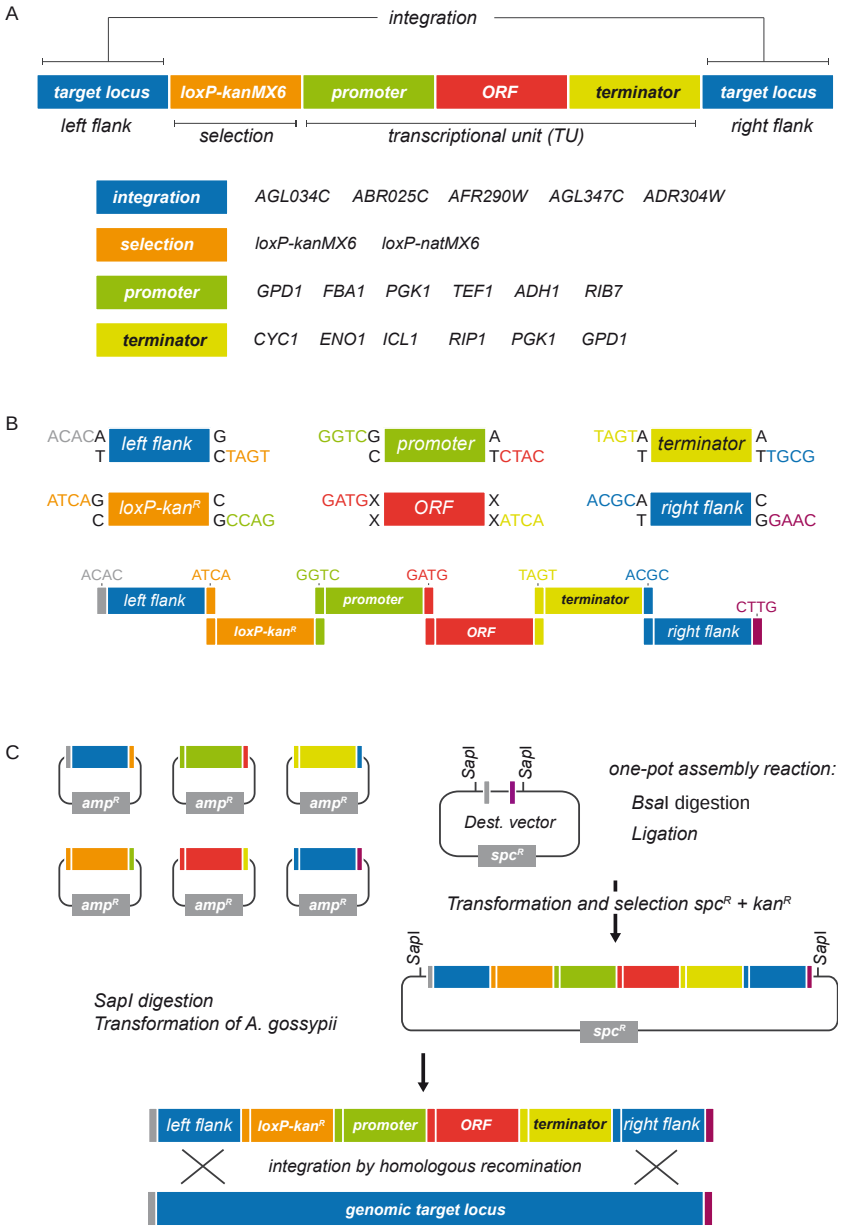


Figure 2. Cassettes for heterologous gene expression in *A. gossypii*. A, scheme of the integration (left and right flanks), selection and expression modules of a typical cassette for the overexpression of heterologous genes in *A. gossypii*. List of currently used modules in the overexpression cassettes. B, modules comprising the overexpression cassette are assembled following a head-to-tail strategy, which is determined by the 4-nt overhangs of each module. C, scheme of the one-pot assembly reaction for the construction and genomic integration of the overexpression cassette.

The possibility of using multiple overexpression modules within a single cassette provides versatility and modularity to the system, i. e. the ability of using either the same or different promoter/terminator sequences for the expression of the genes of interest. Hence, the generation of a combinatorial library of GG modules contributes to the development of a valuable toolbox in *A. gossypii*, which can be employed for a large number of applications in metabolic engineering and synthetic biology. Indeed, the use of different combinations of promoters, with different

transcriptional activity and regulatory features, can be decisive for the optimization of metabolic fluxes in *A. gossypii.* Accordingly, to validate a multigene GG methodology in *A. gossypii*, we implemented a complex metabolic pathway for the production of LC-PUFAS.

As stated above, a strain overexpressing the endogenous Δ12 desaturase is able to accumulate high levels of LA (C18:2), which in turn is the immediate precursor of omega PUFAs (Figure 1) [15, 24] Here we decided to further manipulate the fatty acid profile of that strain by using synthetic biology approaches as a proof of concept for the production of both omega-3 and omega-6 PUFAs in *A. gossypii*. Therefore, genes encoding elongases and desaturases from oleaginous yeasts and algae were selected for heterologous overexpression in *A. gossypii* (Table 1 and Supporting Information File S1).

Table 1. List of heterologous genes used in this study.

ORF	Source	UniProt entry
MaFADS6 - Δ6 desaturase	*Mortierella alpina*	Q76LW8
MaGLELO - Δ6 elongase	*Mortierella alpina*	A9ZLZ4
MaFADS5 - Δ5 desaturase	*Mortierella alpina*	Q589C4
Δ12/Δ15 desaturase	*Fusarium moniliforme*	Q27ZJ6
Δ17 desaturase	*Phytophthora infestans*	D0NE80
PsELO5 - Δ5 elongase	*Pavlova salina*	Q0D2W3
PsFADS4 - Δ4 desaturase	*Pavlova salina*	A0PJ29

The production of ARA has been extensively studied in the oleaginous fungus *Mortierella alpina* with reported titers of ARA production ranging from 0.5-19 g/L [25]. Therefore, genes encoding Δ6 desaturase, Δ6 C18/20 elongase and Δ5 desaturase from *M. alpina* were selected for the heterologous expression in *A. gossypii* (Table 1 and Supporting Information File S1). Overexpression cassettes encompassing three TUs for the reconstitution of the ARA pathway were constructed using the GG assembly method. In addition to the TUs, the overexpression cassettes contained flanking sequences that target the integration of the cassettes at defined genomic locations. These sites were previously validated as integration sites that do not notably affect the expression of an inserted *RIB5* gene used as reporter (Figure S2). In addition, the growth rate of the integration strains was not affected (not shown).

Two approaches were designed to test different promoter activities for the overexpression of the conventional Δ6 desaturase pathway (Figure 1). Hence, three different strong constitutive

promoters from genes involved in the glycolytic pathway (*GPD1*, *PGK1*, *FBA1*) were chosen according to gene expression data from the Ashbya Genome Database [26]. In a first approach, each of the three heterologous genes were expressed under the control of different promoter and terminator sequences: P_{GPD1} and T_{CYC1} for the Δ6 C18/20 elongase, P_{PGK1} and T_{PGK1} for the Δ5 desaturase and P_{FBA1} and T_{ENO1} for the Δ6 desaturase (Figure 3A). Thus, a strain overexpressing the ARA heterologous pathway from *M. alpina*, together with the endogenous Δ12 desaturase from *A. gossypii* was generated and designated as omega-6-GPF. In a second approach, the three TUs for the ARA pathway were placed under the control of the *GPD1* promoter, thus generating the strain omega-6-G (Figure 3B).

Figure 3. Schematic representation of the GG modules used for the production of omega PUFAs in *A. gossypii*. A, omega-6-GPF cassette for the simultaneous expression of a Δ6 C18/20 elongase, a Δ5 desaturase and a Δ6 desaturase under the control of three different promoters. B, omega-6-G cassette for the simultaneous expression of a Δ6 C18/20 elongase, a Δ5 desaturase and a Δ6 desaturase under the control of the promoter P_{GPD1}. C, overexpression cassette for the Δ17 desaturase from *P. infestans*. D, omega-3 cassette for the simultaneous expression of a Δ15 desaturase, a Δ4 desaturase and a Δ5 elongase under the control of the promoter P_{GPD1}. The target loci for genomic integration of the cassettes are indicated (left and right flanks). The sequences of the 4-nt overhangs for the GG assembly of each cassette are shown.

Both the omega-6-GPF and omega-6-G strains were able to transform LA into the intermediates of ARA biosynthesis, in contrast to the strain overexpressing only the endogenous Δ12 desaturase (Figure 4). The engineered strains were able to produce between 24-35% of total PUFAs (12.1-

18.3 mg/g of DCW). Moreover, the strain omega-6-G showed a 4.4% ± 0.5% of ARA with regard to the total fatty acid content and a 24.1% of omega-6 fatty acids, thus confirming that the conventional Δ-6 pathway of *M. alpina* is functional in *A. gossypii* (Figure 4 and Table S1). However, the strain omega-6-GPF showed an accumulation of LA and very low levels of GLA and DGLA, which indicate that the transformation of LA into GLA represents a metabolic bottleneck in this strain. Indeed, the gene expression analysis of both strains revealed that the genes encoding Δ6 desaturase and Δ5 desaturase showed much lower expression levels in the strain omega-6-GPF (Figure 5).

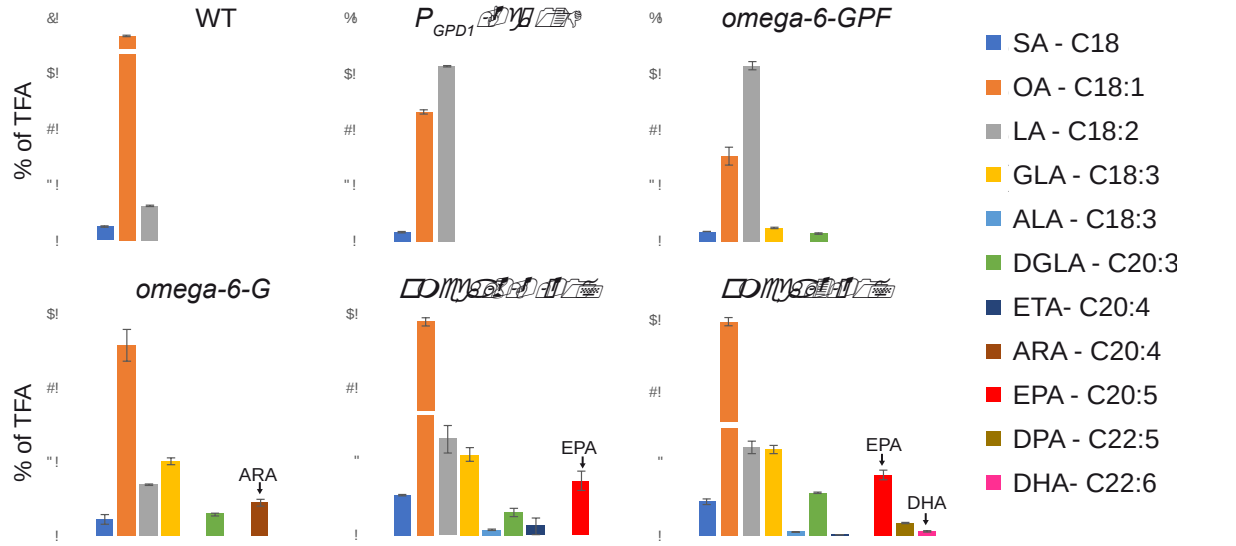


Figure 4. Fatty acids composition (% TFA) of the engineered strains of *A. gossypii.* The production of ARA, EPA and DHA is indicated. The bars represent the average of three independent experiments (see Table S3 for numerical data). The error bars indicate the standard deviations.

In agreement with these data, our preliminary RNAseq data from the wild-type strain showed that *GPD1* is expressed 7-fold higher than *FBA1* (Supporting Information File S2). These results indicate that the accumulation of LA in the omega-6-GPF strain must be caused by an insufficient expression of the Δ6 desaturase from the promoter P_{FBA1}, which is restored with the use of the promoter P_{GPD1} in the omega-6-G strain, thus highlighting the importance of choosing the optimal combination of transcriptional activities for each gene to be expressed. In this regard, common challenges for synthetic biology such as the metabolic bottlenecks and the accumulation of toxic intermediates can be managed by altering transcriptional activity with the use of strong/weak promoters. Consequently, we decided to use the strong constitutive promoter of the *GPD1* gene for the manipulation of the omega-3 pathway.

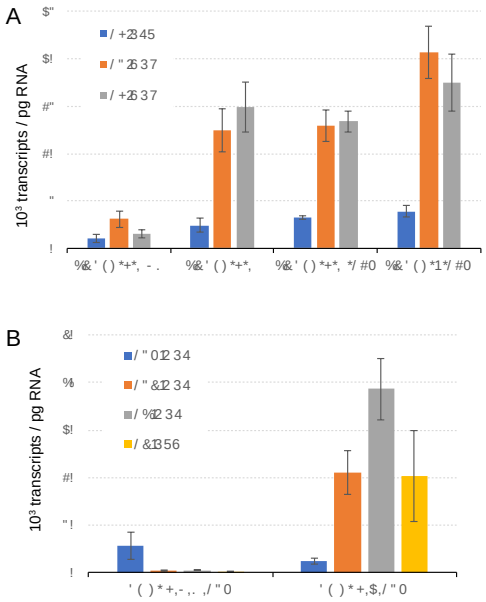


Figure 5. Gene expression analysis of the engineered strains of *A. gossypii.* A, transcription levels of genes from the Δ6 desaturase pathway in the engineered strains. B, transcription levels of heterologous genes expressed in the omega-3 producing strains. The error bars indicate the standard deviations.

Our strategy to implement the omega-3 production in *A. gossypii* was intended first to transform all the omega-6 intermediates into omega-3 by the overexpression of a Δ17 desaturase, and second to direct metabolic flux from OA into the production of omega-3 FA. Accordingly, we used an overexpression cassette containing a Δ17 desaturase from *Phytophthora infestans*, which is able to convert a wide range of omega-6 substrates into omega-3 PUFAs (Figure 1 and Table 1) [27]. The codon optimized ORF encoding the Δ17 desaturase from *P. infestans* was expressed under the control of the *GPD1* promoter and the *ICL1* terminator (Figure 3C). In addition, we used an overexpression cassette for the heterologous reconstitution of the omega-3 DHA pathway (Figure 1), which comprised three additional TUs: omega-3 Δ12/Δ15 desaturase from *Fusarium moniliforme* [28], and Δ5 elongase and Δ4 desaturase from *Pavlova salina* [29-30] (Figure 3D and Table 1). It has been reported that the heterologous expression of the omega-3 Δ12/Δ15 desaturase from *F. moniliforme* resulted in high ALA content in *Y. lipolytica* and soybean [28]. Besides, we selected both the Δ5 elongase and Δ4 desaturase from *P. salina* based on the ability of this marine microalga to produce high levels of EPA and DHA [29-30]. Accordingly, two strains were generated that harbored the omega-6 pathway together with the Δ17 desaturase (*omega-6-Δ17*) and the complete omega-6/omega-3/Δ17 pathway (*omega-3-Δ17*), respectively. The analysis of the lipids

profile revealed that the strain *omega-6-Δ17* was able to produce the omega-3 EPA as well as the intermediates ALA and ETA, with a total omega-3 content of about 5% of the total fatty acid content (Figure 4 and Table S1). In addition, the strain *omega-3-Δ17* showed higher levels of EPA (4.1% ± 0.3%) and, moreover, it was able to produce both the omega-3 DPA and DHA PUFAs (Figure 4 and Table S1). The total PUFA production reached 20.5% of the total fatty acids in this strain (Figure 4 and Table S1). The lipid profile of the strain *omega-6-Δ17* indicates that the overexpression of a Δ17 desaturase is critical for the transformation of omega-6 substrates into omega-3 PUFAs, which is confirmed by the total conversion of ARA into EPA. Also, it is noteworthy that the Δ17 desaturase from *P. infestans* may be able to transform LA into ALA. In this regard, the gene expression analysis of the omega-3 producing strains showed that the level of expression of the Δ17 desaturase does not constitute a bottleneck for the transformation of omega-6 substrates into omega-3 PUFAs (Figure 5). In contrast, the low levels of both DPA and DHA in the *omega-3-Δ17* strain, which cannot be attributed to a deficient transcription of the omega-3 DHA pathway (Figure 5), show that the conversion of EPA is not efficient in *A. gossypii*, thus suggesting the use of alternative Δ5 elongase and Δ4 desaturase heterologous genes.

Previous works have also shown different strategies for the simultaneous production of EPA and DHA: in *Phaeodactylum tricornutum*, a unicellular marine diatom, the heterologous co-expression of Δ6 desaturase and Δ5 elongase from *Ostreococcus tauri* resulted in 18% EPA and 11% DHA of TFA [31]; in *E. coli*, the overexpression of the EPA/DHA pathway gene cluster from *Shewanella baltica* produced about 0.2-0.4% DHA and 14-31% EPA of TFA [32-33]; more recently, it has been reported that transgenic plants from *Camelina sativa*, a member of the Brassicacae, were able to accumulate about 4% of both DHA and EPA [34]. However, most of these species have a low growth rate, thus generating low biomass yields. Other works reported high yields for the production of EPA exclusively, using either the conventional Δ6-pathway or the alternative Δ-8-pathway (see [4] and references therein). Notably, both strain engineering and optimization of downstream processing have been carried out in *Y. lipolytica* to achieve a 25% of EPA in the yeast biomass [35].

Our work describes the development of a powerful synthetic biology tool for the industrial fungus *A. gossypii*, which will accelerate the construction of engineered strains and will permit the use of

combinatorial approaches to optimise metabolic fluxes otherwise unaffordable. In addition, here we proved the methodology useful for the production of high-value nutritional lipids. This represents a proof of concept about the production of PUFAs in *A. gossypii*, which is an excellent biotechnological chassis with several advantages: rapid growth and biomass generation, high productivity and accumulation of lipids, efficient utilization of waste streams from industrial processes for fermentation and the strong capacity of overproducing vitamins, nucleosides and lipds, which can be used for food and feed purposes [9, 11-13, 16-19]. Thus, we foresee that additional engineering of the pathway in new *A. gossypii* strains, together with the optimization of culture conditions and downstream processing, will allow at considering *A. gossypii* as a good candidate for the sustainable production of PUFAs.

Methods

A. gossypii strains and growth conditions. The *A. gossypii* ATCC 10895 strain was used and considered a wild-type strain. Other *A. gossypii* strains used in the study are listed in Table S2. *A. gossypii* cultures were initiated with spores (10^6 spores per liter) and carried out at 28°C in MA2 rich medium [7]. Cultures for PUFA production were grown for 3 days at 28°C. *A. gossypii* transformation, its sporulation conditions and spore isolation were as described previously [7]. Concentrations of 250 mg/L for geneticin (G418) (Gibco-BRL) were used where indicated.

Golden Gate Assembly Method. All the modules for the assembly of the overexpression cassettes were PCR-amplified either using *A. gossypii* genomic DNA or synthetic codon-optimized ORFs (Integrated DNA Technologies, USA) (see Supporting Information File S1 and Table S3 for sequences). PCR primers included sequences of 4-nucleotide (nt) overhangs flanked by *Bsa*I recognition sites, which serve for the assembly of the whole overexpression cassette in a single-step, one-pot reaction, into a destination vector carrying compatible 4-nt overhangs (Figure S1). The PCR products consisting of the module sequence plus the specific 4-nt overhangs flanked by *Bsa*I recognition sites are cloned into the pBluescript-SK II vector, thus generating a Golden Gate library of exchangeable modules for integration, selection and expression (a summary of available modules is shown in Figure 2A). To ensure an organized and efficient assembly of all the modules that comprised the overexpression cassette, the 4-nt sticky ends of each module are designed following a head-to-tail strategy. Accordingly, the assembly of the modules can only occur in a controlled order and orientation into a destination vector that contains a spectinomycin resistance marker (spc^R) and compatible 4-nt sticky ends flanked by *Sap*I recognition sites (Figure 2B-C). For the assembly of the overexpression cassette, equimolar amounts of each plasmid (typically 100ng) were used in a one-pot assembly reaction with the following conditions on a thermocycler: 30 cycles of 37ºC for 3 min and 16ºC for 4 min; 50ºC for 5 min and 80ºC for 5 min.

The assembled plasmids were selected in spectinomycin/kanamycin-containing LB plates and confirmed by restriction analysis and DNA sequencing. Then, the overexpression cassettes were cleaved by *Sap*I digestion and used for *A. gossypii* transformation (Figure 2C). Positive clones were selected in G418-containing medium. Homokaryon clones were obtained by sporulation of

the primary transformants. The correct genomic integration of each overexpression cassette was confirmed by analytical PCR followed by DNA sequencing. The *loxP* repeated inverted sequences present in the *loxP-KanMX-loxP* marker enabled the selection marker to be eliminated and subsequently reused by expressing a Cre recombinase, as described elsewhere [14].

Lipid extraction and analysis. Lipid extraction for gravimetric quantitative analysis was performed as previously described [12]. The mycelial biomass was collected by filtration, lyophilized and equal volumes of methanol and chloroform were added to the mycelium powder and mixed vigorously by vortex. Then, ½ volume of H_2O was added and mixed again. After centrifuging for 5 min. at 2000 r.p.m., the lower organic phase was collected and the total fatty acids content was determined gravimetrically after evaporation of organic solvents.

Lipid extraction and analysis for GC-MS was carried out as described elsewhere [12]. Briefly, lyophilized biomass was resuspended in 1 mL of 97.5% methanol and 2.5% sulfuric acid with an internal standard. The samples were incubated at 80ºC for 1.5 hours and the transesterification reaction was stopped by the addition of 1.5 mL H_2O. Then, 0.45 mL of hexane were added and the mixture was vigorously stirred. The upper phase was recovered after centrifugation for 5 min. at 2500 r.p.m. Methyl esters of fatty acids dissolved in hexane were analysed on a gas chromatograph coupled to a mass spectrometer (GC-MS). GC-MS was carried out using the GC17 Shimazdy GC and Shimazdy QP5000 MS. A column DB-5 (30 m, 0.25 mm, 25 µm) was used. The conditions were as follows: helium was used with a flow of a 1.3 mL/min as a carrier gas, with a Split-ratio 60:1. The injector temperature was 270ºC and the interface temperature was 290ºC (initial temperature of 90ºC for 5 minutes, a ramp of 12ºC/min to 190ºC, and a ramp of 4ºC/min to 290ºC). The fatty acids were identified by comparison with the methyl esters of fatty acids of standard commercial sample (FAME32; Supelco). The total quantification of fatty acids was carried out following the method of standard internal pattern using 50 µg of heptadecanoic acid C17:0 (Sigma).

Quantitative Real-Time PCR. Quantitative real-time PCR (qRT-PCR) was performed with a LightCycler 480 real-time PCR instrument (Roche), using SYBR Green I master mix (Roche) and following the manufacturer's instructions. Total RNA samples were obtained as described

previously [10] and cDNA samples were prepared using the Transcriptor First Strand cDNA Synthesis Kit (Roche). All real-time PCR reactions were performed in duplicate and in two independent experiments. Quantitative analyses were carried out using the LightCycler 480 software.

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Author Contribution: JLR conceived the pivotal idea of the study, designed the experiments and supervised the work. RL-A performed most of the experiments. AJ performed some experiments. AJ, JLR and RL-A drafted the manuscript and AJ wrote the paper.

Conflict of Interest: The authors declare that they have no competing interests

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Supporting Information

Figure S1. Example of a *Bsa*I-based Golden Gate assembly of two DNA fragments into a destination vector. The overhangs sticky ends generated by *Bsa*I digestion are highlighted in red, green and blue colours.

Figure S2. Validation of genomic integration sites. A *RIB5* wild-type allele integrated at different genomic loci is able to restore the synthesis of riboflavin in a *rib5*Δ mutant.

Table S1. Omega fatty acids profile in the engineered strains of *A. gossypii*.

Table S2. *A. gossypii* strains used in this study.

Table S3. List of primers used for the amplification of the GG modules.

Supporting Information File S1. Sequences of the codon-optimized synthetic genes used in this work.

Supporting Information File S2. Gene expression clustering from RNAseq of the wild-type strain of *A. gossypii*.

References

1. Ledesma-Amaro, R.; Nicaud, J. M., Yarrowia lipolytica as a biotechnological chassis to produce usual and unusual fatty acids. *Prog Lipid Res* **2015,** *61*, 40-50.
2. Swanson, D.; Block, R.; Mousa, S. A., Omega-3 fatty acids EPA and DHA: health benefits throughout life. *Adv Nutr* **2012,** *3* (1), 1-7.
3. Bellou, S.; Triantaphyllidou, I.-E.; Aggeli, D.; Elazzazy, A. M.; Baeshen, M. N.; Aggelis, G., Microbial oils as food additives: recent approaches for improving microbial oil production and its polyunsaturated fatty acid content. *Current Opinion in Biotechnology* **2016,** *37*, 24-35.
4. Mao, X.; Liu, Z.; Sun, J.; Lee, S. Y., Metabolic engineering for the microbial production of marine bioactive compounds. *Biotechnol Adv* **2017,** *35* (8), 1004-1021.
5. Tavares, S.; Grotkjaer, T.; Obsen, T.; Haslam, R. P.; Napier, J. A.; Gunnarsson, N., Metabolic engineering of Saccharomyces cerevisiae for production of Eicosapentaenoic Acid, using a novel {Delta}5-Desaturase from Paramecium tetraurelia. *Appl Environ Microbiol* **2011,** *77* (5), 1854-61.
6. Xue, Z.; Sharpe, P. L.; Hong, S. P.; Yadav, N. S.; Xie, D.; Short, D. R.; Damude, H. G.; Rupert, R. A.; Seip, J. E.; Wang, J.; Pollak, D. W.; Bostick, M. W.; Bosak, M. D.; Macool, D. J.; Hollerbach, D. H.; Zhang, H.; Arcilla, D. M.; Bledsoe, S. A.; Croker, K.; McCord, E. F.; Tyreus, B. D.; Jackson, E. N.; Zhu, Q., Production of omega-3 eicosapentaenoic acid by metabolic engineering of Yarrowia lipolytica. *Nat Biotechnol* **2013,** *31* (8), 734-40.
7. Jiménez, A.; Santos, M. A.; Pompejus, M.; Revuelta, J. L., Metabolic engineering of the purine pathway for riboflavin production in Ashbya gossypii. *Applied and Environmental Microbiology* **2005,** *71* (10), 5743-5751.
8. Jiménez, A.; Santos, M. A.; Revuelta, J. L., Phosphoribosyl pyrophosphate synthetase activity affects growth and riboflavin production in Ashbya gossypii. *BMC biotechnology* **2008,** *8*, 67.
9. Ledesma-Amaro, R.; Serrano-Amatriain, C.; Jiménez, A.; Revuelta, J. L., Metabolic engineering of riboflavin production in Ashbya gossypii through pathway optimization. *Microb Cell Fact* **2015,** *14* (1), 163.
10. Mateos, L.; Jiménez, A.; Revuelta, J. L.; Santos, M. A., Purine biosynthesis, riboflavin production, and trophic-phase span are controlled by a Myb-related transcription factor in the fungus Ashbya gossypii. *Appl Environ Microbiol* **2006,** *72* (7), 5052-60.
11. Revuelta, J. L.; Ledesma-Amaro, R.; Lozano-Martinez, P.; Diaz-Fernandez, D.; Buey, R. M.; Jiménez, A., Bioproduction of riboflavin: a bright yellow history. *J Ind Microbiol Biotechnol* **2017,** *44* (4-5), 659-665.
12. Díaz-Fernández, D.; Lozano-Martinez, P.; Buey, R. M.; Revuelta, J. L.; Jiménez, A., Utilization of xylose by engineered strains of Ashbya gossypii for the production of microbial oils. *Biotechnol Biofuels* **2017,** *10*, 3.
13. Ledesma-Amaro, R.; Lozano-Martinez, P.; Jiménez, A.; Revuelta, J. L., Engineering Ashbya gossypii for efficient biolipid production. *Bioengineered* **2015,** *6* (2), 119-23.
14. Ledesma-Amaro, R.; Santos, M. A.; Jiménez, A.; Revuelta, J. L., Strain Design of Ashbya gossypii for Single-Cell Oil Production. *Appl Environ Microbiol* **2014,** *80* (4), 1237-44.
15. Ledesma-Amaro, R.; Santos, M. A.; Jiménez, A.; Revuelta, J. L., Tuning single-cell oil production in Ashbya gossypii by engineering the elongation and desaturation systems. *Biotechnol Bioeng* **2014,** *111* (9), 1782-91.
16. Lozano-Martinez, P.; Buey, R. M.; Ledesma-Amaro, R.; Jiménez, A.; Revuelta, J. L., Engineering Ashbya gossypii strains for de novo lipid production using industrial by-products. *Microb Biotechnol* **2017,** *10* (2), 425-433.
17. Ledesma-Amaro, R.; Buey, R. M.; Revuelta, J. L., Increased production of inosine and guanosine by means of metabolic engineering of the purine pathway in Ashbya gossypii. *Microb Cell Fact* **2015,** *14*, 58.
18. Ledesma-Amaro, R.; Buey, R. M.; Revuelta, J. L., The filamentous fungus Ashbya gossypii as a competitive industrial inosine producer. *Biotechnol Bioeng* **2016,** *113* (9), 2060-3.

19. Ledesma-Amaro, R.; Jiménez, A.; Santos, M. A.; Revuelta, J. L., Biotechnological production of feed nucleotides by microbial strain improvement. *Process Biochemistry* **2013,** *48* (9), 1263-1270.
20. Serrano-Amatriain, C.; Ledesma-Amaro, R.; Lopez-Nicolas, R.; Ros, G.; Jiménez, A.; Revuelta, J. L., Folic Acid Production by Engineered Ashbya gossypii. *Metab Eng* **2016**.
21. Aguiar, T. Q.; Silva, R.; Domingues, L., Ashbya gossypii beyond industrial riboflavin production: A historical perspective and emerging biotechnological applications. *Biotechnol Adv* **2015,** *33* (8), 1774-86.
22. Gattiker, A.; Rischatsch, R.; Demougin, P.; Voegeli, S.; Dietrich, F. S.; Philippsen, P.; Primig, M., Ashbya Genome Database 3.0: a cross-species genome and transcriptome browser for yeast biologists. *BMC Genomics* **2007,** *8*, 9.
23. Ledesma-Amaro, R.; Kerkhoven, E. J.; Revuelta, J. L.; Nielsen, J., Genome scale metabolic modeling of the riboflavin overproducer Ashbya gossypii. *Biotechnol Bioeng* **2013**.
24. Ratledge, C., Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. *Biochimie* **2004,** *86* (11), 807-815.
25. Ji, X. J.; Ren, L. J.; Nie, Z. K.; Huang, H.; Ouyang, P. K., Fungal arachidonic acid-rich oil: research, development and industrialization. *Crit Rev Biotechnol* **2014,** *34* (3), 197-214.
26. Ashbya Genome Database. http://agd.unibas.ch/Ashbya_gossypii/index.html.
27. Xue, Z.; He, H.; Hollerbach, D.; Macool, D. J.; Yadav, N. S.; Zhang, H.; Szostek, B.; Zhu, Q., Identification and characterization of new Delta-17 fatty acid desaturases. *Appl Microbiol Biotechnol* **2013,** *97* (5), 1973-85.
28. Damude, H. G.; Zhang, H.; Farrall, L.; Ripp, K. G.; Tomb, J. F.; Hollerbach, D.; Yadav, N. S., Identification of bifunctional delta12/omega3 fatty acid desaturases for improving the ratio of omega3 to omega6 fatty acids in microbes and plants. *Proc Natl Acad Sci U S A* **2006,** *103* (25), 9446-51.
29. Robert, S. S.; Petrie, J. R.; Zhou, X. R.; Mansour, M. P.; Blackburn, S. I.; Green, A. G.; Singh, S. P.; Nichols, P. D., Isolation and characterisation of a delta5-fatty acid elongase from the marine microalga Pavlova salina. *Mar Biotechnol (NY)* **2009,** *11* (3), 410-8.
30. Zhou, X. R.; Robert, S. S.; Petrie, J. R.; Frampton, D. M.; Mansour, M. P.; Blackburn, S. I.; Nichols, P. D.; Green, A. G.; Singh, S. P., Isolation and characterization of genes from the marine microalga Pavlova salina encoding three front-end desaturases involved in docosahexaenoic acid biosynthesis. *Phytochemistry* **2007,** *68* (6), 785-96.
31. Hamilton, M. L.; Haslam, R. P.; Napier, J. A.; Sayanova, O., Metabolic engineering of Phaeodactylum tricornutum for the enhanced accumulation of omega-3 long chain polyunsaturated fatty acids. *Metab Eng* **2014,** *22*, 3-9.
32. Amiri-Jami, M.; Abdelhamid, A. G.; Hazaa, M.; Kakuda, Y.; Griffths, M. W., Recombinant production of omega-3 fatty acids by probiotic Escherichia coli Nissle 1917. *FEMS Microbiol Lett* **2015,** *362* (20).
33. Amiri-Jami, M.; Griffiths, M. W., Recombinant production of omega-3 fatty acids in Escherichia coli using a gene cluster isolated from Shewanella baltica MAC1. *J Appl Microbiol* **2010,** *109* (6), 1897-905.
34. Usher, S.; Han, L.; Haslam, R. P.; Michaelson, L. V.; Sturtevant, D.; Aziz, M.; Chapman, K. D.; Sayanova, O.; Napier, J. A., Tailoring seed oil composition in the real world: optimising omega-3 long chain polyunsaturated fatty acid accumulation in transgenic Camelina sativa. *Sci Rep* **2017,** *7* (1), 6570.
35. Xie, D.; Miller, E.; Sharpe, P.; Jackson, E.; Zhu, Q., Omega-3 production by fermentation of Yarrowia lipolytica: From fed-batch to continuous. *Biotechnol Bioeng* **2017,** *114* (4), 798-812.