Engineering Ashbya gossypii strains for de novo lipid production using industrial by-products

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Summary
Ashbya gossypii is a filamentous fungus that naturally overproduces riboflavin, and it is currently exploited for the industrial production of this vitamin. The utilization of A. gossypii for biotechnological applications presents important advantages such as the utilization of low-cost culture media, inexpensive downstream processing and a wide range of molecular tools for genetic manipulation, thus making A. gossypii a valuable biotechnological chassis for metabolic engineering. A. gossypii has been shown to accumulate high levels of lipids in oil-based culture media; however, the lipid biosynthesis capacity is rather limited when grown in sugar-based culture media. In this study, by altering the fatty acyl-CoA pool and manipulating the regulation of the main Δ9 desaturase gene, we have obtained A. gossypii strains with significantly increased (up to fourfold) de novo lipid biosynthesis using glucose as the only carbon source in the fermentation broth. Moreover, these strains were efficient biocatalysts for the conversion of carbohydrates from sugarcane molasses to biofuels, able to accumulate lipids up to 25% of its cell dry weight. Our results represent a proof of principle showing the promising potential of A. gossypii as a competitive microorganism for industrial biodiesel production using cost-effective feed stocks.

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Microbial Biotechnology (2017) 10(2), 425–433
doi:10.1111/1751-7915.12487

Funding Information
This work was supported in part by BASF and by grant BIO2014-56930-P to José Luis Revueltas. Rubén M Buey is supported by a ‘Ramón y Cajal’ contract from the Spanish Ministerio de Economía y Competitividad.

Introduction
During the last years, studies unravelling the lipid metabolic pathways in microorganisms have boosted the application of systems metabolic engineering to produce biofuels that could be used to produce biofuels and oleochemicals (Beopoulos et al., 2011). Lipid-derived fuels from renewable or even waste feedstocks and advanced biofuels avoid the severe inconveniences of first- and second-generation biofuels such as competition with food industry, dependence on climate season and longer processing cycle (Stephanopoulos, 2007; Beopoulos et al., 2011; Peralta-Yahya et al., 2012). Lipid biofuels provide a sustainable alternative for fossil fuels that might help to reduce the carbon footprint. Additionally, bio-based oleochemicals could substitute petroleum-based chemically synthesized compounds with interest in pharma, food and polymer industries (Ledesma-Amaro et al., 2014b). Thus, the combination of an engineered microorganism host and a cost-effective feedstock is nowadays a major challenge to produce biofuels and oleochemicals in an environmentally and economically feasible manner.

The model microorganism Saccharomyces cerevisiae and the oleaginous yeast Yarrowia lipolytica have been extensively manipulated by means of rational metabolic engineering approaches to optimize lipid production (Vorapreeda et al., 2012; Blazeck et al., 2014; Run-guphan and Keasling, 2014; Kavšek et al., 2015). Other oleaginous microorganisms such as Rhodosporidium toruloides have also been studied for fatty acid-derived products because of its natural ability to accumulate triacylglycerol (Fillet et al., 2015).

Ashbya gossypii is a filamentous fungus first identified for its natural capacity to overproduce riboflavin (vitamin B2) and, currently, more than half of the worldwide riboflavin industrial production relies on A. gossypii fermentation (Stahmann et al., 2000; Schwechheimer et al., 2016). A. gossypii is a very convenient fungus for industrial use because it can be readily grown in industrial waste-based culture media. These media include low-cost oils (Schwechheimer et al., 2016), glycerol (Ribeiro et al., 2012) or sucrose (Pridham and Raper, 1950), the main carbon source of sugarcane molasses (Hashizume et al., 1966). This and other advantages have stimulated the use of A. gossypii not only for industrial scale riboflavin production, but also for nucleoside production.
We have previously reported *A. gossypii* strains with compromised lipid β-oxidation which are able to accumulate lipids up to 70% of the cell dry weight when grown in culture media supplemented with 2% oleic acid. Nevertheless, when grown in glucose-based media without lipid supplementation, these strains only accumulated up to 10% of their cell dry weight (Ledesma-Amaro *et al.*, 2014a). The development of efficient biocatalysts for the production of biolipids requires not only the conversion of low-cost oily feedstocks into high-value oils, but also a high-yield conversion of carbohydrates to biolipids. In this regard, major bottlenecks exist in the biosynthesis of lipids due to feedback inhibition of lipidogenic enzymes (Fig. 1): acyl-CoA esters regulate the activity of the fatty acid synthase (FAS), the acetyl-CoA carboxylase (*ACC1*) and the Δ9 desaturase (*OLE1*; Chen *et al.*, 2014; Neess *et al.*, 2015). Furthermore, saturated fatty acids also exert a negative effect over the *ACC1* enzyme, thus regulating their own synthesis (Qiao *et al.*, 2015).

Here, we aimed at developing of *A. gossypii* strains that significantly increased lipid production using sugar-based culture media through the manipulation of two major bottlenecks in lipid metabolism: (i) altering the fatty acyl-CoA pool and the subsequent feedback inhibition of lipidogenic genes and (ii) manipulating the regulation of the main Δ9 desaturase encoded by the *OLE1* gene (Fig. 1). We show that rewiring the regulation of lipogenesis can increase significantly the conversion of carbohydrates to lipids in *A. gossypii*. In addition, we demonstrate that engineered strains of *A. gossypii* are able to produce biolipids when grown on a very simple culture medium consisting of sugarcane molasses and tap water. Our results represent a proof of principle showing the promising potential of *A. gossypii* as a competitive microorganism for industrial biolipid production using cost-effective feedstocks.

**Results**

Aiming at generating *A. gossypii* strains with improved lipid de novo biosynthesis, using glucose as the only carbon source, we have manipulated two known bottlenecks for lipid metabolism (Fig. 1): (i) insertion of the first double bond in palmitic and stearic acid by Δ9 desaturases and (ii) alteration of the intracellular concentration of the fatty acyl-CoA pool.

*OLE1* endogenous regulation is a bottleneck for the de novo lipid biosynthesis in *A. gossypii*

*Ashbya gossypii* has two identified and characterized Δ9 desaturases codified by the genes *AgOLE1* and...
AgOLE2 that are responsible for the insertion of the first desaturation in stearic and palmitic acid (Fig. 1; Lozano-Martinez et al., 2016). The simultaneous overexpression of these two genes in A. gossypii only slightly increased total fatty acid accumulation in glucose-based medium (up to 1.2-fold with respect to wild type; Lozano-Martinez et al., 2016). Thereby, contrary to what has been reported for Y. lipolytica (Qiao et al., 2015), the overexpression of AgOLE1/OLE2 is not enough to significantly improve the lipid de novo biosynthesis in A. gossypii.

We then decided to study AgOLE1 regulation.

In S. cerevisiae, MGA2 – and its homologous SPT23 – are the main regulators of OLE1: Mga2p has been shown not only to activate OLE1 transcription (Zhang et al., 1999; Jiang et al., 2001, 2002; Auld et al., 2006) but also to stabilize OLE1 mRNA transcript when the cells are grown in fatty acid free medium and destabilize it when the cells are exposed to unsaturated fatty acids (Kandasamy et al., 2004). MGA2 codifies for an endoplasmic reticulum membrane protein (Mga2p), but the C-terminal proteolytic cleavage converts Mga2p into a cytoplasmic protein that can be transported to the nucleus (Martin et al., 2007; Liu et al., 2015). The deletion of MGA2 in S. cerevisiae has modest effect on cell fitness, but the double knockout of MGA2 and its homologous SPT23 results in an inviable mutant in the absence of unsaturated fatty acids in the culture medium (Zhang et al., 1999). The overexpression of the cleaved version of MGA2 in S. cerevisiae led to a 1.2-fold increase in triacylglycerides with respect to wild type (Kaliszewski et al., 2008), most probably due to OLE1 overexpression (Chellappa et al., 2001).

Prompted by these results, we decided to investigate the effect on lipid accumulation of the manipulation of the gene ACR165W (AgMGA2), the Ashbya’s homologue of S. cerevisiae MGA2/SPT23 (Dietrich et al., 2004). In glucose-based media, AgMGA2 disruption (mga2Δ) caused a slight increase in total lipid accumulation, in contrast to its overexpression (P<sub>GPD</sub>-MGA2) that showed a significant increase with respect to the wild-type strain (Table 1). These results agree with previous reports in S. cerevisiae, where the MGA2 orthologues stabilize OLE1 mRNA transcript when the cells are grown in fatty acid free medium (Kandasamy et al., 2004). On the other hand, MGA2 might destabilize OLE1 mRNA transcript when the cells are exposed to unsaturated fatty acids, as reported for S. cerevisiae (Kandasamy et al., 2004), which would result in decreased lipid accumulation (Table 1).

Interestingly, AgMGA2 disruption does not confer auxotrophy for unsaturated fatty acids (not shown), in contrast to what has been described for S. cerevisiae (Kandasamy et al., 2004). This might indicate that MGA2 might be required for maximal transcriptional activation of OLE1/2, but it is possible that OLE1/2 might be expressed at basal levels in the absence of MGA1. In this context, MGA2 deletion would not confer auxotrophy for unsaturated fatty acids. At this point, we do not know if there are additional genes implied in OLE1/2 transcriptional activation, but no additional paralogues of MGA2 exist in the genome of A. gossypii.

The increase in lipid accumulation was significantly higher when a C-terminal truncated version of AgMGA2 was expressed (mga2<sup>-1C-term</sup>), reaching more than 10% of the cell dry weight (Table 1), similarly to what has been reported for S. cerevisiae (Kaliszewski et al., 2008). Remarkably, in media supplemented with oleic acid, P<sub>GPD</sub>-MGA2 strains showed a strong decrease in total lipid accumulation with respect to the wild type, in contrast to both the mga2Δ and mga2<sup>-1C-term</sup> strains that showed no significant differences with the wild type (Table 1).

The fatty acyl-CoA pool is a bottleneck for the de novo lipid biosynthesis in A. gossypii

We next decided to study how the alteration of the fatty acyl-CoA pool could influence the de novo lipid biosynthesis in A. gossypii. The intracellular acyl-CoA pool is extensively regulated by the counteraction of acyl-CoA synthetases and acyl-CoA thioesterases (Black and DiRusso, 2007; Chen et al., 2014). Acyl-CoA-thioesterases catalyse the conversion of activated fatty acids into free fatty acids, which is the reverse reaction catalysed by fatty acyl-CoA synthetases (Fig. 1).

We first disrupted the main fatty acyl-CoA synthetase in A. gossypii: AgFAA1, which is the homolog of FAA1 and FAA4 in S. cerevisiae. Our results showed that AgFAA1 is essential when the only carbon source in the

| Strain                  | TFA (%), MA2-8G | TFA (%), MA2-1G-2O |
|-------------------------|----------------|-------------------|
| Wild type               | 5.30 ± 0.4     | 23.53 ± 0.4       |
| P<sub>GPD</sub>-MGA2    | 8.62 ± 0.3     | 15.61 ± 2.1       |
| ΔMGA2                   | 6.63 ± 0.1     | 24.08 ± 1.5       |
| MGA2-<sup>-1</sup>C-term| 10.47 ± 0.1    | 24.63 ± 0.9       |
| P<sub>GPD</sub>-FAA1    | 5.90 ± 0.9     | 11.55 ± 1.9       |
| ΔFAA1                   | 2.17 ± 0.4     | 12.88 ± 2.1       |
| P<sub>GPD</sub>-TES<sup>Δ</sup> | 7.89 ± 0.4 | n.d.              |
| P<sub>GPD</sub>-ACOT5<sup>Δ</sup> | 9.85 ± 0.1 | n.d.              |
| MGA2-<sup>-1</sup>C-term/P<sub>GPD</sub>-ACOT5<sup>Δ</sup> | 20.01 ± 0.2 | n.d.              |
culture medium is oleic acid (data not shown) and its disruption (\textit{faa1}Δ strain) significantly decreased lipid accumulation as compared to the wild type when grown on glucose-containing media (Table 1). This finding further supports that \textit{AgFAA1} is the main acyl-CoA synthetase in \textit{A. gossypii}, in agreement with the phenotype of the \textit{S. cerevisiae} \textit{faa1}Δ/\textit{faa4}Δ strain, which is also unable to grow in fatty acid-based media (Black and DiRusso, 2007). However, the marked decrease in lipid accumulation in the strain \textit{faa1}Δ in glucose-based media differs from previous results reported for \textit{S. cerevisiae}, where lipid accumulation was not significantly changed with respect to wild type (Færgeman \textit{et al.}, 2001; Black and DiRusso, 2007; Chen \textit{et al.}, 2014). On the other hand, our results agree with the sharp decrease observed when the strain \textit{faa1}Δ of \textit{Y. lipolytica} is grown on oleic acid-containing media (Dulermo \textit{et al.}, 2015). This might indicate that \textit{AgFAA1} has additional functions, apart from the one shown in Fig. 1, in the lipid metabolism of \textit{A. gossypii} that remain unknown. Interestingly, \textit{AgFAA1} overexpression (\textit{PGPD-FAA1}) also decreased lipid accumulation in \textit{A. gossypii} when the strain is grown in media containing 2% of oleic acid, despite it has no significant effects in glucose-based media (Table 1). This might happen because in media-containing oleic acid \textit{FAA1} overexpression might greatly increase the cytoplasmic levels of fatty acyl-CoA that would result in the inhibition of lipidogenic genes and, therefore, the observed decrease in lipid accumulation. Moreover, this result agrees with previous reports describing that the alteration of the intracellular acyl-CoA pool has an inhibitory effect on lipidogenic genes (Færgeman and Knudsen, 1997) and also induced the expression of the genes involved in lipid degradation (Færgeman \textit{et al.}, 2001).

We then intended to alter the fatty acyl-CoA pool by the overexpression of acyl-CoA-thioesterase genes. Acyl-CoA esters are known to repress fatty acid synthesis (Fig 1) by inhibiting several lipidogenic enzymes, such as \textit{FAS}, \textit{ACC1} and \textit{OLE1} (Bortz and Lynen, 1963; Sumper and Träuble, 1973; Choi \textit{et al.}, 1996; Færgeman and Knudsen, 1997). Thereby, we hypothesized that the decrease in the fatty acyl-CoA intracellular pool (by conversion to free fatty acids) could upregulate lipid accumulation. To test this hypothesis, we constructed two \textit{A. gossypii} strains that ectopically overexpress in their cytoplasm two peroxisomal acyl-CoA thioesterase enzymes: (i) \textit{PGPD-TEST1}\textsuperscript{\textit{cyt}} that overexpress \textit{A. gossypii} \textit{TES1} (the homologue of \textit{TES1} in \textit{S. cerevisiae}) and (ii) \textit{PGPD-ACOT5}\textsuperscript{\textit{cyt}} that overexpress \textit{Mus musculus} \textit{ACOT5}, which has been previously shown to increase the accumulation of free fatty acids in \textit{S. cerevisiae} (Chen \textit{et al.}, 2014). Both genes encode fatty acyl-CoA thioesterases involved in fatty acid degradation in the peroxisome (Jones and Gould, 2000; Westin \textit{et al.}, 2004; Maeda \textit{et al.}, 2006; Chen \textit{et al.}, 2014) and contain the ‘SKL’ prototypical C-terminal peroxisomal targeting signal (PTS) that it is both necessary and sufficient for directing cytosolic proteins to peroxisomes (Gould \textit{et al.}, 1987). Interestingly, total fatty acid quantification of both strains grown in glucose-based medium showed a significant increase (up to twofold) with respect to the wild type (Table 1), suggesting that an excess of cytoplasmatic fatty acyl-CoA thioesterase activity results in a decreased acyl-CoA pool that relieves the feedback inhibition of lipidogenic genes (Fig. 1). The excess of free fatty acids in the cytoplasm can be readily excreted to the culture media and, accordingly, the two engineered strains excreted approximately fivefold more fatty acids (118.25 ± 0.4 and 122.38 ± 0.4 mg l\textsuperscript{−1} for \textit{PGPD-TEST1}\textsuperscript{\textit{cyt}} and \textit{PGPD-ACOT5}\textsuperscript{\textit{cyt}} respectively) than the wild type (22.00 ± 0.6 mg l\textsuperscript{−1}).

We next combined the two most favourable modifications (Table 1) into a single strain (\textit{MGA2-1C-term/PGPD-ACOT5}\textsuperscript{\textit{cyt}}) and observed an additive effect of both modifications (Table 1). Remarkably, this \textit{A. gossypii} strain accumulates more than 20% of its dry cell weight.

\textit{Sugarcane molasses are a very convenient carbon source for biolipid production in \textit{A. gossypii}}

The results obtained with the strain \textit{MGA2-1C-term/PGPD-ACOT5}\textsuperscript{\textit{cyt}} together with the advantages for industrial use convert \textit{A. gossypii} into a very promising microorganism for biolipid production using sugar-based culture media formulations. We therefore studied the use of convenient culture media for industrial use. To this end, we tested sugarcane molasses as the unique carbon source for the culture media of \textit{A. gossypii}.

Molasses, from sugarcane or beet, mainly contains fructose, sucrose and glucose. This sugar is an industrial by-product from sugar manufacturing, and it is considered an ideal raw material for cheap medium culture formulations (Chao \textit{et al.}, 2013). Indeed, sugarcane molasses have been previously proved to be acceptable carbon sources for lipid production in \textit{Y. lipolytica} (Gadjos \textit{et al.}, 2015), as well as for ethanol and butanol production in \textit{S. cerevisiae} (Ni \textit{et al.}, 2012; Arshad \textit{et al.}, 2014).

Remarkably, in contrast to the wild-type strain which slightly decreased lipid accumulation in molasses with respect to glucose-based medium, the \textit{MGA2-1C-term/PGPD-ACOT5}\textsuperscript{\textit{cyt}} \textit{A. gossypii} engineered strain increased lipid accumulation up to 25% of its dry cell weight (Fig. 2). We then quantitatively characterized the lipid profile of the engineered strain when grown in glucose and molasses-based culture media. As can be observed in Fig. 3, there are no significant changes between both media. The strain \textit{MGA2-1C-term/PGPD-ACOT5}\textsuperscript{\textit{cyt}}
showed a slight increase in saturated C16:1 and C18:1, in detriment of C16:0 and C18:0 fatty acids (Fig. 3), as an expected consequence of the upregulation of the Δ9 desaturase AgOLE1.

Altogether, by manipulating two genes, we have obtained an A. gossypii strain able to accumulate up to 25% of its dry cell weight in a very convenient culture media composed of sugarcane molasses and tap water. Furthermore, we envisage that this number can be readily increased through a systematic optimization of the culture medium composition as well as the fermentation conditions. Eventually, further genetic manipulations of this strain by means of random and/or rational modifications could also increase this number.

Discussion

Ashbya gossypii has a large capacity to accumulate lipids when grown in media-containing oleic acid (Ledesma-Amaro et al., 2014a). Encouraged by this result, we aimed at further manipulating A. gossypii to optimize the de novo lipid biosynthesis and have obtained strains with increased de novo lipid biosynthesis, rather than lipid accumulation. These manipulations enable the utilization of A. gossypii as an efficient biocatalyst for the production of biolipids from sugar-based by-products such as molasses. The most productive strain contained only two modifications that resulted in additive effects on lipid accumulation: a truncated version of MGA2, a main regulator of OLE1 (the main Δ9 desaturase in A. gossypii) and a heterologously expressed murine thioesterase gene, MmACOT5. These modifications are expected to increase the expression of OLE1 and promote the lipid biosynthetic process. In addition, the expression of a heterologous thioesterase is expected to decrease the cytoplasmic pool of fatty acyl-CoA, thus alleviating the feedback inhibition mechanisms of that this metabolite exerts on lipogenesis. Up to our knowledge, this is the first report on the combination of the manipulation of these two genes in microorganisms to enhance de novo lipid biosynthesis. We envisage that further manipulations will readily increase the percentage of lipid accumulation and, indeed, significant efforts are being directed at present in our laboratory towards this aim.

Although the achieved amount of lipid accumulation is not as high as that reported for S. cerevisiae and/or Y. lipolytica (Kamisaka et al., 2013; Blazeck et al., 2014), it must be stressed here that A. gossypii shows important advantages for industrial production of lipids compared to these yeasts that make of it a promising competitive candidate to be taken into account. First, the biomass from a filamentous fungus can be easily separated from culture media by convenient filtration or sedimentation techniques, easy to implement at industrial level (Zheng et al., 2012). Second, large-scale fermentations are nowadays used for riboflavin production, demonstrating the suitability of this fungus for industrial scale-up. Third, A. gossypii hyphae suffer autolysis in the late stationary phase, and triglycerides could be
easily recovered by centrifugation, avoiding costly cell-disruption processes. Therefore, our results represent a proof of principle showing that A. gossypii is a promising and convenient microorganism that deserves the further investigation of its potential use as a convenient industrial biolipid producer.

On the other hand, one of the disadvantages of using microbial hosts for lipid production is the global cost of the process, which can be notably diminished with the use of alternative feed stocks such as industrial by-products. Thereby, the efficient utilization of alternative sources of carbon will have important economic advantages for the scale-up of lipid production with A. gossypii using a cheap and convenient culture media. Sugarcane molasses represent a cheap industrial by-product consisting on sucrose (up to 50%), nitrogen source, proteins, vitamins and amino acids among others. The use of molasses presents important advantages with respect to other waste products such as lignocellulosic biomass, which needs a costly pre-treatment for its consumption by microorganisms (Stephanopoulos, 2007; Taherzadeh and Karimi, 2008). Interestingly, A. gossypii can grow on molasses without any further modification, contrary to what happens in Y. lipolytica that needs the ectopic overexpression of invertases to degrade sucrose (Gadjos et al., 2015). Thereby, the engineered MGA2-1C-term/PUDP-ACOT5\textsuperscript{\textsuperscript{34}} strain is a promising candidate that deserves future attention.

The lipid profile of the engineered oleaginous strain has a composition in monounsaturated, polyunsaturated and saturated methyl esters that correlate with good biodiesel properties, that is neither high levels of polyunsaturated nor long-chain saturated FAs (Ramos et al., 2009). Thereby, this strain accumulates significant amounts of lipids suitable for biodiesel production. Furthermore, we have recently reported that the modification of the lipid profile by manipulating the elongation and desaturation systems enhances biodiesel properties in A. gossypii (Ledesma-Amaro et al., 2014b). Thus, future experiments in our laboratory will be focused on the modification of the lipid profile in our engineered oleaginous strain.

Altogether, our results demonstrate that A. gossypii is a very promising industrial microorganism that uses a cost-effective feedstock to \textit{de novo} synthetize significant amounts of biolipids that can be used for producing biofuels in an environmentally and economically feasible manner.

**Experimental procedures**

**A. gossypii strains, media and growth conditions**

The A. gossypii strain ATCC10895 was used and considered wild-type strain. The strains were cultured at 28°C using MA2 rich medium during 7 days (Fürster et al., 1999). MA2 is composed of yeast extract, bactopeptone, agar, water and glucose. In this study, for lipid accumulation, the C/N ratio was increased using 8% of glucose (MA2-8G) as carbon source instead of 2%, which is the standard formulation. Alternatively, 1% glucose and 2% oleic acid, previously emulsified by sonication in the presence of 0.02% Tween-40 (MA2-1G-2O), were used. For experiments with molasses, media was prepared with 8% sugarcane molasses (kindly provided by AB Azucarera Iberia S.L.), 0.1% of yeast extract and tap water (MA2-8M). A. gossypii transformation, sporulation conditions and spore isolation have been described elsewhere (Santos et al., 2005). Briefly, DNA was introduced into A. gossypii by electroporation, and primary transformants were isolated in selective medium. Homokaryon transformant clones were obtained by sporulation of the primary heterokaryon transformants and isolated on antibiotic-containing plates with 250 mg l\textsuperscript{-1} of gentamicin (G418). Liquid cultures were initiated from spores and were incubated on an orbital shaker at 200 r.p.m at 28°C.

**Gene manipulation of A. gossypii**

Gene deletion and overexpression were carried out by the construction of recombinant integrative cassettes (Ledesma-Amaro et al., 2014a,b). For gene deletion, a replacement cassette with selection marker (loxP-KanMX-loxP module for G418 resistance) was used. This selection marker is flanked by the repeated inverted sequences loxP, which enable the elimination of the selection marker by the expression of a Cre recombinase (Ledesma-Amaro et al., 2014a). The deletion of the C-terminal part of AgMGA2 was performed by substituting this region by a G418 antibiotic resistance marker. For gene overexpression, a module based on the A. gossypii glycerol 3-phosphate dehydrogenase promoter (P\textsubscript{GPD}) and phosphoglycerate kinase (T\textsubscript{PGK1}) terminator sequences, recombinogenic flanks and the antibiotic selectable marker loxP-KanMX-loxP, was integrated at the STE12 locus. DNA constructs were obtained using Golden Gate methodology (Enger et al., 2008). Genome integration of the deletion and overexpression modules was confirmed by analytical PCR and DNA sequencing.

To ectopically express TES1 and ACOT5 in the cytosol of A. gossypii, we removed the C-terminal prototypical peroxisomal targeting signal (PTS) that it is both necessary and sufficient for directing cytosolic proteins to peroxisomes (Gould et al., 1987). The signal ‘SKL’ that both TES1 and ACOT5 contain is a prototypical PTS and, thereby, its removal avoids peroxisome localization.
Lipid extraction and quantification

Triacylglycerols were extracted and trans-methylated from lyophilized biomass using a modification of the method described by Bligh and Dyer (Bligh and Dyer, 1959). Approximately 200 μg of dried mycelia was mixed with 1 ml of 97.5% methanol/2.5% sulfuric acid and incubated at 80°C for 90 min. The transesterification reaction was stopped by the addition of 1 ml of distilled water. The extraction was performed by mixing the samples with 0.5 ml of hexane and recovery of the upper phase was stopped by the addition of 1 ml of distilled water. This work was supported in part by BASF and by grant BIO2014-56930-P to José Luis Revuelta. Rubén M Buey is supported by a ‘Ramón y Cajal’ contract from the Spanish Ministerio de Economía y Competitividad. Patricia Lozano-Martínez is supported by a postgraduate fellowship (FPI Program) from the Spanish Ministerio de Economía y Competitividad. We thank María Dolores Sánchez and Silvia Domínguez for excellent technical help.

Conflict of interest

The authors declare that they have no competing interests.

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