Journal of Biotechnology 184 (2014) 27–38

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Over-expression of Dof-type transcription factor increases lipid production in *Chlamydomonas reinhardtii*

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ARTICLE INFO

Article history:
Received 30 October 2013
Received in revised form 6 May 2014
Accepted 7 May 2014
Available online 17 May 2014

Keywords:
Biofuel
Chlamydomonas reinhardtii
Lipid
Transcription factor

ABSTRACT

The high demand for less polluting, newer, and cheaper fuel resources has increased the search of the most innovative options for the production of the so-called biofuels. *Chlamydomonas reinhardtii* is a photosynthetic unicellular algae with multiple biotechnological advantages such as easy handling in the laboratory, a simple scale-up to industrial levels, as well as a feasible genetic modification at nuclear and chloroplast levels. Besides, its fatty acids can be used to produce biofuels. Previous studies in plants have found that the over expression of DOF-type transcription factor genes increases the synthesis and the accumulation of total lipids in seeds. In this context, the over-expression of a DOF-type transcription factor in *C. reinhardtii* was applied as approach to increase the amount of lipids. The results indicate higher amounts (around 2-fold) of total lipids, which are mainly fatty acids, in the genetically *C. reinhardtii* modified strains when compared with the non-genetically modified strain. In order to elucidate the possible function of the introduced Dof-type transcription factor, we performed a transcription profile of 8 genes involved in fatty acid biosynthesis and 6 genes involved in glycerolipid biosynthesis, by quantitative real time (qRT-PCR). Differential expression profile was observed, which can explain the increase in lipid accumulation. However, these strains did not show notable changes in the fatty acid profile. This work represents an early effort in generating a strategy to increase fatty acids production in *C. reinhardtii* and their use in biofuel synthesis.

1. Introduction

DNA-binding-with-one-finger (Dof) transcription factors constitute a family of proteins typically composed of 200–500 amino acids and defined as DNA-binding proteins having a highly conserved DOF domain in plants. This domain has been previously reported by Yanagisawa (2002) as: CPRCASRDTKFCYYNNYNTSQPRHFCKGCRRYWTKGGTLRNVPVGGGTRK. All DOF proteins have one copy of this DOF domain generally located in the N-terminal region, however diverged amino-acid sequences outside the DOF domain have been reported (Yanagisawa, 2002).

The conserved DOF domain seems to provide all DOF proteins with a similar DNA-binding specificity. This domain presents four conserved cysteine residues but the amino acid sequence of the

Abbreviations: AS, acetosyringone; BHT, butylated hydroxytoluene; dNTPs, deoxyribonucleotides; DOF, DNA-binding-with-one-finger; dcw, dry cell weight; FAMES, fatty acid methyl esters; FA, fatty acids; GC-FID, gas chromatography with flame ionization detector; Kan-r, kanamycin resistant; LB, Luria Bertani; ML, maximum likelihood; ME, Minimum Evolution; NJ, neighbor-joining; PUFA, polyunsaturated fatty acids; TAG, triacylglycerides; TAP, Tris-acetate-phosphate; WT, wild type; *BCR1*, biotin carboxylase; *FAT1*, acyl carrier protein thioesterase; *PGP1*, phosphatidylglycerophosphate synthase; *KAS2*, β-ketoacyl-ACP synthase; *SQD2*, sulfolipid synthase; *ENR1*, enoyl-ACP-reductase; *HAD1*, β-hydroxyacyl-ACP dehydratase; *SQD1*, UDP-sulfoquinovose synthase; *CDS1*, CDP-DAG synthetase; *ACP1*, acyl carrier protein; *KAS3*, β-ketoacyl-ACP synthase; *DGD1*, digalactosyldiacylglycerol synthase; *BCX1*, β-carboxyltransferase; KAR1, β-ketoacyl-ACtP reductase; MGD1, monogalactosyldiacylglycerol synthase.

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http://dx.doi.org/10.1016/j.jbiotec.2014.05.003
0168-1656/

DOF domain and the arrangement of the cysteine (C_2C_2-type zinc finger) residues differ from other zinc fingers (Yanagisawa, 1995, 2000, 2004).

Many DOF proteins have already been discovered in both monocots and dicots plants. The first DOF protein was found as a DNA-binding protein of maize (ZmDof1). The DNA-binding domain in ZmDof1 includes a C_2C_2-type zinc-finger-like motif. Recently, more than 100 cDNA's encoding a DOF domain from maize and other plant species have been isolated, including: soybean, tomato, sorghum, tobacco, wild strawberry, *Arabidopsis*, *Medicago truncatula*, purple false brome, pumpkin, potato, wheat, rice, among others (Wang et al., 2006; Yanagisawa and Izui, 1993; Shimofurutani et al., 1998).

Dof transcription factors have been suggested to participate in a variety of biological processes focused on plants including the expression of genes associated with carbon assimilation (Yanagisawa, 2000; Yanagisawa and Sheen, 1998), phytochrome signaling, seed maturation and germination, the salicylic acid response (Ward et al., 2005), and the function of the stomata guard cells (Plesch et al., 2001). Imaizumi et al. (2005) and Skirycz et al. (2006) reported the involvement of Dof transcription factors in photoperiodic flowering and biosynthesis of glucosinolates (a group of secondary metabolites), respectively.

However, Dof-binding sequences, which are abundantly distributed in the promoter region of many genes related to lipid biosynthesis, functioning as nuclear transcriptional activator through direct interaction with DNA. This finding prompted to suggest that *Dof* genes might be involved in the process of lipid biosynthesis. Wang et al. (2007) demonstrated that a Dof-type transcription factor derived from soybean (GmDof4 and GmDof11) increased the content of total fatty acids and lipids in seeds of *Arabidopsis* transgenic plants. They suggested that such *Dof* genes may increase the lipid content by upregulating genes that are associated with the biosynthesis of fatty acids.

Nowadays, there is an critical need to develop technologies for sustainable production of renewable energy. The first generations of biofuels, primarily produced from food crops, and mostly from oily seeds, compete for arable land and freshwater resources. Moreover these crops could compromise the biodiversity of previous natural landscapes. These concerns have increased the interest in developing the second and third generation biofuels using lignocellulosics and microalgae, instead of food crops, which potentially offer great opportunities in the long-term (Chisti, 2007; Schenk et al., 2008).

In general, triacylglycerides (TAG) are used as energy storage in microalga and, once extracted, can be easily converted into biodiesel through transesterification reactions (Chisti, 2007). Lipids produced by microalgae can be grouped into two categories: storage lipids (having non-polar properties) and structural lipids (having polar properties) like phospholipids. Storage lipids, mainly in the form of TAG, are predominately constituted of saturated fatty acids (FA) and can be transesterified to produce biodiesel. Structural lipids typically present a high content of polyunsaturated fatty acids (PUFA), which are essential nutrients for aquatic animals and human. Polar lipids (phospholipids) and sterols are an important structural component of cell membranes acting as a selective permeable barrier (Stephens et al., 2010). In general TAG are synthesized when energy from light is absorbed and stored in cytosolic lipid bodies, then these bodies can be utilized for polar lipid synthesis under no-light (dark) conditions. Microalgal TAG are generally composed of saturated and monounsaturated FA (Thompson, 1996).

Within the last few decades, the concept of lipid induction in microalga has been intensively addressed to increase TAG production. Nutrient starvation, temperature and salinity stresses, culture pH and heavy metals concentration, light irradiation, UV irradiance, and genetic engineering have been the strategies used for lipid induction (Sharma et al., 2012; Msanne et al., 2012; James et al., 2011). As of now, these different lipid induction techniques have not been compared to each other (Stephens et al., 2010).

In this work, a strategy supported by genetic engineering tools has been investigated. This approach consisted in a synthesis and posterior incorporation of a DOF-type transcription factor to increase fatty acids (as TAG) synthesis and accumulation in the microalga *Chlamydomonas reinhardtii* by an over-expression of such transcription factor.

2. Materials and methods

2.1. Bioinformatic search for Dof gene in C. reinhardtii

In the complete (wild type) *C. reinhardtii* genome only one putative Dof gene is discernible; nevertheless a specific localization of the DOF sequence has not been reported. In order to achieve this goal, an exhaustive search of the domain reported by Yanagisawa (2002) into the entire genome of *C. reinhardtii* was performed. The genome of *C. reinhardtii* was obtained from the Chlamy Center (http://www.chlamy.org/) through local alignments using CLUSTAL W (Thompson et al., 1997).

A phylogenetic tree was constructed analyzing the DOF identified sequences from a variety of representative organisms from different taxonomic groups like *Physcomitrella patens* (gi373249021) [most ancient vascular plant], *Ostreococcus lucimarinus* (gi 144576424), *Micromona* ssp (gi226520519) and *Micromonas pusilla* (gi226457837) [marine algae], *Volvox carteri* (gi302853223) [fresh water algae], *Populus tomentosa* (gi212725381), *Vitis vinifera* (gi297733967), *Jatropha curcas* (gi256387096), *Glycine max* (gi112363375), *Corylus heterophylla* (gi344190178), *Arabidopsis thaliana* (gi18394978) and *Prunus persica* (gi333795952) [all of these classified as dicotyledonous angiosperm], *Hordeum vulgare* (gi148473094) and *Sorghum bicolor* (gi 242034487) [both classified as monocot angiosperm]. The sequences were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and Chlamy Center (http://www.chlamy.org/) websites. The amino acid sequences of the Dof genes from the organisms listed above were deduced through the "Translate tool" at ExPASy Proteomics Server (http://www.expasy.ch/). Identification of a homologous region in all these protein sequences, that spanned the classical DOF-binding domain, was performed through a multiple alignment using CLUSTAL W (Saitou and Nei, 1987). The alignment of these homologous regions prior to the phylogenetic analysis was also carried out using CLUSTAL W.

The Mega software (http://www.megasoftware.net/) was used to generate a phylogenetic tree, which was optimized by the Maximum Likelihood (ML) method based on the JTT matrix-based model (Saitou and Nei, 1987). The bootstrap consensus tree was inferred from 500 replicates (Tamura et al., 2011).

2.2. Design of the synthetic gene and plasmid construction

A synthetic gene encoding a soybean DOF-type transcription was selected (GenBank Accession Number DQ857261.1). The codon sequences were optimized for *C. reinhardtii* nucleus and synthesized by GenScript Corp. (Piscataway, NJ), decreasing the possibility of having destabilizing mRNA structures. Flanking restriction sites for the *Xba*I and *Bam*HI enzymes were also included to facilitate subcloning in the pBI-121 binary vector. This vector harbors the selection *nptII* gene and the CaMV 35S promoter. *Escherichia coli* strain TOP10 was used for the maintenance and multiplication of the plasmids. A positive clone named pBI-Dof was selected, by restriction analysis and sequencing, and used for the transformation. All these procedures were performed using standard