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Production of xylitol by recombinant microalgae

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ABSTRACT

Microalgae have received significant attention recently as a potential low-cost host for the production of next-generation biofuels and natural products. Here we show that the chloroplast genome of the eukaryotic green microalga *Chlamydomonas reinhardtii* can be genetically engineered to produce xylitol through the introduction of a gene encoding a xylose reductase (XR) from the fungi *Neurospora crassa*. Increased levels of heterologous protein accumulation and xylitol production were achieved by synthesizing the XR gene in the chloroplast codon bias and by driving expression of the codon-optimized XR gene using a 16S/atpA promoter/5′-UTR fusion. These results demonstrate the feasibility of engineering microalgae to produce xylitol, and show the importance of codon optimizing the XR gene and using the 16S/atpA promoter/5′-UTR fusion to express XR in the chloroplast of *C. reinhardtii*.

1. Introduction

Xylitol is a five-carbon sugar alcohol that is used as an artificial sweetener in the food and confectionary industries. Xylitol is roughly as sweet as sucrose and can be used as a sucrose substitute for diabetics (Emodi, 1978). In a series of field studies, a daily intake of xylitol was found to reduce tooth decay for high risk children and adolescents (Makinen et al., 1981). Consumer products such as toothpaste, chewing gum, mouthwash, and nasal spray are increasingly using xylitol in their formulations. A chemical method, involving the hydrogenation of xylose using a Raney nickel catalyst, is generally used for the industrial production of xylitol; however, this method presents several safety and environmental concerns because it requires high pressures and involves a toxic catalyst (Aminoff et al., 1978).

More recent studies have focused on developing a safer, more environmentally friendly microbial route for the production of xylitol. These studies have primarily focused on investigating natural xylose-fermenting yeasts such as *Candida tropicalis* and *Candida parapsilosis*, or genetically engineering yeast strains such as *Saccharomyces cerevisiae* (Moon et al., 2002). For instance, high conversion of xylose to xylitol (>95%) was obtained by transforming *S. cerevisiae* with the gene encoding the xylose reductase (XR) from *Pichia stipitis* (Hallborn et al., 1991). XR catalyzes the first step in D-xylose metabolism, reducing xylose to xylitol, and using NAD(P)H as a cofactor. In addition to various yeast strains, the recombinant expression of XR to facilitate xylitol production has been reported in the bacterium *Escherichia coli* (Cirino et al., 2006), but to date, no research efforts have focused on using microalgae for xylitol production. Compared to bacterial and yeast strains, microalgae may offer an attractive alternative for producing xylitol based on its simple growth requirements, generally regarded as safe (GRAS) designation, and long history as a source of food and food additives.

Microalgae are a diverse group of prokaryotic and eukaryotic photosynthetic microorganisms that have useful applications in the food, nutritional, cosmetic, pharmaceutical, and biofuels industries (Coragliotti et al., 2011; Olaizola, 2003). Algae offer an attractive platform for the production of a variety of bioproducts because of their rapid growth rate, cost effective culturing, genetic manipulability, and ease of scale-up (Rasala et al., 2010). Among microalgae strains, *Chlamydomonas reinhardtii* is a popular model algae used in the study of photosynthesis and flagella function. This singled-cell eukaryotic green alga is genetically well characterized and has a significant molecular toolkit. All three genomes (nuclear, chloroplast, and mitochondrial) of *C. reinhardtii* have been fully sequenced and transformation methods that target each of these genomes have been developed (Specht et al., 2010). The chloroplast genome, in particular, is an attractive target for genetic engineering because the chloroplast has been shown to support high levels of heterologous protein accumulation (as high as 2-20% of total soluble protein (TSP)) and foreign DNA can be stably integrated at a specific location within the chloroplast genome (Rasala and Mayfield, 2011).

In this study, we investigated the potential of using the microalga *C. reinhardtii* as a platform for the production of xylitol. The chloroplast genome of *C. reinhardtii* was transformed with a gene encoding a XR from the filamentous fungus *Neurospora crassa*. Strategies involving codon-optimizing of XR gene and using a hybrid-fusion promoter were also tested in an effort to increase heterologous XR protein levels in the chloroplast of *C. reinhardtii*.

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2. Materials and methods

2.1. Strains and media

C. reinhardtii strain 137c (mt+) was obtained from Chlamydomonas Center (Duke University, Durham, NC). The plasmid pRS426m-xylose and the yeast strain *S. cerevisiae* YSG50 were obtained from Huimin Zhao at the University of Illinois at Urbana-Champaign. All algal cultures were grown to late logarithmic phase (typically 5-7 days) in tris-acetate-phosphate (TAP) medium containing 100 μg/ml kanamycin under fluorescent white light (80 $\mu mol\, m^{-2}\, s^{-1}$ as measured by a Field Scout Quantum Meter (Plainfield, IL)) at 23 °C on a rotary shaker at 100 rpm. *E. coli* DH5α was used for recombinant DNA manipulations and *E. coli* BL21 (DE3) was used as an expression host. *S. cerevisiae* YSG50 strain was grown in yeast extract–peptone–dextrose plus adenine (YPAD) medium at 30 °C.

2.2. Plasmid construction

Codon optimization of the *XR* gene was performed using software developed in-house. This program was written in the C++ programming language and optimizes a target gene sequence by substituting the most frequently used *C. reinhardtii* chloroplast codons (data obtained from http://www.kazusa.or.jp/codon (Nakamura et al., 2000)). The coding sequence of the codon optimized *XR* gene was ordered from Integrated DNA Technologies (Coralville, IA, USA) and designated *optXR*. The *optXR* gene was cloned into the *Nde*I/*Hind*III sites of the pET26b expression vector.

The plasmids pTJ322-XR, pTJ322-optXR, and pTJ322-16S/optXR were constructed using a method as described by Noor-Mohammadi et al. (2012). The primers used to construct the three plasmids are shown in supplementary table 1. The 5′ UTRs (*psbA*, 251 bp; *atpA*, 556 bp), 3′ UTRs (*psbA*, 400 bp; *atpA*, 400 bp) and gene *aphA6* (780 bp) were amplified via PCR from the plasmid pTJ322-aphA6-aadA. The *XR* gene (969 bp) was amplified from the plasmid pRS426m-xylose. The 16S rRNA sequence (Genbank accession number X03269.1; 219 bp) was amplified from chloroplast genomic DNA isolated from *C. reinhardtii* using a Wizard Genomic DNA isolation kit (Promega, Madison, WI, USA). The constructed plasmids were then subjected to restriction digestion analysis as described by Noor-Mohammadi et al. (2012).

2.3. Chloroplast transformations

For chloroplast transformations, a 50 ml culture of *C. reinhardtii* was grown to late logarithmic phase in the presence of 0.5 mM 5′-fluoro-2′-deoxyuridine (Sigma-Aldrich, St. Louis, MO, USA). Cells were harvested by centrifugation and resuspended in 4 ml of TAP medium. These cells were then spread onto TAP/agar plates containing 100 μg/ml kanamycin. Chloroplast transformations were performed by particle bombardment (PDS-1000/He; Bio-Rad, Hercules, CA, USA) using DNA-coated gold particles (S550d; Seashell Technologies, San Diego, CA, USA). Particle bombardment parameters for the PDS-1000/He system were as follows: chamber vacuum of 28 in. Hg, helium pressure of 1350 psi, distance of 9 cm, and 3 mg of 0.55 μm gold particles coated with 10 μg of plasmid DNA. The particle-bombarded plates were placed under constant light for 1-2 weeks until transformed algae colonies appeared. Primary transformants were restreaked 4-5 times on TAP plates containing 100 μl/ml kanamycin until homoplasmic lines were identified.

2.4. Protein expression, immunoblotting, and protein purification

The plasmid pET26b-optXR was transformed into *E. coli* strain BL21 (DE3). Transformed cells were grown in LB media with shaking at 37 °C. XR protein expression was induced by adding 0.5 mM isopropyl B-D-thiogalactopyranoside (IPTG) and placing the culture in a 30 °C shaker until an OD_{600} of ∼0.6 was achieved. The cells were harvested by centrifugation, resuspended in 20 mM Tris–HCl pH 8.0, then placed in a −80 °C freezer. After 1 h, the cells were thawed at room temperature, the cellular debris was removed by centrifugation, and the resulting supernatant was used to isolate the XR protein by affinity gel purification. Purification of FLAG-tagged XR protein was carried out using EZview™ Red ANTI-FLAG® M2 Affinity Gel (Sigma, St. Louis, MO) according to the manufacturer's instructions.

Algal cell cultures were grown to late log phase in TAP media. Cells were harvested by centrifugation, and resuspended in 1 ml 20 mM Tris–HCl pH 8.0. The resuspended cells were lysed by sonication using a Misonix sonicator (Farmingdale, NY) with the amplitude set at 30% and with a pulse sequence of 7 s on and 10 s off, for 5 min. Samples were then centrifuged at 14,000 × *g* for 30 min at 4 °C and the resulting supernatant used in western blot analysis. The protein concentration of the total soluble protein was measured using the BioRad Protein Reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. Approximately 3 μg total soluble protein from each sample was separated on a 12% sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE) and then transferred onto a methanol-treated PVDF (polyvinylidene fluoride, Millipore, Billerica, MA, USA) membrane. The PVDF membrane was blocked with 5% milk in TBST buffer (20 mM Tris, 150 mM NaCl, 0.02% Tween 20, pH 7.5) for 1 h at room temperature. The blocked membrane was incubated at 4 °C overnight in TBST solution containing 1:1000 dilution of a rabbit anti-FLAG primary antibody (Cell Signaling Technology, Danvers, MA, USA), washed three times with TBST for 5 min, incubated with 1:2000 dilution of a horseradish peroxidase linked rabbit anti-rabbit IgG secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for 1 h, then the membrane was washed with TBST for 30 min. A working solution of Pierce ECL Substrate (Thermo Scientific, Rockford, IL, USA) was prepared according to the manufacturer's instructions and added to the membrane for 1 min. The membrane was removed from the substrate and analyzed by an Alpha Innotech FluorChem® HD2 imager and the software AlphaEaseFC™.

2.5. Quantitative RT-PCR analysis of mRNA accumulation

Cells were grown in TAP media under light until late log phase and then harvested by centrifugation at 4000 × *g*. Total RNA was isolated from the harvested cells using the Plant RNA Reagent Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The concentration of extracted RNA was measured using a Thermo Scientific NanoDrop 2000c spectrophotometer and the integrity of the RNA was checked by agarose gel electrophoresis. Ten micrograms of total RNA was treated with DNase (Ambion Turbo DNA-free, Austin, TX, USA) to remove any contaminating genomic DNA. A Bio-Rad's iScript cDNA Synthesis kit was used to synthesize cDNA from 1 μg of DNase-treated total RNA. The synthesized cDNA was diluted 4-fold for use in the qPCRs. Each 20 μl qPCR contained 10 μl Bio-Rad iQ™ SYBR Green Supermix, 0.5 μM oligonucleotides, and 2 μl diluted cDNA. Real-time qPCR was performed in triplicate for each sample using a StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). Thermocycling conditions consisted of a two-step sequence with an annealing/extension temperature of 60 °C, followed by a melt curve to monitor for primer dimers. "No reverse transcriptase" for each sample was performed to monitor for possible genomic DNA contamination and "no template controls" for each primer pair were also performed in triplicate. The endogenous chloroplast gene *rbcL* of *C. reinhardtii* was used as a reference gene. Standard curves were generated for the heterologous genes *XR*, *optXR*, and the