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Effects of rare codon clusters on the expression of a high-turnover chloroplast protein in *Chlamydomonas reinhardtii*

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ABSTRACT

Expression of foreign proteins in chloroplasts has become an important field of plant genetic engineering. Optimized codon usage is generally thought to increase translational efficiency, but high speed translation of codon bias-adjusted mRNAs can also result in protein misfolding due to a lack of rare codons. In order to analyze the effect of rare codons on a native chloroplast protein in vivo, we modified the D1 subunit of photosystem II by fusing small peptides with different codons into a loop region which tolerates insertions without loss of function. Because of its high-turnover properties, the D1 protein represents an excellent test object to investigate the impact of rare codons on its translation. We choose codons for amino acids Arg, Leu, Ser, Ala and Gly which are rarely used and compared translation of the modified D1 proteins with the respective mutant proteins containing insertions with frequently used codons. Our data indicate that only rare Arg codons drastically affect synthesis of the D1 protein and cluster of rare Ser-codon can induce strategic ribosomal pausing sites.

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1. Introduction

Codon usage is specific not only for the organism but also for the intracellular genome type under investigation. Generally, there is a positive correlation between tRNA content and codon usage (Grantham et al., 1980, 1981; Gouy and Gautier, 1982; Ikemura, 1985), i.e. the choice between synonymous codons corresponds to the most frequent tRNA species in the cell and correlates with protein expression levels (Ikemura, 1981, 1985; Sharp and Li, 1987; Kanaya et al., 1999). Many studies in various organisms have shown that an optimized codon usage increases the translational efficiency (Morton and Levin, 1997; Duret and Mouchiroud, 1999; Coghlan and Wolfe, 2000; Castillo-Davis and Hartl, 2002) and non-adapted codon usage results in a lower expression level. This selection for translational efficiency could be a reason for intragenic differences in codon bias (Sharp and Li, 1986) and the phenomenon of codon usage is discussed to be a result of mutation and selection (Morton, 1993).

One example for optimized codon selection and adjustment to tRNA content is the *psbA* gene which encodes the D1 subunit of photosystem II on the chloroplast genome. While the plastome has a high A-T content and plastidic codon bias appears to be a function of composition bias, the *psbA* gene shows a preference for NNC over NNT codons and therefore a strong adaptation to the tRNA content of the plastids (Morton, 1993; Morton and Levin, 1997). The atypical codon usage of the *psbA* gene leads to an increased translational efficiency and ensures a smooth and continuous expression of the D1 protein. This is necessary since D1 is the protein with the highest turnover in chloroplasts: it is constantly damaged in the light by reactive oxygen species, proteolytically degraded and rapidly re-synthesized de novo in order to maintain photosynthetic electron flow (Nixon et al., 2010).

The chloroplast of the green alga *Chlamydomonas reinhardtii* has recently gained interest as a bioreactor for the production of foreign proteins like antibodies and hormones (Cardi et al., 2010; Johanningmeier and Fischer, 2010; Specht et al., 2010). A deeper understanding of how specific codons can affect translation should improve our understanding of the chloroplast translation apparatus of the alga and can help to solve problems encountered in expressing cloned genes. Although rare codons are generally considered to have a negative effect on protein production (Kane, 1995), there are recent reports on positive effects as well (Clarke and Clark, 2008; Rosano and Ceccarelli, 2009; Angov et al., 2011). Thus, a major goal of this work was to study in vivo the effect of rare codons and codon clusters on the expression of a highly abundant chloroplast protein. We chose to analyze the D1 subunit of photosystem II as an example for a polypeptide that is synthesized in high amounts and is cotranslationally inserted into the thylakoid membrane (Marín-Navarro et al., 2007). The *psbA* gene can be easily modified by site-specific mutagenesis procedures and introduced into the plastome by particle gun transformation using suitable deletion mutants. Here we

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Table 1
Sequences of the *psbA* primers and mutagenic primers used for generating PCR constructs for transformation.

Primer	Sequence 5′–3′
A	GGGTCGTGAGTGGGAATTATCTTTCCG
B	GCTGGTAACCTTCTATAGAATTATGAACAATAATATTATTTG
C	GCTGGTTACCGTAGTGGATGCATATCAAAATTTAG
D	GCTAGAGTTAGTTGAAGCTAAGTCTAGAGGGA
E	GGTTAC(codon insertion)CGTTT
F	AAACG(codon insertion)GTAACC
G	GAGTGGGAATTATCTTTCCGTTTAGGTAT
H	AAGTCTAGAGGGAAGTTG

describe the generation and analysis of D1 mutants containing small peptide insertions in the D-de loop region of the D1 protein employing different synonymous codons.

2. Materials and methods

2.1. Algal strains and transformation

Liquid cultures of *C. reinhardtii* strains IL (Johanningmeier and Heiss, 1993) and Del1 (Preiss et al., 2001) were grown under continuous light (50 $\mu E\,m^{-2}\,s^{-1}$) in Tris–acetate–phosphate medium (TAP; Harris, 1989) at 22 °C. Due to a 425 bp deletion in the *psbA* gene the Del1 strain is unable to grow photoautotrophically and needs acetate as an external carbon source. Del1 cells were transformed with a particle gun using PCR-generated *psbA* fragments. Transformants were selected for photosynthetic growth on minimal HS medium as described by Dauvillee et al. (2004). Correct *psbA* gene modifications in transformants were verified by DNA-sequencing (Seqlab, Göttingen).

2.2. Polymerase chain reactions

PCR fragments for transformation of mutant Del1 were generated using plasmid pSHc5 containing the intronless *psbA* gene and 3′-flanking regions as a starting template (Preiss et al., 2001). Primer combinations A/B and C/D (Table 1) generated two non-overlapping *psbA* fragments using a regime of 30 cycles of 95 °C denaturation (1 min), 55 °C annealing (45 s), 72 °C extension (30 s) with a 5 min denaturation step at 95 °C in the first cycle and a 10 min extension step at 72 °C in the final cycle. Gel-purified fragments were combined and used as template in a second PCR with mutagenic primers E/F containing the desired insertions and flanking *psbA* specific sequences and *psbA* specific primers A/D for generating the mutant construct. The PCR protocol followed the above regime, except that the optimal annealing temperature for this reaction was determined using a gradient cycler. The mutagenic primers contain overlapping regions and were applied in one-fifth of the concentrations of the *psbA* specific primers. PCR products were again gel-purified and amplified with *psbA* specific primers G/H using the PCR protocol as described above, yielding the final fragment for particle gun transformation without further purification.

2.3. PSII efficiency

PSII efficiency was measured by chlorophyll fluorescence (Fv/Fm) using excitation at 695 nm (high pass) and 780 nm (low pass) cutoff filters with the Photon System Instrument FluorCam 800MF (Brno, Czech Republic) as described by Nedbal et al. (2000). Cells in the mid log phase with an optical density of one at 750 nm were exposed to 1500 $\mu E\,m^{-2}\,s^{-1}$ white light, transferred into a 96-well plate and incubated in the dark before measurement.

2.4. Western blot analyses

For the preparation of total cellular protein, algae were grown in TAP medium under continuous light. Cells from the mid log phase equivalent to 1500 μg chlorophyll were harvested by centrifugation at 1000 × *g* for 5 min at room temperature. The pellet was dissolved in 100 ml fresh TAP medium, incubated in the dark for 1 h and exposed to high light (2000 $\mu E\,m^{-2}\,s^{-1}$) for 180 min. At each time point protein samples equivalent to 3 μg chlorophyll were solubilized at room temperature in 75 μl buffer A (0.1 M Na_2CO_3), 50 μl buffer B (5% SDS w/v, 30% Saccharose w/v) and 6.25 μl Mercaptoethanol for 25 min on a shaker, separated by SDS–PAGE (Schägger and von Jagow, 1987), transferred onto nitrocellulose membranes (BA-85, Schleicher and Schüll, Dassel, Germany) as described previously (Towbin et al., 1979) and immunodecorated with antibodies against D1 protein (chicken anti-PsbA 1:10,000, rabbit anti chicken 1:10,000, Agrisera, Sweden) using the alkaline phosphatase detection system. Western blots were quantitated by AIDA software (Advanced image data analyzer v 4.03, Raytest, Germany).

2.5. In vivo labeling

Pulse labeling experiments were performed as described previously (Preiss et al., 2001). In short, cells were grown in sulfate reduced TAP medium for 3 days. Mid-log phase cells equivalent to 90 μg of chlorophyll were harvested by centrifugation at 1000 × g for 5 min at room temperature and resuspended in TAP medium with all sulfate salts substituted by chloride salts at a chlorophyll concentration of 200 μg/ml chlorophyll. After incubation for 2 h at room temperature, cycloheximide (10 μg/ml final concentration) was added to inhibit cytoplasmic protein synthesis 15 min before the pulse. The pulse was started by adding 100 μCi/ml of [35S]-sulfate (2 mCi/ml). Cells were incubated at 21 °C under white light (80 $\mu E\,m^{-2}\,s^{-1}$) for 20 min in a water bath. Protein samples equivalent to 9 μg of chlorophyll were removed at the indicated time points and immediately precipitated in ice cold acetone (80% final concentration). Samples were centrifuged at 16,000 × *g* for 10 min at 4 °C and the dried pellet solubilized in 2× loading buffer (100 mM Tris–HCl (pH 6.8), 200 mM DTT, 4% w/v SDS, 0.2% w/v Bromophenol Blue, 20% w/v Glycerol). After incubation at 95 °C for 10 min, 6 M urea (final concentration) was added and samples corresponding to 3 μg of chlorophyll were separated by SDS/PAGE in gels containing 6 M urea. Gels were fixed for 1 h in 45% methanol, 9% acetic acid, dried in vacuum and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale CA, USA). Quantitation was performed using the software IMAGE QUANT v. 1.2 by the same company.

3. Results

3.1. Codon test system

In order to determine possible effects of specific codons on a major chloroplast translation product, we made in-frame fusions of single amino acids and peptides of different length into the large stromal loop of the D1 protein (Fig. 1). This region has been shown to tolerate various insertions without loss of function (Kless et al., 1994; Johanningmeier et al., 2006) and allows to set up an in vivo codon test system to study changes in production of the test protein in comparison with the control protein. The insertion site is located between amino acids Y237 and R238 within an extended loop connecting transmembrane helix D and the parallel helix de.

According to the codon usage table for *C. reinhardtii* plastids, certain codons for amino acids Arg, Leu, Ser, Ala and Gly are most

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