A putative cytochrome *c* biogenesis gene in *Synechocystis* sp. PCC 6803

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Abstract A gene (*orf334*) with homology to chloroplast *ycf5* (*ccsA*) was isolated from the cyanobacterium *Synechocystis* PCC 6803. The mRNA level of *orf334* decreases in the dark and increases rapidly upon illumination. Transcription is initiated 69 nucleotides upstream of the start site of translation. The deduced amino acid sequence of *orf334* has limited identity with bacterial proteins involved in cytochrome *c* biogenesis. Sequence comparison indicates differing pathways of cytochrome *c* biogenesis in cyanobacteria/chloroplasts and Gram⁻ positive bacteria versus proteobacteria and mitochondria. Insertional inactivation of the *orf334* gene gave rise to a heterozygous mutant, i.e. complete absence of the *orf334* product seems to be lethal to the cell.

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Key words: *Synechocystis*; Cyanobacterium; Chloroplast; Cytochrome *c*

1. Introduction

C-type cytochromes are ubiquitous electron transfer proteins of photosynthesis, respiration and denitrification. They are localized at the periplasmic side of the bacterial cytoplasmic membrane, in the intermembrane space within mitochondria and in the lumen of thylakoids. *C*-type cytochromes contain one or several protoheme IX molecules covalently bound via thioether linkages to the apoprotein [1].

Several components of the bacterial cytochrome *c* biogenesis pathway have been identified. Whereas a membrane-anchored ABC-type transporter translocates heme through the membrane, cytochrome *c* holoenzyme is formed in the periplasmic space [2,3]. Subunits of the putative transporter are encoded in the *cycVWorf263XY* operon of *Bradyrhizobium japonicum* [4] and in the *helABCDX* operon of *Rhodobacter capsulatus* [5]. A second group of cytochrome *c* biogenesis genes is clustered in the *ccl1/ccl2*-operon of *R. capsulatus* and the *cycHJKL*-operon of *B. japonicum*, respectively [2,5–7]. These genes encode proteins that are suggested to form a scaffold to bring the apoproteins and heme together for subsequent linkage or may act as components of a multi-subunit cytochrome heme lyase.

Cyanobacteria contain the *c*-type cytochromes *f* and c_6. Both are involved in photosynthetic electron transport. Whereas the membrane-associated cytochrome *f* is present also in photosynthesizing plastids of algae and plants, the occurrence of soluble cytochrome c_6 is confined to cyanobac-

teria and some algae [1,8]. Although recent studies have provided new insight, the process of cytochrome maturation in cyanobacteria and plastids remains poorly understood [9–11]. Mutational analyses with the green alga *Chlamydomonas reinhardtii* indicate that, as in bacteria, multiple genetic loci are involved (for review, see [3]). One locus has been attributed to the chloroplast *ycf5* gene (also designated *ccsA*) [12]. *Ycf5* is conserved in the plastome of red and green algae, diatomeae, cryptomonads, liverworts and vascular plants. It shows partial sequence identity to the bacterial cytochrome *c* biogenesis genes *helC* and *ccl1*.

A *ycf5*-homologue (*orf334*) has been localized in the genome of the cyanobacterium *Synechocystis* sp. PCC 6803 (this work and [13]). The present report describes the transcription of *orf334* and the attempts to inactivate the gene by insertional mutagenesis. The results are discussed with respect to a possible role of the Orf334 protein in cyanobacterial cytochrome *c* biogenesis.

2. Materials and methods

2.1. Plant material and Synechocystis growth conditions
Seedlings of barley (*Hordeum vulgare* L.) were grown and harvested as described previously [14]. *Synechocystis* sp. PCC 6803 wild-type cells were grown in BG-11 medium at 30°C at a light intensity of ~ 40 μE m^{-2} s^{-1} [15]. Transformation and selection of transformants was carried out as described [16].

For dark incubations, cells were grown to mid-log phase (A$_{730\,nm}$ ~ 0.6), culture flasks were wrapped with aluminum foil and incubated at 30°C. After 24 h of dark incubation, the aluminum foil was removed and cells were exposed to light (~ 40 μE m^{-2} s^{-1}) for 5, 10, 30 and 120 min, respectively.

2.2. DNA techniques, library screening and nucleotide sequencing
Genomic DNA of *Synechocystis* PCC 6803 was isolated according to Franche and Damerval [17]. Total barley DNA was extracted following the protocol of Rogers and Bendich [18]. PCR amplification of a barley *ycf5*-subfragment was done with the 5′ primer, 5′-TGGTTAATGATGCATGTCAGT-3′ and 3′ primer, 5′-CCCAAG-CTTCATTAGCCCA-3′ as described previously [19]. The barley gene fragment with a size of 347 bp was cloned into vector pBluescript/KS⁺ (Stratagene) and sequenced. DNA probes were labelled with the Megaprime kit (Amersham Buchler), hybridization was done in 0.25 M Na$_2$HPO$_4$, pH 7.2, 7% SDS at 55°C or 65°C.

A *Lambda* EMBL 3 genomic library of *Synechocystis* sp. PCC 6803 was screened with the barley *ycf5* fragment. Two positive phage clones were isolated, subfragments were inserted into pBluescriptII/KS⁺ (Stratagene) vector and were sequenced by the double-stranded dideoxy chain-termination method [20] with the Sequenase kit version 2.0 (United States Biochemicals). Nucleotide and protein sequences were analyzed using PC/Gene (IntelliGenetics, Inc. Betagen, Mountain View, CA) and Husar-GCG-software (Deutsches Krebsforschungszentrum Heidelberg, Germany).

2.3. RNA techniques
Total *Synechocystis* RNA was isolated as described [21]. Size fractionation of isolated RNA and transfer to Hybond N membranes

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(Amersham Buchler) were performed according to standard procedures [22]. Hybridization was carried out in 0.12 M Na_2HPO_4, pH 7.2, 0.25 M NaCl, 7% SDS and 50% formamide at 42°C. Uniformly labelled RNA probes were obtained by in vitro transcription with the MAXIscript kit (Ambion). Isolation of full-length transcripts and RNase protection assays were performed as described [23]. Primer extension was carried out with 30 μg DNase-treated total *Synechocystis* RNA and AMV reverse transcriptase (Promega) according to the instructions of the manufacturer. Primer 5'-TCAGCAAAGCA-CAAGGAAGGCAG-3' was end-labelled using T4 polynucleotide kinase (Amersham Buchler).

3. Results

3.1. Cloning of a cyanobacterial ycf5 homologue and sequence analysis

A barley *ycf5* subfragment was used to screen a *Lambda* EMBL 3 genomic library of the cyanobacterium *Synechocystis* sp. PCC 6803 (see Section 2). After subcloning and sequence analysis, an open reading frame of 1005 nucleotides was localized on a 2.8-kb *Bgl*II fragment. It encodes a protein of 334 amino acids (*orf334*; designated as *ccsA* in the Cyanobase databank, Kazusa DNA Research Institute, Japan). The molecular mass of the protein was calculated to be 36.1 kDa. The deduced polypeptide of *orf334* showed 42–60% identity with that of chloroplast *ycf5*. The cyanobacterial Orf334 had the highest scores of similarity with Ycf5-proteins from algae (71–75%). A comparison with plant sequences showed the best coincidence with liverwort Ycf5 (71%). The cyanobacterial protein is slightly more similar to Ycf5 of dicots (65–68%) than to those of monocots (62–63%).

The *orf334* gene codes for a hydrophobic polypeptide with seven predicted trans-membrane regions. The hydrophobicity profile was strikingly similar to that of the Ycf5-proteins of plastids (not shown). The C-termini of the deduced proteins were conserved from cyanobacteria to vascular plants. They all contain a putative heme-binding site W-G-X-X-W-X-W-D [2]. This motif has been detected in the cytochrome *c* biogenesis components HelC and CcI1 of *R. capsulatus* [5] and in related bacterial and mitochondrial proteins. However, an alignment of all Orf334 with the entire HelC and CcI1 polypeptides showed only 42–49% similarity. A higher degree of similarity was found with Orf327 from *Mycobacterium leprae* (58%) and with OrfX16 from *Bacillus subtilis* (54%) (Smith, D.R. and Robison, K., GenBank accession No. U00018; [24]). Both open reading frames encode proteins of 35.8 kDa and 39.7 kDa, respectively, with hydrophobicity patterns very similar to Orf334.

3.2. Insertional inactivation

Southern blot analysis of DNA digested with *Hin*dIII, *Eco*RV and *Bgl*II revealed the presence of one copy of *orf334* in the genome of *Synechocystis* sp. PCC 6803 (not shown). For a functional characterization of the *orf334* encoded protein, the cartridge mutagenesis technique was applied. A *Bam*HI site at position 813 of the coding region of *orf334* was used to insert the kanamycin resistance-cartridge from the pUC-4K vector (Pharmacia). Kanamycin resistant transformants were obtained and single colonies were purified by serial streaking on increasing concentrations of the antibiotic (final concentration 40 μg/ml). To verify the insertion of the disrupted copy of *orf334* into the genome of *Synechocystis*, chromosomal DNA of transformants was digested with *Bgl*II. A DNA blot was probed with an internal fragment
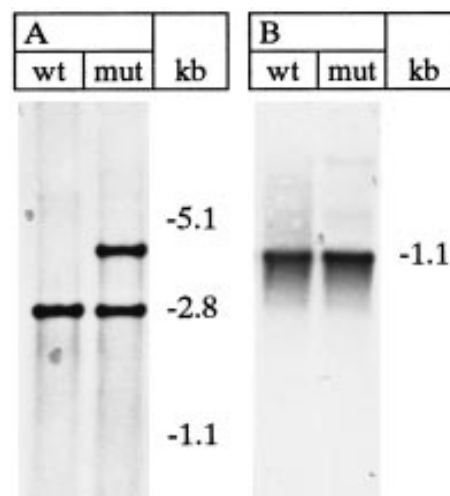

Fig. 1. Southern blot (A) and Northern blot (B) analysis of wild-type and a *orf334* mutant. (A) Genomic DNA of *Synechocystis* sp. PCC 6803 wild-type (wt) and mutant cells (mut) was digested with *Bgl*II, transferred to Nylon filter and probed with an internal *Alu*I-fragment of *orf334*. DNA molecular size markers are given at the right in kilobases. (B) Total RNA was isolated from mid-log phase cultures of wild-type (wt) and mutant cells (mut) and hybridized with the same probe as in (A). The size of the transcript is indicated at the right in kilobases.

of *orf334* (Fig. 1A). Open reading frame 334 is localized on a 2.8-kb *Bgl*II fragment in wild-type cells. An insertion of the kanamycin cartridge (size of 1.2 kb) into *orf334* should produce a *Bgl*II fragment of 4.0 kb. The detection of both the 2.8 and 4.0-kb fragments in mutant cells reflected the hemizygous status of the transformants. Further selection of single colonies at kanamycin concentrations up to 100 μg/ml did not result in a homozygous *orf334*-mutant. All hemizygous mutants grew well under photoautotrophic and photoheterotrophic conditions. Northern blot analyses showed no differences in the accumulation of *orf334*-mRNA between normal and mutated cells. In either case, *orf334* is transcribed into a message of 1.1 kb (Fig. 1B).

3.3. Transcription of orf334 and transcript mapping

Transcript levels of several cyanobacterial genes respond to light [25]. In order to monitor whether light affected expression of *orf334*, RNA was isolated from *Synechocystis* cells grown in the dark and from cells returned to light (see Section 2). Northern blots were probed with either an internal fragment of *orf334* or, as a reference, with an internal fragment of *psbB* – the gene encoding the chlorophyll *a*-binding protein CP47 of photosystem II (Fig. 2). After 4 h of darkness, the transcript accumulation of *orf334* was slightly reduced. A 24 h incubation without light resulted in a low, but still detectable mRNA level. When dark grown cultures were illuminated, the initial accumulation rate of *orf334* was restored within 5 min. The *psbB* gene of *Synechocystis* sp. PCC 6803 is transcribed into a mRNA of 1.8 kb. 4 h of darkness were sufficient to diminish the *psbB* transcripts to an undetectable level. 5 min of illumination led to a rapid increase of *psbB* mRNA accumulation, similar to the effect on *orf334*. After a further transient rise over another 5 min, the amount of *psbB* message is reduced to the initial level. The quantity of rRNA did not change during the dark/light shift (not shown).

A computer analysis of the 5' region of the cyanobacterial

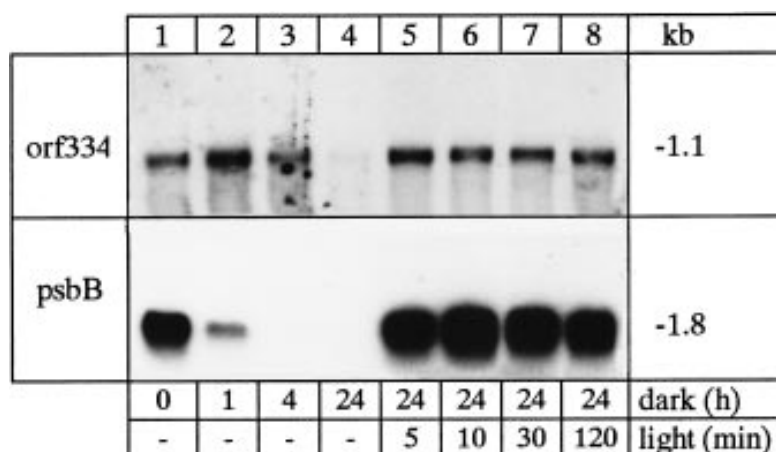|	1	2	3	4	5	6	7	8	kb
orf334									-1.1
psbB									-1.8
dark (h)	0	1	4	24	24	24	24	24	
light (min)	-	-	-	-	5	10	30	120	

Fig. 2. Light-dependent transcript accumulation of *orf334* and *psbB*. Light grown cultures of *Synechocystis* PCC 6803 (lane 1) were transferred into darkness for 1, 4 and 24 h (lanes 2–4). After 24 h of dark incubation, the cultures were illuminated for 5, 10, 30 and 120 min (lanes 5–8). RNA was isolated and probed with both an internal *Alu*I-fragment of *orf334* and an internal *Bam*HI/*Sma*I-fragment of the *psbB* gene. The sizes of the transcripts are given at the right in kilobases.

open reading frame gave no indication for the presence of a typical *E. coli*-like σ^{70} promoter. A putative −35 region (5′-GTGATA-3′) could be predicted 105 nucleotides upstream of the initiation codon. Therefore, we applied RNase protection assays to determine the transcriptional start site of *orf334*. RNA of *Synechocystis* was hybridized with a uniformly labelled antisense probe and digested with RNaseA and RNaseT1. The 5′ end of the probe corresponded to position 168 of the *orf334* coding region, the 3′ end was localized 170 nucleotides upstream of the initiation codon. A RNA molecule of approximately 230 nucleotides was protected from degradation (Fig. 3A). Since 168 nucleotides of the protected RNA fragment cover the coding region, the transcriptional start was localized about 60 nucleotides upstream of the initiation codon of *orf334*. This result was verified by primer extension analysis (Fig. 3B), indicating that transcription is initiated 69 nucleotides upstream of the translational start site. A putative ribosome-binding motif (5′-GGA-3′) precedes the start codon by six nucleotides. A potential stem-loop structure with a calculated free energy value of $\Delta G'$ (25°C) = −6 kcal followed the stop codon at a distance of 95 nucleotides (not shown).

4. Discussion

An open reading frame (*orf334*) with sequence homology to chloroplast *ycf5* has been detected in the genome of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. The cyanobacterial gene was transcribed into a mRNA of 1.1 kb. The start site of transcription was localized 69 nucleotides upstream of the initiation codon. A computer search did not reveal sequences with significant homology to the consensus promoters of *B. subtilis* (σ^{43}) and *E. coli* (σ^{70}) RNA polymerases. Limited similarity to a −35 domain was attributed to the region −32 to −38 of *orf334*. Deviations from the canonical *E. coli* σ^{70} promoter motifs have been reported for several cyanobacterial genes (e.g. [26–28]). Moreover, the prediction of eight sigma factor genes in *Synechocystis* sp. PCC 6803 [13] indicates transcript initiation at several different promoter motifs.

Transcript accumulation of *orf334* is positively affected by

light. A 24 h incubation in the dark reduced the mRNA level of *orf334* to a low but still detectable level. Restoration of illumination led to a rapid increase of the accumulation rate. These data suggest that the *orf334* product is required by dark-grown cells and in higher amounts after illumination. It remains to be shown, however, whether these alterations in mRNA accumulation are due to transcriptional regulation or changes in message stability. There is no sequence motif present 5′ to *orf334* that has been reported to be involved in photoregulation of transcription in cyanobacteria [29,30].

The deduced amino acid sequence of *orf334* is about 20% identical and 49% similar to those of the proteobacterial cytochrome *c* biogenesis genes *helC* and *ccl1*, respectively. The similarity between HelC, Ccl1 and Orf334 is mainly confined to a region around the putative heme-binding site. A higher degree of overall similarity exists between Orf334 and the products of two genes from Gram-positive bacteria – *orfX16* (also designated *resC*) from *B. subtilis* and *orf327* from *M. leprae*. The translated proteins contain the putative heme-binding site and have similar sizes to Orf334 with 352 and 327 amino acids, respectively. *HelC*-like sequences encode proteins of approximately 240 amino acids and *ccl1*-like sequences encode polypeptides of about 650 amino acids. These data may reflect a larger evolutionary distance between cyanobacteria and proteobacteria than between cyanobacteria and the Gram-positive bacteria *Bacillus* and *Mycobacterium*. Alternatively, *orfX16* and *orf327* of Gram-positive bacteria and *orf334* of *Synechocystis* and the *ycf5* genes of chloroplasts encode a component of the cytochrome *c* biogenesis pathway that is lacking in proteobacteria. In line with that hypothesis, the total genome of the proteobacterium *Haemophilus influenzae* does not contain an *orf334* related gene other than *helC* *(ccmC), ccl1 (ccmF)* and the *ccl1* homologue of the nitrite reductase biogenesis pathway *(nrfE)* [31]. Furthermore, there is only one gene in the entire genome of *Synechocystis* PCC 6803 with significant similarity to *helC* and *ccl1*, namely *orf334*. If *orf334* does not substitute all three genes in *Synechocystis*, then *helC*-like, *ccl1*-like or *nrfE*-like sequences should be significantly different from the proteobacterial ones or they are completely absent. This raises the question whether other components of proteobacterial cytochrome *c*
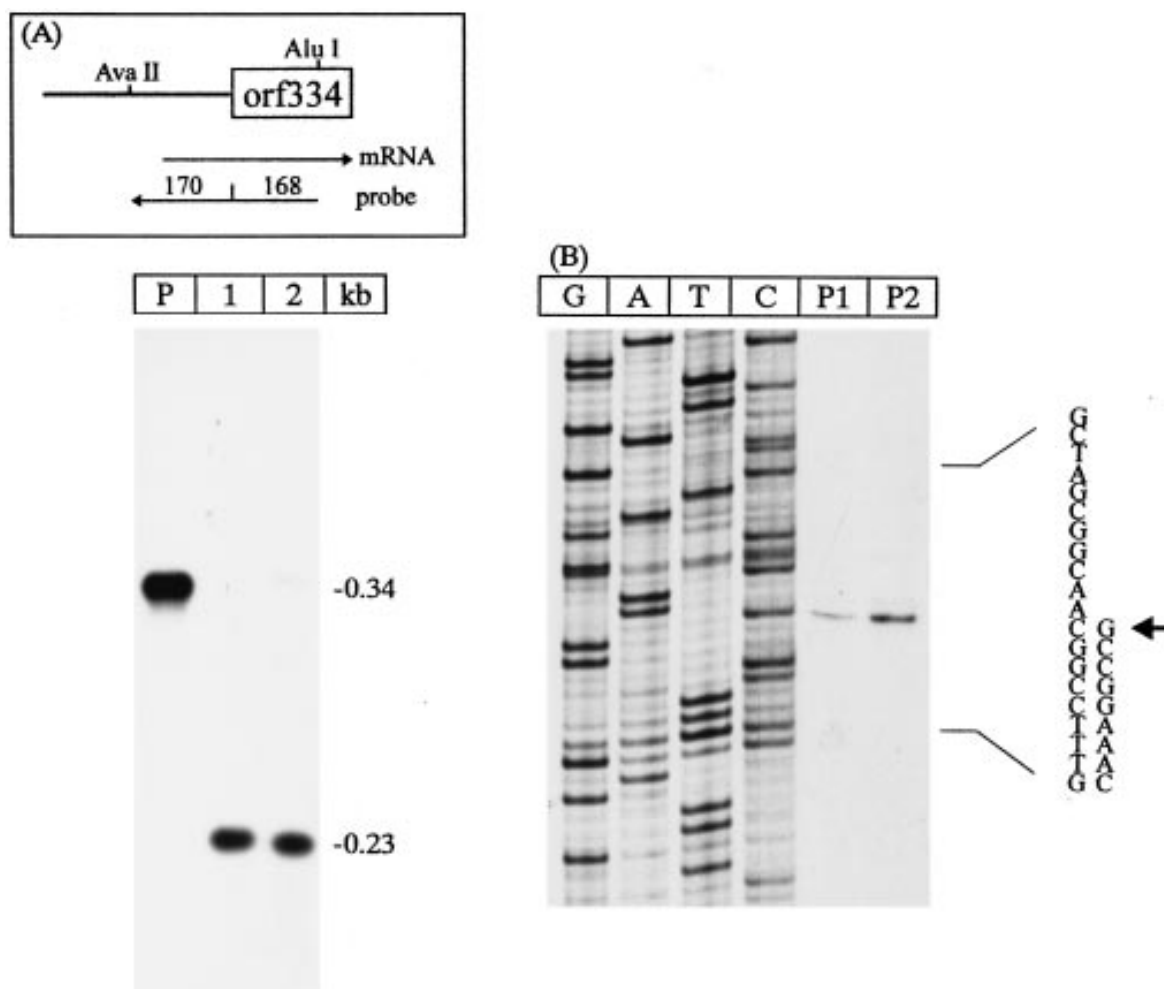
Fig. 3. RNase protection (A) and primer extension analysis (B) of the 5′ UTR of *orf334*. (A) A labelled antisense probes (P) of 340 bases was hybridized to *Synechocystis* RNA. Hybridization was followed by digestion with two different RNase concentrations: (1) 7 μg RNase A, 25 U RNase T1, (2) 3.5 μg RNase A, 12.5 U RNase T1. Sizes of the probe and of the protected RNA are given in kilobases at the right. The position of the probe is shown in the scheme. (B) Primer extension was done with primer P_E (see Section 2). Extension products of two independent reactions (P1), (P2) are shown. Sequencing ladders of corresponding DNA fragments served as a reference. The transcriptional start site is indicated by an arrow.

biogenesis (*helA*, *helB* or *ccl2*) are encoded in the genome of *Synechocystis*. Although there are several genes with sequence identity to *helA*, neither a *helB* homologue nor a *ccl2* homologue was detectable. The *helA* gene encodes the ATP-binding subunit of the putative ABC-type heme translocator [5]. As the ATP-binding regions of different transporters share significant sequence identity [32], the *helA*-like sequences of *Synechocystis* probably mirror more the presence of other ABC-transporters than reflect the occurrence of a *helA*-related gene. Taken together, different components seem to participate in the final steps of cytochrome *c* biogenesis in proteobacteria/mitochondria versus cyanobacteria/chloroplasts and Gram-positive bacteria.

Insertional inactivation of *orf334* gave rise to a hemizygous mutant only. Since *c*-type cytochromes are known to participate in both photosynthetic and respiratory electron transport in cyanobacteria [33], we expect that disruption of all *orf334* gene copies would lead to a loss of all *c*-type cytochromes and, eventually, to the death of the cell. Interestingly, a similar approach with *orfX16* in *B. subtilis* failed. No *orfX16⁻*-mutant was selectable by insertional mutagenesis

[34]. Hence, the gene product of *orfX16* appears to be essential for *B. subtilis* as with *orf334* of *Synechocystis*.

Approximately 50% of the *orf334* gene copies contained a kanamycin cassette insertion in the hemizygous mutant. Nevertheless, the mRNA accumulation of *orf334* was similar in wild-type and mutant cells, i.e. transcription and/or transcript stability is upregulated in mutant cells and compensates for the loss of gene copy number. Thus, the *orf334* gene product should be present at wild-type level also in hemizygous cells. In line, a heme staining of *c*-type cytochromes did not show any difference between the hemizygous mutant and wild-type (not shown). Our observation of a low, but detectable level of the *orf334* transcript in dark grown cells and of a higher level in light-grown cells supports the idea of a participation of the *orf334* product in the biosynthesis pathway of *c*-type cytochromes.

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