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Expression levels of domestic cDNA cassettes integrated in the nuclear genomes of various *Chlamydomonas reinhardtii* strains

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We attempted to overexpress three types of expression cassettes, each of which contained a different open reading frame (ORF) of domestic *Chlamydomonas* cDNAs. Each ORF was strongly driven by an artificial hybrid promoter. We used two wild-type *Chlamydomonas* strains (i.e., CC-124 and CC-125) and two mutant strains [i.e., UV-mutated (UVM) 4 and UVM11] that have been reported to have a high potency for expressing nondomestic nuclear transgenes. We found that the 1-deoxy-D-xylulose-5-phosphatesynthase (DXS1), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR1), and squalene synthase (SQS) cassettes were not readily overexpressed in the wild-type strains at levels where the products were clearly detectable by Western blotting using a monoclonal antibody. In contrast, Western blot-positive *SQS* cassette transformants were frequently detected in the UVM4 and UVM11 strains, i.e., at an approximately 4.5 times higher frequency than that in the CC-124 wild-type strain. Moreover, transformants that accumulated large amounts of the SQS protein were obtained frequently in the UVM4 and UVM11 strains, i.e., the frequency was approximately 2.2 times higher than that in the CC-124 strain. However, a position effect of the integrated expression cassette was obviously detected not only in the wild-type but also in UVM strains. This suggests that the epigenetic repression mechanism of transgenic genes was not completely knocked out, even in the UVM strains. Further improved *Chlamydomonas* strains are essential to facilitate high-throughput screening of transformants that express nuclear transgenes at a high level.

[**Key words:** cDNA expression cassettes; *Chlamydomonas reinhardtii*; Nuclear transformation; UV-mutated strains; Overexpression]

The unicellular green alga, *Chlamydomonas reinhardtii*, is expected to be a potentially low-cost host for generating various products, e.g., biofuel, pharmaceutical terpenoid, and vaccine, because of its rapid phototrophic growth potency and the availability of several advanced molecular techniques (1). However, the nuclear expression levels of nondomestic cDNA genes are disappointingly poor (2,3). Unsuitable codon usage in the open reading frame (ORF) is one of the proven causes of this problem (4). The random integration of transgenes in *C. reinhardtii* has been analyzed using the RESDA-PCR method (5).

Moreover, the integration position of the construct dramatically affects the expression level (1,6), which is referred to as the position effect. Recently, Neupert et al. (7) developed *C. reinhardtii* UV-mutated (UVM) strains to overcome the inhibited expression of foreign genes. These cell wall-deficient strains, UVM4 and UVM11, harbor unknown and unmapped mutation(s), and it has been suggested that the epigenetic transgene suppression mechanisms have been successfully knocked out in these mutants. To the best of our knowledge, high expression in these UVM strains has only been demonstrated for green fluorescent protein (GFP) and yellow fluorescent protein (YFP) (7). Moreover, all of the reported genes are nondomestic genes, and their codon usage was not optimized for *C. reinhardtii*. This suggests that we might readily obtain transformants that accumulate high levels of target proteins if endogenous genes could be introduced into the UVM strains, because their codon usage would not interfere with their translation.

1-Deoxy-D-xylulose-5-phosphatesynthase (DXS1) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR1) proteins are expected to be key enzymes involved with isoprene synthesis in the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (8), while squalene synthase (SQS) protein catalyzes the first enzymatic step in the central isoprenoid pathway during sterol and triterpenoid biosynthesis (9). These three genes are unique and essential and contain a limited number of rare codons, which probably have no harmful effects on their translation levels.

In this study, we examined the expression levels of the cDNA cassettes of *DXS1* and *SQS* in the wild-type strain CC-124, and that of DXR1 in the wild-type strain CC-125. We also introduced the *SQS* expression cassette into the UVM4 and UVM11 strains to compare the expression levels of the wild-type strain and the UVM mutants.

MATERIALS AND METHODS

Construction of transformation vectors The cassettes of *Hsp70A*/*RbcS2* promoter (containing the first intron of *RbcS2*) and *RbcS2* terminator were cloned into pUC18 (without the *BamH*I restriction enzyme site) as a *Sac*I/*Kpn*I fragment to generate the pHsp70A/RbcS2-plasmid. This artificial promoter also contains the first intron of *RbcS2* because of its enhancer activity (10). A gp64-tag sequence (11) was integrated into the respective constructs for analysis with Western blotting. The

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fragments containing the gp64-tag sequences were as follows: gp64 tag 1: 5′-GATGCTCGAGTCCTGGAAGGACGCGTCCGGCTGGTCCCATATGTAATAAAGTACTACTAG TGGATCCCCGCT-3′ and gp64 tag 2: 5′-GATGCTCGAGAGATCTACACCGACTAGTTCC TGGAAGGACGCGTCCGGCTGGTCCTAATAAGGATCCCCGCT-3′. These sequences were synthesized and cloned into the *Xho*I/*BamH*I site of pHsp70A/RbcS2 plasmid to construct the pHsp70A/RbcS2-gp64 tag 1 and pHsp70A/RbcS2-gp64 tag 2 plasmids, respectively.

DXS1, *DXR1*, and *SQS* cDNA were amplified using polymerase chain reaction (PCR) with high-fidelity PrimeSTAR HS DNA polymerase (Takara, Japan), according to the manufacturer's instructions. The specific sets of primers used for each amplification reaction were as follows: DXS1fw (5′-AAGAGATCTATGCTGCGTGGTGCTG-3′) and DXS1rev (5′-AAGACTAGTCGCTTGCAGCGCTGA-3′) for *DXS1*, which were generated by introducing *Bgl*II and *Spe*I sites at the 5′ ends, respectively (the restriction endonuclease sites are underlined); DXR1fw (5′-AAGCTCGAGATG CAGTCCGCGCAGC-3′) and DXR1rev (5′-AAGACTAGTGGCCATGGCCAGCACC-3′) for *DXR1*, which were generated by introducing *Xho*I and *Spe*I sites at the 5′ ends, respectively; and SQSfw (5′-CATATGGGAAAGTTAGGGGAGCTACTCTC-3′) and SQSrev (5′-GGATCCTCACGCACGCCGCA-3′) for *SQS*, which were generated by introducing *Nde*I and *BamH*I sites at the 5′ ends, respectively. The amplification conditions were as follows: 98°C for 1 min, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 15 s, and extension at 72°C for 2 min using a Thermal Cycler 2720 (Applied Biosystems, Foster City, CA, USA).

The *DXS1* cDNA fragment (~2.2 kb) was inserted into the pHsp70A/RbcS2-gp64 tag 1 plasmid as a *Bgl*II/*Spe*I fragment, generating the recombinant plasmid pHsp70A/RbcS2-gp64-DXS1. The *DXR1* cDNA fragment (~1.4 kb) was inserted into the pHsp70A/RbcS2-gp64 tag 1 plasmid as a *Xho*I/*Spe*I fragment, generating the plasmid pHsp70A/RbcS2-gp64-DXR1. The *SQS* cDNA fragment (~1.4 Kb) was inserted into the pHsp70A/RbcS2-gp64 tag 2 plasmid as a *Nde*I/*BamH*I fragment, generating the plasmid Hsp70A/RbcS2-gp64-SQS. The pHyg3 plasmid, which contained *aphVII* and conferred hygromycin resistance (12), was double digested with *Hind*III and subcloned into the Hsp70A/RbcS2-gp64-DXS1 plasmid in the sense orientation to generate the *DXS1* overexpression construct. The pSI103 plasmid, which contained *aphVIII* and conferred paromomycin resistance (13), was double digested with *Xba*I and *Kpn*I and then subcloned into the Hsp70A/RbcS2-gp64-DXR1 plasmid in the sense orientation to generate the *DXR1* overexpression construct. The pSP124S plasmid, which contained *ble* and conferred zeocin resistance (14), was double digested with *Xba*I and *Kpn*I and then subcloned into the Hsp70A/RbcS2-gp64-SQS plasmid in the sense orientation to generate the *SQS* overexpression construct. The DNA sequences of all the constructs were confirmed by direct sequencing using the dideoxy chain termination method (15). The final *DXS1*, *DXR1*, and *SQS* overexpression constructs are shown schematically in Supplementary Fig. S1.

Strains and transformation conditions *C. reinhardtii* CC-124 (wild-type, mt^-) and CC-125 (wild-type, mt^+) were provided by the *Chlamydomonas* Resource Center (Duke University, USA), while the UVM4 and UVM11 strains (7) were kindly provided by Dr. R. Bock (MPI-MP, Germany). Unless stated otherwise, cells were cultivated mixotrophically at 25°C in Tris-acetate phosphate (TAP) medium (16) under constant cool white fluorescent light (84 μmol photons m^{-2} s^{-1}) with gentle shaking. Before transformation, vectors were linearized with a single restriction enzyme and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA), according to the manufacturer's protocols. Approximately 300 ng linearized plasmid DNA was used during each transformation experiment by electroporation, as described previously (17). Transformants of the *DXS1*, *DXR1*, and *SQS* overexpression constructs were selected directly on 1.5% TAP agar plates containing hygromycin B (30 mg L^{-1}), paromomycin sulfate (20 mg L^{-1}), or zeocin (15 mg L^{-1}), respectively, and the plates were incubated under continuous fluorescent light (20 μmol m^{-2} s^{-1}) at 25°C.

PCR screening of cotransformants Transformants with antibiotic resistance were screened to detect the presence of the expression cassettes using a promoter-specific forward primer and a gene-specific reverse primer, the locations of which are shown in Supplementary Fig. S1. Genomic DNA was isolated from independent transformants using the CTAB method (18). The specific sets of primers used to examine construct integration were as follows: forward, 5′-AAAATGGCCAGGTGAGTCGACG-3′and reverse, 5′-GATTGACCGAAGCCTCCGCG-3′ for *DXS1*; forward, 5′-CGTTTCCATTTGCAGGCTCGAGA-3′ and reverse, 5′-AGCAGCTTCA CGTTGGAGCC-3′ for *DXR1*; and forward, 5′-TTGCAGGATGCTCGAGTCCTG-3′ and reverse, 5′-CCACGTCCAGTGCCATGTCA-3′ for *SQS*. The amplification conditions were as follows: 95°C for 5 min; followed by 30 cycles at 95°C for 15 s, 54°C for 30 s, and 72°C for 30 s; and a final step at 72°C for 7 min using the Thermal Cycler 2720 (Applied Biosystems).

Western blot screening of transformants Total soluble protein extracts were prepared by resuspending pelleted 5.0×10^6 *Chlamydomonas* cells in 50 μL of 2X SDS sample buffer (100 mM Tris, pH 6.8; 4% SDS; 1 mM DTT; and 30% glycerol). The samples were denatured at 95°C for 7 min. Subsequently, 5-μL aliquots of the supernatants from the denatured cell lysates were separated using 10% SDS–PAGE, transferred using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA) to polyvinylidene difluoride (PVDF) membranes (Hybond P; GE Healthcare, UK), and incubated overnight with standard transfer buffer (25 mM Tris, 20% methanol, 0.01% SDS, and 192 mM glycine). Immunobiochemical protein detection was performed using a monoclonal anti-BV envelope gp64 primary antibody (1/5000) (Santa Cruz, CA, USA) and ECL peroxidase-labeled anti-mouse secondary antibody (1/20,000) (Sigma–Aldrich, Canada) with an ECL detection system (GE Healthcare).

RESULTS AND DISCUSSION

All of the vectors used for transformation contained one of the three expression cassettes with a marker gene cassette in the same strand (Supplementary Fig. S1). The gp64 epitope tag for analysis with Western blotting was introduced at the N-terminal end in SQS, whereas it was introduced at the C-terminal end in DXS1 and DXR1 (Supplementary Fig. S1), given that the N-terminal transit peptide region of DXS1 and DXR1 proteins are removed post-transnationally to localize these proteins in plastids.

The linearized plasmids for *DXS1* or *SQS* were introduced into the CC-124 strain (mt^-) to allow overexpression, whereas the linearized plasmid for *DXR1* was introduced into the CC-125 strain (mt^+). The antibiotic resistance characteristics used for selection guaranteed the integration of the marker gene in the transformants, but this does not guarantee the cointegration of the cDNA expression cassette in the transformants. To exclude transformants without the cointegrated expression cassette of the target gene, we performed PCR to confirm the integration of the promoter region. PCR was performed using a set of primers, which were specific to 3′ end of the *Hsp70A/RbcS2* promoter and the N-terminal region of the cDNA expression cassette. For the PCR-positive transformants, we assumed that the intact cDNA cassette was integrated into the genome with the marker gene cassette; therefore, we tentatively counted these transformants as being successfully cotransformed (Table 1). The PCR results showed that the cotransformation ratios (number of PCR-positive transformants/number of transformants analyzed) for *DXS1* with *aphVII* (introduced into the CC-124 strain), *DXR1* with *aphVIII* (introduced into the CC-125 strain), and *SQS* with *ble* (introduced into the CC-124 strain) were approximately 42.4% (112/264), 21.8% (55/252), and 51.9% (109/210), respectively (Table 1). The relatively lower cotransformation ratio of the *DXR1*

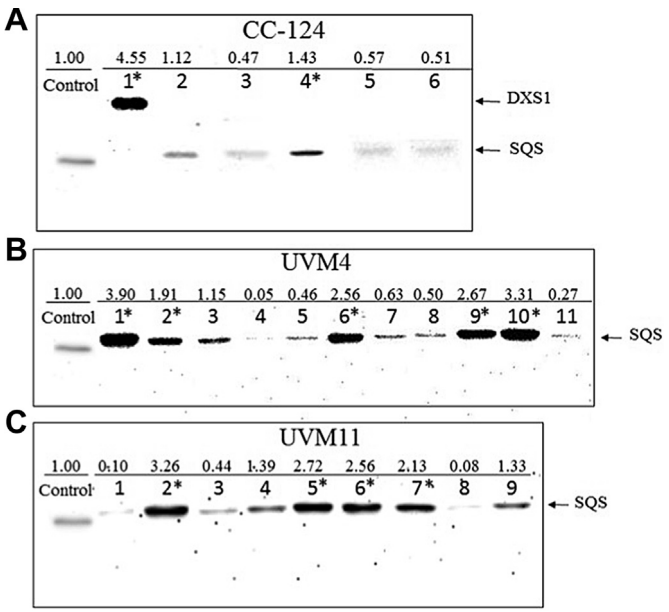

FIG. 1. Identification of DXS1 and SQS protein-positive transformants by Western blotting. The DXS1- and SQS-positive transformants in the CC-124 strain (A) and SQS-positive transformants in the UVM4 (B) and UVM11 (C) strains are shown. The bands were quantified using the Quantity One 1-D Analysis Program (Bio-Rad). The band intensities are shown as relative intensity units in their respective gels. Band intensity values greater than 11 were defined as strongly expressing transformants in this study. Asterisk indicates transformants that expressed the target proteins at high levels.

TABLE 1. Expression of *SQS* cDNA casette in various *Chlamydomonas reinhardtii* strains.

Gene name	Host strain	Cotransformation ratio[a]	Western blot-positive ratio[b]	Highly expressing transformant ratio[c] relative to western blot-positive ratio
SQS	CC-124 (WT)	109/210 (51.9%)	5/109 (4.6%)	1/5 (20.0%)
	UVM4	52/87 (59.8%)	11/52 (21.2%)	5/11 (45.5%)
	UVM11	51/87 (58.6%)	9/51 (17.6%)	4/9 (44.4%)

WT, wild-type.
[a] Number of PCR-positive transformants/number of transformants analyzed.
[b] Number of Western blot-positive transformants/number of PCR-positive transformants.
[c] Number of high-protein accumulation transformants/number of Western blot-positive transformants.

cassette was probably because of the characteristics of the CC-125 strain. Recently, it was reported that the optimal transformation efficiency, using the electroporation method (19), for the CC-125 strain was approximately 66% of that for the CC-124 strain.

Western blotting was performed to detect the protein accumulation levels of the PCR-positive transformants. The results showed that the Western blot-positive ratios (number of Western blot-positive transformants/number of PCR-positive transformants) for the *DXS1* and *SQS* transformants in the CC-124 strain were 0.89% (1/112) and 4.6% (5/109), respectively (Table 1 and Fig. 1A). However, no DXR1-positive transformants were found among 55 PCR-positive transformants in the CC-125 strain (data not shown). The low expression levels of the target protein in the wild-type strains were in agreement with previous reports (1–3). The sensitivity of Western blotting performed using a monoclonal antibody for gp64-tag was sufficiently high to detect the protein derived from gp64-tag-containing *aphVII* transformants (data not shown).

Encouraged by the successful overexpression of three non-endogenous fluorescence-related genes in the UVM4 and UVM11 strains (7,20), we examined the overexpression of the *SQS* cassette in the UVM strains. The PCR results showed that the estimated cotransformation ratios for *SQS* in the UVM 4 and UVM11 strains were approximately 59.8% (52/87) and 58.6% (51/87) (Table 1), respectively, which were only approximately 8% and 7% higher than those in the CC-124 strain. These results were not consistent with the results of Neupert et al. (7), who showed that the cotransformation ratios of the full-length *GFP* cassette in the genomes of the UVM4 and UVM11 strains were about five times higher than that of the control strain (CW-15, mt^{+}, *arg7*).

However, the Western blotting results detected dramatic differences between the UVM and wild-type strains. The Western blot-positive ratios for SQS in the UVM 4 and UVM11 strains were approximately 21.2% (11/52) and 17.6% (9/51) (Table 1 and Fig. 1B, C), respectively, which were about five and four times higher than the ratio in the CC-124 strain (4.6%, 5/109). Moreover, the high-protein accumulation ratios (number of high-protein accumulation transformants/number of Western blot-positive transformants) of the Western blot-positive clones were 45.5% (5/11) for the UVM4 strain and 44.4% (4/9) for the UVM11 strain (Table 1 and Fig. 1B, C). These ratios were approximately two times higher than those in the CC-124 strain (20.0%, 1/5) (Table 1 and Fig. 1A).

Unstable transgene expression has been observed frequently in *C. reinhardtii* (2). However, the *SQS* transformants generated from the UVM strains were cultured on antibiotic-containing plates for over 5 months, and they exhibited stable expression (data not shown). In addition, the growth rates of all the SQS protein-expressing transformants of the UVM4 and UVM11 strains did not differ significantly from the CC-124 strain (data not shown) in mixotrophic conditions (in TAP medium) with a 16/8-h light/dark cycle or continuous light. These results suggest that the UVM strains are highly suited to the endogenous cDNA transgene expression of moderate-molecular-weight proteins, and they are not restricted to the expression of low-molecular-weight proteins such as GFP.

The *SQS* cDNA cassette originated from domestic mRNA; therefore, its codon usage and substitution in the ORF must have been optimized by evolution. Thus, *SQS* may have fewer translational disadvantages than foreign genes. However, Fig. 1 shows that the SQS protein accumulation levels were not uniformly high even in the UVM strains, which was also the case in the CC-124 strain. It is reasonable to suspect that translational repression works uniformly against all *SQS* mRNAs, irrespective of the genomic locations of the *SQS* that generated the *SQS* mRNA. Therefore, the observed differences in the levels of accumulated SQS protein must have been because of the variable transcriptional levels of the *SQS* located in different genomic regions.

Neupert et al. (7) reported that all PCR-positive *GFP* transformants (9/9) had uniformly high protein accumulation levels. Based on this fact, they suggested the successful knock out of the transcriptional repression pathways of nuclear transgenes in the UVM strains. However, our results of *SQS* expression showed that epigenetic transcriptional repression was alleviated dramatically, although they were not completely free of it even in the UVM strains. This might have been due to the fact that the mutated gene(s) had not lost their activity completely, because these UVM strains were generated by UV treatments to introduce nucleotide mutations. The observed inconsistent characteristics of the UVM strains mentioned above might have been due to the different characteristics of the promoters used to drive the cDNAs, i.e., *GFP* was driven by either *PsaD* or *RbcS2*, whereas *SQS* was transcribed by the *Hsp70A/RbcS2*-hybrid promoter (21).

Further improvements are required to obtain truly epigenetic repression-free *Chlamydomonas* strains. The development of such strains would make *Chlamydomonas* an attractive host for producing biofuel and biopharmaceuticals.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2013.10.025.

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