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Robust expression of heterologous genes by selection marker fusion system in improved *Chlamydomonas* strains

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***Chlamydomonas* is a very attractive candidate plant cell factory. However, its main drawback is the difficulty to find the transformants that robustly express heterologous genes randomly inserted in the nuclear genome. We previously showed that domestic squalene synthase (*SQS*) gene of *Chlamydomonas* was much more efficiently overexpressed in a mutant strain [UV-mediated mutant (UVM) 4] than in wild type. In this study, we evaluated the possibility of a new mutant strain, *met1*, which contains a tag in the maintenance type methyltransferase gene that is expected to play a key role in the maintenance of transcriptional gene silencing. The versatile usefulness of the UVM4 strain to express heterologous genes was also analyzed. We failed to overexpress *CrSSL3* cDNA, which is the codon-adjusted squalene synthase-like gene originated from *Botryococcus braunii*, using the common expression cassette in the wild-type CC-1690 and UVM4 strains. However, we succeeded in isolating western blot-positive transformants through the combinational use of the UVM4 strain and ble2A expression system of which expression cassette bears a fused ORF of the target gene and the antibiotic resistance gene *ble* via the foot-and-mouth disease virus (FMDV) self-cleaving 2A sequence. It is noteworthy that even with this system, huge deviations in the accumulated protein levels were still observed among the UVM4 transformants.**

[**Key words:** Transgene silencing; Position effect; Squalene synthase-like gene; *Botryococcus braunii*; Methyltransferase gene]

Recently, microalgae have attracted much attention as plant cell factories for the production of various commercial products, including biofuels, pharmaceutical terpenoid, nutraceuticals, and therapeutics (1–3). However, until now, only limited transgenic products have been commercialized mainly because of the difficulty in improving strains for abundant accumulation of a product of interest above the required levels.

The unicellular green alga, *Chlamydomonas reinhardtii*, has been a prominent model organism for genetic studies primarily because of the efficient genetic transformation techniques for mitochondria, chloroplasts, and nucleus (4–7). The genome (nuclear, plastid, and mitochondria) of *C. reinhardtii* has been fully sequenced (8), and large chemical and insertional mutant libraries have been established. On the other hand, the major disadvantage of *C. reinhardtii* is the poor expression of transgenes from the nuclear genome (9–11). The molecular mechanism(s) of this is still uncovered, and a possible reason for this is closely related to the strong transcriptional silencing against transgenes (2,3,11), which is mediated by both DNA methylation and DNA methylation-independent pathways (12,13).

Over the past two decades, several advanced strategies have been developed to improve the expression of transgenes in *Chlamydomonas*, i.e., codon optimization (9–11), utilization of endogenous intron(s) (14), and development of artificial strong promoter (15). Recently, UV-mediated mutant (UVM) strains of *Chlamydomonas* have been isolated for improved transgene expression. The UVM strains harbor unknown and unmapped mutation(s), and it has been suggested that their epigenetic transgene suppression mechanisms have been successfully knocked out (16).

Moreover, a novel nuclear expression system was reported to robustly express heterologous genes (2). The system utilizes the foot-and-mouth disease virus (FMDV) 2A self-cleaving peptide to transcriptionally fuse a transgene open reading frame (ORF) to the antibiotic resistance marker gene *ble* (referred to as ble2A system in this paper). The FMDV 2A peptide, which is a short peptide with approximately 20 amino acid sequences, mediates ribosome-skipping reaction during translation (17). Because of this reaction, when 2A is fused between two ORFs to generate a single ORF, the resulting products are the two discrete proteins with the short 2A peptide sequence fused to the C-terminus of the first protein product, whereas the following protein has only one amino acid of the peptide covalently attached to the N-terminus. The efficient ability of the ble2A system to improve the heterologous expression of transgenes compared with the traditional nuclear expression vector in which the expression of the interest and selection marker

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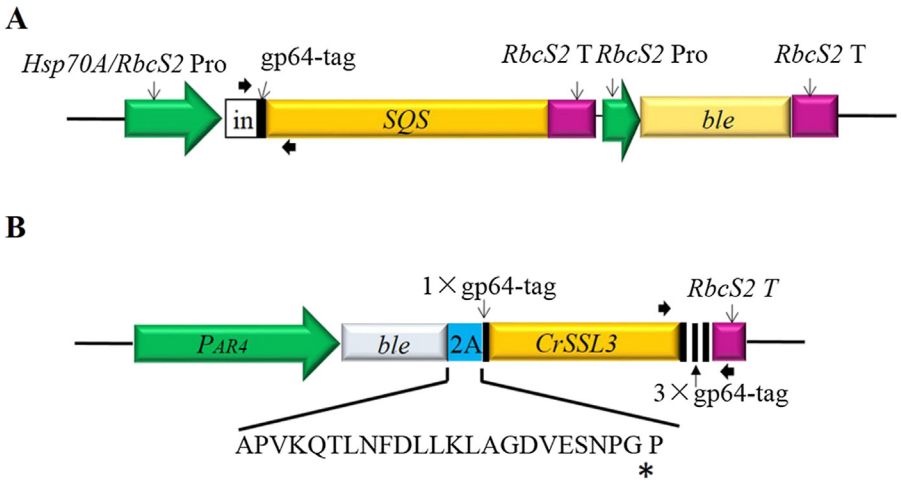

FIG. 1. Schematic representation of the transformation vectors. (A) Expression cassette for *Chlamydomonas SQS* cDNA and *ble* marker gene. In, first intron of the *Chlamydomonas RbcS2* gene; *RbcS2* T, *Chlamydomonas RbcS2* terminator; gp-64-tag, gp-64 gene sequence for epitope tag peptide. The arrows show the location of the PCR primers used for the cotransformation assay. (B) Expression cassette for the codon-adjusted *Botryococcus braunii SSL3* cDNA. *PAR4*, *Hsp70A*/*RbcS2* promoter modified to contain four copies of the first intron of *RbcS2*; 2A, FMDV 2A peptide, *CrSSL3*, codon-optimized *SSL3* cDNA. The amino acid sequence of 2A is denoted by an asterisk to indicate the cleavage site.

genes controlled under independent promoters has been reported (2,18).

Previously, we demonstrated that the *Chlamydomonas* squalene synthase (*SQS*) cDNA was much more efficiently expressed in the UVM4 and UVM11 than in wild-type strains (19). Recently, using the *Chlamydomonas* MmeI-based insertion site Sequencing (ChlaMmeSeq) method (20), an insertional mutant of *Cre10.g461750* was isolated. This gene encodes DNA methyltransferase 1 (*Dnmt1*) (20), which is expected to be involved in the maintenance of DNA methylation patterns (21,22). Robust transcriptional gene silencing through DNA methylation is one of the major pathways for stable repression of transgenes. In this study, to expand the platform for efficient expression of various transgenes, we evaluated the potency of this tag-inserted strain by comparing domestic *SQS* gene expression levels in four strains CC-124 (wild-type), UVM4, UVM11, and the insertional mutant of *Cre10.g461750* ("*met1*", see below). Moreover, to evaluate the ability of the ble2A system for overexpression of codon-optimized transgenes, a codon-adjusted *SQS*-like 3 gene (*CrSSL3*) and *CrSSL1*, which originated in *Botryococcus braunii*, were heterogeneously expressed in wild-type strains and in the UVM4 strain. These *SSL* genes are key enzymes for the biosynthesis of botryococcene in *B. braunii* B-race (23). The expression levels of these enzymes were closely analyzed by western blotting using a monoclonal antibody against gp-64 epitope.

MATERIALS AND METHODS

Construction of the transformation vectors The construction of the transformation vector containing *SQS* expression cassette was shown in detail by Kong et al. (19). For the construction of the PAR4::ble-2A-SSL::term expression vector, the *ble* sequence, which contained one copy of the *RbcS2* intron 1, was fused in frame to the codon-optimized FMDV 2A coding sequence (2), and synthesized as an *Xba*I-*Nde*I/*Kpn*I fragment. The ble-2A fragment was inserted into a pSTBlue-1 plasmid (EMD Biosciences, USA) as *Xba*I/*Kpn*I fragment, generating the recombinant plasmid pSTBlue-1-ble-2A. The *Hsp70A* promoter fragment was amplified by polymerase chain reaction (PCR) with high-fidelity PrimeSTAR HS DNA polymerase (Takara, Japan) from the pALM32 plasmid (24) using primers *Xba*I-Hsp70A-F (5′-AATCTAGAGACGGCGGGG-3′) and *Nde*I-*Hind*III-Hsp70A-R (5′-CATATGAACTGAAGCTTGAGTGGTTATGTA-3′). This fragment was inserted into the pSTBlue-1-ble-2A plasmid as a *Xba*I/*Nde*I fragment, generating the recombinant plasmid pHsp70A-ble-2A.The fragment containing the sequence of *RbcS2* 3′ untranslated region (UTR) terminator was excised from the pHsp70A/RbcS2-cgLuc plasmid (25) by *BamH*I-*Kpn*I digestion and cloned into pHsp70A-ble-2A, resulting in the recombinant plasmid pHsp70A-ble-2A-term. For the construction of four parallel copies of the first intron (intron 1) of *RbcS2*, the sense and antisense single-stranded oligonucleotides intron 1-left (5′-CAGGTGAGTCGACGAGCAAGCCCG GCGGATCAGGCAGCGTGCTTGCAGATTTGACTTGCAACGCCCGCATTGTGTCGACGAA GGCTTTTGGCTCCTCTGT-3′) and intron 1-right (5′-TGCCTGCAGGAATTCGA TTGGTCTTGGCCATCCTGCAAATGGAAACGGCGACGCAGGGTTAGATGCT GCTTGAGACAGCGACAGAGGAGCCAAAAGCCTT-3′), respectively, were synthesized, annealed, and used as the templates to generate the fragment of intron 1 using the following primers: intron 1-left-F (5′-AAGCTTGATTGTCATGGC CAGGTGAGTCGACGAGCAAG-3′) and intron 1-right-R (5′-CCATGGGATATC GCATGCCTGCAGGAATTCGATTG-3′) by employing the overlap extension PCR (OE-PCR) (26) method. The fragment was then used as the template to amplify four parallel copies of intron 1 using the following primers: *Hind*III-c1-F (5′-AAGTAAAAGCTTGATTGTCATGGCCAG-3′) and *Sac*I-c1-R (5′-AAGTAAGAGCTCCCATGGGATATCGCATGC-3′) for intron 1-copy 1; *Sac*I-c2-F (5-AAGTAAGAGCTCGATTGTCATGGCCAGGTG-3′) and *Xba*I-c2-R (5′-AAGTAATCTAGACCATGGGATATCGCATGC-3′) for intron 1-copy 2; *Xba*I-c3-F (5′-AAGTAATCTAGAGATTGTCATGGCCAGGTG-3′) and *Sac*I-c1-R for intron 1-copy 3; *Sac*I-c2-F and *Kpn*I-c4-R (5′-AAGTAAGGTACCCCATGGGATATCGCATGC-3′) for intron 1-copy 4. These four fragments of copies of intron 1 were double digested using the introduced restriction enzymes and then ligated using the Mighty Mix DNA ligation kit (Takara) to generate four parallel copies of intron 1 of *RbcS2* as *Hind*III/*Kpn*I fragment. The *RbcS2* promoter fragment was generated by PCR from pHsp70A/RbcS2-cgLuc plasmid using the primers *Kpn*I-RbcS2-Pro-F2 (5′-TAAGGTACCCCGGGCGCGCCA-3′) and *Nde*I-RbcS2-Pro-R2 (5′-CTTGGCCATATGTTTAGATGTTGAGTGACT-3′). The obtained fragment containing four copies of intron 1 and *RbcS2* promoter were digested by the *Hind*III-*Kpn*I and *Kpn*I-*Nde*I restriction enzyme couples, respectively, and then inserted into the *Hind*III/*Nde*I sites of pHsp70A-ble-2A-RbcS2 plasmid, generating the recombinant plasmid PAR4::ble-2A::term expression vector.

The codon usage of *CrSSL3* and *CrSSL1* cDNAs was optimized for *Chlamydomonas* (http://www.kazusa.org.jp/codon), and the codon-adjusted 1 × gp64-tag (27) sequence was attached at the N-terminus. These sequences were synthesized as *Xho*I/*Kpn*I fragments. For the construction of the GS-linker-3 × gp64-tag fragment, the sense and antisense single-stranded oligonucleotides GS-linker-1 × gp64-tag (5′-ATGGGCGGCAGCGGCGGCGGCAGCGGCGGCGGCAGCGGCTCCTGGAAGGACGC-GAGCGGCTGGAGCATCAGCGGCTCCTGGAAGGA-3′) and 2 × gp64-tag (5′-CGTGCCCTCAGTGGATCCTTATTAGCTCCAGCCGCTCGCGTCCTTCCAGGAGCCGCTC-CAGCCGCTCGCGTCCTTCCAGGAGCCGCTGAT-3′) were synthesized, annealed, and used as the templates to generate the *Kpn*I-GS-linker-3 × gp64-tag-*BamH*I fragment by employing the OE-PCR method with the following primers: *Kpn*I-GS-linker-3 × gp64-tag-F (5′-AAGTAAGGTACCATGGGCGGCAGCGGC-3′) and *BamH*I-3 × gp64-tag-R (5′-CGTGCCCTCAGTGGATCCTTATTA-3′). The fragments of the *SSL3* and *SSL1* cDNA cassettes and GS-linker-3 × gp64-tag were double digested by the *Xho*I-*Kpn*I and *Kpn*I-*BamH*I restriction enzyme couples, respectively, and then cloned into the PAR4::ble-2A::term vector as an *Xho*I/*BamH*I fragment to generate the *SSL3* transformation vector that is schematically shown in Fig. 1. The DNA sequences of all the constructs were confirmed by direct sequencing using the dideoxy chain termination method (28).

***C. reinhardtii* strains, growth, and transformation conditions** The *C. reinhardtii* strains CC-124 (wild type, mt^-) and CC-1690 (wild type, mt^+) were provided by the *Chlamydomonas* Resource Center (MN, USA), the C-9 strain (wild