Short Communication

An improved ARG7-based selection cassette with highly efficient transformation rates and a small size suitable for complex expression constructs

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ABSTRACT

In *Chlamydomonas reinhardtii*, the ARG7 locus constitutes one of the first and most widely used selection markers. Until now it has been available only as a large, intractable genomic fragment unsuitable for incorporation in complex expression constructs and exhibiting poor transformation efficiency. Here we succeed in significantly improving the ARG7 nuclear marker by placing the short arginosuccinate lyase (ASL) cDNA under its own promoter and 5' UTR and by including the first ASL intron. We test the new ARG7 marker in three different arginine auxotrophic strains and show highly superior transformation efficiencies compared to the conventional genomic ARG7 marker. The plasmid carrying the shorter ARG7 selection cassette has been deposited at the *Chlamydomonas* Resource Center under the name pHR11.

INTRODUCTION

Chlamydomonas reinhardtii has emerged as a leading model system to explore the potential of micro-algae as a low-cost platform for the production of recombinant proteins, biofuels, nutraceuticals, and other natural products. The suite of available molecular tools for genetically modifying *C. reinhardtii* is unparalleled in comparison to other micro-algae, and includes a number of positive selection markers. These marker genes were typically cloned as genomic fragments (nitrate reductase 1 [1], oxygen-evolving enhancer protein 1 (OEE1) [2], arginosuccinate lyase [3]). Despite subsequent advances in molecular biology techniques, the current publicly available versions of these markers are typically still large, intractable genomic fragments, which are unsuitable for incorporation in complex expression constructs due to their size, and which generally exhibit poor transformation efficiencies.

One of the most frequently used markers for nuclear transformation in *C. reinhardtii* is the endogenous arginosuccinate lyase gene (ASL, EC 4.3.2.1). This enzyme performs the last step in the arginine biosynthesis pathway and rescues arginine auxotrophic strains that have been mutated at the ARG7 locus [3]. The current publicly available pARG7.8 plasmid at the *Chlamydomonas* Resource Center contains a 7.8kb genomic fragment, which has widely been used as a co-transformed selectable marker [4,5,6]. In a previous study, the cDNA fragment of the ASL gene was isolated to create a shorter selection marker that would simplify DNA manipulation procedures and increase transformation efficiency [7]. Sequence analysis of the cloned cDNA revealed an extra exon and intron [7] upstream of the previously predicted 13 coding exons in the pARG7.8 plasmid, and a 3' UTR region that was 468bp longer [8]. Both cDNA versions – one containing all 14 exons and the other lacking the first exon and intron – complemented arginine auxotrophic strains, but

did not exhibit improved transformation efficiency compared to that of the larger ARG7.8 genomic fragment [7]. These cDNA versions were devoid of all introns and were placed under the control of various promoters and 5' UTR regions, which were also devoid of introns (Fig. 1) [7].

In this study, we generate a transformation construct containing the ASL cDNA under its own endogenous promoter and a 5' UTR that contains the first exon and intron of the gene (Fig 1). The construct is approximately half the size of the publicly available pARG7.8 construct and shows superior transformation efficiencies in three different arginine auxotrophic strains, with transformation rates at least 10 fold higher than the genomic fragment.

MATERIALS AND METHODS

Plasmids, strains and growth conditions

Arginine auxotrophic strains CC1819, CC1820, and CC1826, and wild type CC1010 were obtained from the *Chlamydomonas* Resource Center (chlamycollection.org, University of Minnesota). Strain names correspond to the Resource Center's nomenclature. Plasmid pArg7.8 was obtained from the *Chlamydomonas* Resource Center. Cultures were grown with constant shaking under light in standard tris-acetate-phosphate media (TAP); arginine auxotrophs were supplemented with 100mg/L L-arginine (Sigma, St. Louis, MO, USA).

RNA isolation, cDNA generation and PCR

RNA was isolated using the RNA Bee reagent (Amsbio, Lake Forest, CA, USA) following the manufacturer's instructions. cDNA was generated using the RevertAid Reverse Transcriptase kit (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's instructions and recommendations for GC-rich templates. The best results were obtained with oligo(dT)₁₈ primers. The PfuX7 polymerase [9] was used together with the Phusion polymerase GC buffer (New England Biolabs, Ipswich, MA, USA) and DMSO at a final concentration of 4% v/v.

Cloning ARG7 CDS

Fragments up to 1700bp were amplified from cDNA. Full-length cDNA for ARG7 was assembled seamlessly from smaller fragments using the USER fusion technique. The ARG7 cDNA was amplified in three fragments: the promoter and 5' UTR (forward primer: LS160, GGGTTTAAUGTCGACGAGGAGGAGGTGC; reverse primer: LS161, ATGACGCUGAGACGAGGGG), the entire coding sequence (forward primer: LS162, AGCGTCAUGGCCCAGGCAGCTGC; reverse primer: LS163, ACCCGCTCAGUGCTGTCCCTCCGC), and the 3' UTR (forward primer: LS164, ACTGAGCGGGUCGGGGGAGGG; reverse primer: LS165, GGTCTTAAUATGTAACATGTTCTATTTAAAAAGACTGCCC). The three fragments were assembled

102 into the USER cloning compatible vector Hcr1 (pBluescript into which the USER cloning cassette
103 (GCTGAGGGTTTAATTAAGACCTCAGC) has been inserted into the *EcoRI* and *PstI* restriction sites as
104 previously described [10]) by USER fusion as previously described [11], using approximately 100ng of
105 vector and equimolar amounts of each fragment with 1unit USER enzyme (New England Biolabs) in a total
106 reaction volume of 10 μ l. Reactions were incubated at 37°C for 20min followed by 25°C for 20min.
107 Chemically competent DH5- α *E. coli* were heat shock transformed. Once obtained, the plasmid was verified
108 by full sequencing of the entire cassette. The plasmid was named pHR11 and is deposited at the
109 *Chlamydomonas* Resource Center. A GenBank annotated sequence file of the entire plasmid is also
110 deposited.

111 *Transformation conditions*

112 Strains were grown to mid-late log phase (3-5x10⁶cells/ml) before harvesting by centrifugation at 2500rpm
113 and resuspension at 3x10⁸cells/ml in *Chlamydomonas* MaxEfficiency transformation reagent (Life
114 Technologies). 250 μ L of the resuspension was added to 4mm cuvettes, along with 1 μ g of linearized (*PvuI*)
115 plasmid DNA. Cuvettes were incubated at room temperature for 5min, on ice for 5min, and electroporated in
116 a Bio-Rad Gene Pulser Xcell with the following settings: 800V, 25 μ F, infinite resistance. Cuvettes were
117 immediately placed in a room-temperature water bath for 5min, and transferred to 50mL conical tubes
118 containing 10mL of TAP plus 40mM sucrose. Cells were incubated with gentle shaking for 14-18 hours,
119 spun down (2000rpm, 10min), resuspended in 800 μ L TAP with 40mM sucrose, and plated onto selection.
120 Colonies appeared after about 5 days. For each arginine auxotrophic strain, 3 cuvettes were transformed per
121 construct and pooled into a single recovery suspension, which was subsequently plated onto three plates. For
122 each experiment, the sum of colonies from the three plates was recorded. This procedure was repeated three
123 independent times where a new liquid culture was grown as indicated above and used for transformation.

124

125 **RESULTS**

126 **The cDNA-derived ARG7 marker exhibits higher transformation efficiency**

127 The cDNA sequence of ASL has been shown experimentally to contain 14 exons. The first exon begins with
128 a start codon but is suggested to comprise part of the non-coding 5' UTR [7]. In an attempt to improve the
129 ARG7 selection marker we cloned the last 13 exons of the ASL gene (1422bp) and full length 3' UTR
130 (632bp) from wildtype *C. reinhardtii* cDNA and placed the resulting fragment (2054bp) under the control of
131 the 1425bp genomic fragment lying upstream of the 13 coding exons of the ASL gene. The 1425bp fragment
132 contains the first exon and intron and is henceforth called the ARG7 promoter; the entire ARG7 selection
133 construct generated in this study was named pHR11 (Fig. 1). The 1425bp fragment is identical to that
134 controlling the expression of the genomic ARG7 marker in the pARG7.8 plasmid. The transformation
135 efficiency of pHR11 was compared experimentally to that of pARG7.8 in three different arginine

136 auxotrophic strains: CC1819, CC1820, and CC1826. For all three strains, colonies were obtained on TAP
137 media (devoid of exogenous arginine) when complemented by either pHR11 or pARG7.8. In all three
138 strains, pHR11 generated far greater numbers of colonies per μg DNA (Table 1). In CC1826, in particular,
139 approximately 40 times more colonies per μg DNA were generated by pHR11 compared to pARG7.8. In
140 comparison, pHR11 produced approximately 7 and 10 fold more colonies in CC1819 and CC1820,
141 respectively. The pHR11 construct is approximately half the size of pARG7.8, so these increases in
142 efficiency greatly exceed what might be expected based on molar differences in DNA concentration alone.

143

144 **DISCUSSION**

145 In this study, we improve upon the ARG7 selection marker, which is one of the most widely used selection
146 markers for nuclear transformation of *C. reinhardtii*. One aim was to reduce the size of the marker to
147 facilitate molecular manipulations and inclusion in complex vector constructs while simultaneously
148 increasing transformation efficiency. Both aims were achieved by the pHR11 vector, which contains the last
149 13 exons of the ASL cDNA driven by the endogenous ARG7 promoter and the full length 3' UTR. The
150 smaller size of the selection cassette in the pHR11 (3479bp) makes it more amenable for incorporation in
151 complex DNA constructs compared to the longer cassette in the pARG7.8 vector (7683bp). Further reduction
152 in size by truncating the 3' UTR would likely reduce transformation efficiency as shown previously [7]. To
153 the best of our knowledge the effect of truncating the 1425bp promoter has not yet been investigated. The
154 increase in transformation efficiency by several orders of magnitude (10 to 40 fold depending on strain)
155 when using pHR11 compared to the pARG7.8 plasmid far exceeds what would be expected by a linear
156 correlation to the 2 fold increase in complementing DNA concentration per μg total DNA used per
157 transformation. *C. reinhardtii* cleaves transforming DNA using site specific endonucleases [12]. The
158 regulation and activation of the endonucleolytic activity is still unclear but it is suggested that smaller
159 cassettes may be less susceptible to endonuclease activity [12], which our results support. However, there
160 appear to be other factors involved as the increased transformation efficiency in this study stands in contrast
161 to the low transformation efficiencies obtained by the short ASL cDNA-containing vectors generated in a
162 previous study [7]. In the present study and in the study by Auchincloss *et al.*, the full length 3' UTR was
163 included whereas the pARG7.8 construct contains a truncated 3' UTR. Accordingly, it is not likely that the
164 increased transformation efficiency obtained with pHR11 is due to the inclusion of full length 3' UTR. The
165 higher transformation efficiency correlates with the joining of the shorter ASL cDNA with the endogenous
166 ARG7 promoter and 5' UTR, which contains the first ASL intron. The first intron was not included in the
167 study by Auchincloss *et al.* In general, inclusion of introns in expression constructs has been shown to
168 enhance transgene expression [13]; thus, one explanation for the increased transformation efficiency
169 obtained by the pHR11 may be the inclusion of the first ASL intron in the construct. However, we also

170 produced a construct wherein we fused the 13 last ASL exons to the AR1 promoter [14], which includes the
171 first intron from the RBCS2 gene. This construct failed to complement the arginine auxotrophic strains (data
172 not shown), which indicates that some sequence specificity applies to the expression enhancement by the
173 included intron in pHR11. Additionally, for all the arginine auxotrophic strains generated by UV induced
174 mutagenesis, complementation by the ARG7 marker has been shown to depend on the presence of a second
175 mutation in the genome that appears to affect arginine metabolism by inhibiting arginine degradation and
176 increasing arginine uptake processes [15]. It remains to be investigated whether this (hitherto
177 uncharacterized) mutation also affects the strength of the ARG7 promoter/5' UTR. In a recent study, we
178 showed that the ARG7 promoter in the wildtype CC1010 strain could express the arylsulfatase (ARS)
179 reporter gene as strongly as the chimeric AR1 promoter/5' UTR [16]. Perhaps the strong ARG7 promoter is
180 further enhanced in the UV mutated arginine auxotrophic strains. Although speculative, these suggestions
181 coupled with the observations in this study indicate that increased knowledge of arginine metabolism in *C.*
182 *reinhardtii* could result in further improvements to this selection marker and enhance its utility for
183 heterologous expression.

184

185 Numerous arginine auxotrophic strains have been generated in *C. reinhardtii* and differences in
186 transformation efficiencies among strains using the same construct have been observed previously. For
187 example, complementation of *arg7.8* mutants using pARG7.8 produces colonies faster than in *arg7* and
188 *arg7.3* strains [8]. It has been suggested that the *arg7.8* mutant still produces some of the subunits of ASL, as
189 it has been possible to complement it with truncated non-functional versions of the pARG7.8 plasmid [17].
190 The three arginine auxotrophic strains used in this study harbor different mutations in the ARG7.8 locus
191 (CC1819:*arg7.1*; CC1820:*arg7.2* and CC1826:*arg7.8*) [18]. The large differences in transformation
192 efficiency of CC1819, CC1820 and CC1826 using pARG7.8 and pHR11 indicate that these strains require
193 varying levels of ASL expression for complementation. Thus, it is conceivable that ARG selection could be
194 manipulated to select for only very highly expressed transgenic cassettes.

195

196 CONCLUDING REMARKS

197 In summary, we have generated a superior ARG7 selection marker for *C. reinhardtii* with a small size, which
198 enables easy incorporation into complex expression vectors, and which is capable of complementing arginine
199 auxotrophic strains with much higher transformation efficiency than previous *arg7* complementation
200 constructs. In principle, the same strategy could be used to make shorter versions of other widely used
201 positive selection markers, such as nitrate reductase 1 [1] and oxygen-evolving enhancer protein 1 (OEE1)
202 [2], to improve their transformation efficiency and ease of inclusion into complex expression constructs.

203

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265 **Table 1.** Transformation efficiency of pHR11 vs. pARG7.8 in three arginine auxotrophic strains. Numbers
 266 represent colony numbers per 3μg of DNA from three independent transformation experiments for each
 267 construct (colony count is summed from 3 plates, each from a transformation of 1μg DNA). Error is the
 268 standard deviation between experiments.

	pHR11	pARG7.8
CC1819	5596 ± 455	824 ± 36
CC1820	8363 ± 735	887 ± 119
CC1826	832 ± 31	18 ± 4

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270

271 **FIGURE LEGENDS**

272 **Figure 1. Schematic of original and cDNA-derived ARG7 selectable marker constructs.** A: In pARG7.8
273 (cassette size: 7683bp, plasmid size 11552bp), the ARG7 gene is predicted to contain 13 exons [8];
274 characterization of the ASL cDNA by Auchincloss *et al.* revealed that the sequence upstream of the 13
275 codons contained an extra exon (exon 1) and that the 3' UTR in the pARG7.8 is truncated [7]. B-C:
276 Auchincloss *et al.* generated two ARG7 cDNA markers; B: 14 exons driven by various promoters with a full
277 length ARG7 3' UTR. C: 13 exons driven by various promoters with a full length ARG7 3' UTR. D: The
278 pHR11 vector generated in this study, with a cassette size of 3479bp and full plasmid size of 5975bp. The
279 construct joins the 1425bp ARG7 promoter fragment (including first exon and first intron) with the 13 exons
280 and the full length 3' UTR to generate an ARG7 selection marker with superior transformation rates and
281 smaller size compared to previous versions. Potential in frame translation start codons and fragment sizes are
282 indicated in all constructs. Annotation sizes are not to scale.

283