Utility of a GFP reporter system in the red alga *Cyanidioschyzon merolae*

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*Cyanidioschyzon merolae* is a unicellular red alga found in acidic hot springs. The cell contains one nucleus, one mitochondrion, and one plastid, each with its own genome (Kuroiwa, 1998). The complete sequences of the three genomes have been determined (Matsuzaki et al., 2004; Nozaki et al., 2007; Ohta et al., 1998, 2003). *C. merolae* has been proposed as the primitive phototrophic eukaryote because of its extremely simple cell structure and minimally redundant small genome. Therefore, it is used as a model organism to investigate the basic architecture of photosynthetic eukaryotes.

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has been used as a universal reporter of gene expression and subcellular localization analyses in various organisms (Cubitt et al., 1995; Niwa, 2003; Prasher et al., 1992). In a previous study, we established a method to transiently introduce and express the exogenous gene in *C. merolae* (Ohnuma et al., 2008). In this study, we used the GFP reporter system to analyze subcellular localization of proteins in *C. merolae*.

First, we introduced the available GFP expression vector pTH2 (Niwa, 2003), carrying the GFP gene driven by the cauliflower mosaic virus 35S (CaMV35S) promoter (Fig. 1A), into *C. merolae* as described previously (Ohnuma et al., 2008). However, while the CaMV35S promoter shows strong activity in green plant cells, we did not detect any GFP fluorescence by microscopic analysis, suggesting that the CaMV35S promoter does not function in *C. merolae*. This indicated that some native, and preferably strong, promoters should be used instead.

The nuclear gene apcC (CMO250C) is predicted to encode the phycocyanin-associated rod linker, which is a plastid light-harvesting phycobilisome protein. This gene is highly expressed under light growth conditions (Kanesaki et al., unpublished). Bioinformatic analysis using the TargetP algorithm (http://www.cbs.dtu.dk/services/TargetP/) indicated that the ApcC protein is localized in the plastid. The gene product has a 60-amino acid sequence at the N-terminus that is not found in cyanobacterial apcC genes (Fig. 1B). The SignalP algorithm (http://www.cbs.dtu.dk/services/
SignalP/) predicted that this sequence functions as a plastid targeting signal. To analyze localization of the gene product, we first fused the putative \textit{apcC} promoter region (−564 to −1 with respect to the translation start site) to the \textit{GFP} gene (Fig. 1C, pCG1), and then introduced this gene construct into \textit{C. merolae} cells for transient expression. GFP fluorescence was observed mainly in the cytosol (Fig. 2A–D), indicating that the \textit{apcC} promoter can be used to drive the exogenous \textit{GFP} gene. Next, to experimentally determine the subcellular localization of ApcC, GFP proteins fused to the C-end of the full-length ApcC protein or fused to the C-end of the predicted targeting peptide (Fig. 1C, pCG2 and 3) were transiently expressed in \textit{C. merolae} cells. Fluorescence was clearly observed in the plastid in both cases (Fig. 2E–L), indicating that the N-terminal sequence of ApcC functions as a plastid translocating transit peptide.

To further evaluate the GFP reporter system, we transiently expressed GFP fused to the C-terminus of the previously characterized protein, CENH3 (Maruyama et al., 2007), under the control of the \textit{apcC} promoter (Fig. 1C, pCG4). CENH3 is a histone H3 variant protein specifically localized in the nucleus, and is expressed during anaphase of mitotic cell division. When the GFP-fusion DNA construct was introduced into the cell, GFP fluorescence was likely to be observed in the nucleus, and was co-localized with the DAPI staining of the nuclear DNA (Fig. 3). In contrast to the native CENH3 that appeared only during cell division (Maruyama et al., 2007), CENH3-GFP fluorescence was continually observed throughout the cell cycle, probably because of constitutive expression of the \textit{apcC} promoter. These results demonstrate another application of the GFP reporter system.

In this study, we developed the GFP reporter system in \textit{C. merolae} and used it to determine the subcellular localization of proteins. We demonstrated that the N-terminal transit sequence is essential for plastid localization of ApcC. In addition, we used the system to determine the subcellular localization of CENH3-GFP. This GFP reporter system can be used as a transformation marker, and also as a tool to evaluate protein localization in \textit{C. merolae}. This technique has extensive possibilities for further development of \textit{C. merolae} as a model eukaryotic cell.

\textit{C. merolae} 10D was grown at 40°C in MA2 medium (Ohnuma et al., 2008) under continuous illumination (50 μmol photon m$^{-2}$ s$^{-1}$) with 2% CO$_2$ bubbling.

Sequences of the \textit{apcC} genes and the gene products were obtained from the \textit{C. merolae} genome database (http://merolae.biol.s.u-tokyo.ac.jp/) and KEGG.
The amino acid sequences of ApcC proteins were aligned using Clustal X (version 1.83). The subcellular localization and signal sequences of *C. merolae* ApcC were predicted using TargetP and SignalP algorithms, respectively.

The upstream region of *apcC* was PCR-amplified from the *C. merolae* genome using primers PO250-Hind-f and PO250-Xba-r (Table 1). The amplified fragment was then introduced into the polylinker site of pTH2 (Niwa, 2003) using the attached HindIII and XbaI sites, yielding the plasmid pCG1 (Fig. 1C). To construct the plasmids pCG2 and pCG3 (Fig. 1C), genomic fragments were PCR-amplified using the primer sets [PO250-Hind-f, O250all-Nco-r] and [PO250-Hind-f, O250sp-Nco-r]. Each of the fragments was then introduced into the HindIII-NcoI site of pTH2, yielding pCG2 and pCG3 (Table 1). To construct the plasmid pCG4 (Fig. 1C), the ORF region of CENH3 (CME099C)
amplified by primers E099-Xba-f and E099-Nco-r was cloned into the Xba–Nco site of pCG1.

Plasmids were introduced into C. merolae cells as described previously (Ohnuma et al., 2008).

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References


Table 1. Oligonucleotide primers used for cloning in this study.

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<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)*</th>
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<tr>
<td>PO250-Hind-f</td>
<td>GGGCAAGCTTTTACAATACCGATAGATGAG</td>
</tr>
<tr>
<td>PO250-Xba-r</td>
<td>GCTCTAGACGGTCAAACGAAAGAAACAC</td>
</tr>
<tr>
<td>O250all-Nco-r</td>
<td>GGGCCATGCGTACCTTCGACGCGGTGCCGTTGG</td>
</tr>
<tr>
<td>O250sp-Nco-r</td>
<td>GGGCCATGCGATCATTGCACGCGCGAGATGAC</td>
</tr>
<tr>
<td>E099-Xba-f</td>
<td>GCTCTAGAATGCGGCGGTGTAGAAACC</td>
</tr>
<tr>
<td>E099-Nco-r</td>
<td>GGGCCATGCGAGCTACCCGTCGCGATCGC</td>
</tr>
</tbody>
</table>

*Additional sequences that do not correspond to sequences of relevant genes are shown in italics. Restriction sites are underlined.