Mol Gen Genet (1999) 262: 421–425

ORIGINAL PAPER

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Renilla luciferase as a vital reporter for chloroplast gene expression in *Chlamydomonas*

Received: 12 April 1999 / Accepted: 24 June 1999

Abstract The use of luciferases as reporters of gene expression in living cells has been extended to the chloroplast genome. We show that the luciferase from the soft coral *Renilla reniformis* (Rluc) can be successfully expressed in the chloroplast of *Chlamydomonas reinhardtii*. Expression of the *rluc* cDNA was driven by the promoter and 5′ untranslated regions of the *atpA* gene. Western analysis with an anti-Rluc antibody detected a single polypeptide of 38 kDa in the luminescent cells. This is 3 kDa larger than native Rluc, and suggests that translation of the chimeric mRNA begins at the *atpA* start codon, 29 codons upstream from the *rluc* start site. We also show that the luminescence of the transformants was sufficient to enable imaging of colonies using a cooled CCD camera.

Key words Bioluminescence · *Chlamydomonas* · Chloroplast transformation · Chloroplast translation · Vital reporter

Communicated by R. G. Herrmann

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Introduction

The development of biolistic technology, and the availability of well-characterized, non-photosynthetic *Chlamydomonas reinhardtii* mutants first enabled stable transformation of the chloroplast genome (Boynton et al. 1988). Subsequently, dominant selectable markers were identified, which were either homologous mutant genes that conveyed drug resistance (Boynton et al. 1990; Newman et al. 1990) or the bacterial *aadA* gene, which provides resistance to two different antibiotics (Goldschmidt-Clermont 1991). The *aadA* marker has also been used as a quantifiable reporter (Goldschmidt-Clermont 1991), as has β-glucuronidase (Sakamoto et al. 1993). Assays for GUS and *aadA* activity, however, typically involve killing the cells, thus precluding long-term analysis in living cells.

Reporter genes that can be assayed without serious cell damage are known as "vital" reporters. The best characterized vital reporters are luciferases and green fluorescent proteins (GFPs), both of which are derived from bioluminescent organisms (Hastings et al. 1996). Luciferases also offer other advantages as reporter genes, such as the fact that they are produced from a single gene and require no post-translational processing, and that the assay is highly sensitive. Luciferases from a bacterium (*Vibrio*), firefly (*Photinus pyralis*), and the soft coral *Renilla reniformis* have been successfully expressed from the nuclear genome in plants (Mayerhofer et al. 1995). The purpose of our research was to identify a vital reporter that could be used to study chloroplast gene regulation in *C. reinhardtii*, and possibly other plants. Our results demonstrate that the *Renilla* luciferase cDNA (*rluc*) can be used for this purpose.

Materials and methods

Strains and culture conditions

The H13 mutant of *C. reinhardtii* (obtained from Michel Goldschmidt–Clermont, University of Geneva) was grown mixotroph-

ically (light + acetate) in TRIS-acetate-phosphate medium (TAP) as described previously (Deshpande et al. 1995); agar was added to 2% for solid media. Cells for transformation or biochemical analyses were harvested from liquid culture during exponential growth, unless stated otherwise. Cell number was determined with a hemacytometer (Harris 1989). Selection for transformants was performed under moderately strong light (2500 lux) in TAP-minimal medium. After selection, the transformants were grown in TAP under the same light intensity. The temperature for all growth conditions was 23° C. *E. coli* DH5$_\alpha$ (Life Technologies) was used as the host for plasmid pTZRLuc-1. Lysates were made as described below from overnight cultures induced with 1 mM isopropyl beta-D-thiogalactopyranoside (IPTG) for 4 h.

Plasmid construction and chloroplast transformation

The *rluc* coding region was amplified by PCR from pTZRLuc-1 (Lorenz et al. 1991). The upstream primer (5′-CATGCCATGGGTATGACTTCGAAAGTTTATGATCCAG-3′), annealed to the first 25 nucleotides of the coding region and contained an *Nco*I site. The downstream primer (5′-TTTTCTGCAGTTATTGTTCATTTTTGAGAACTCGCTC-3′), was complementary to the last 27 nucleotides of the coding region, and contained a *Pst*I site. The *Pfu* DNA polymerase was used for amplification under conditions recommended by the supplier (Stratagene). The temperature regime was 30 cycles of 94° C for 1 min, 46° C for 2 min and 72° C for 2 min. The product (948 bp) was digested with *Nco*I and *Pst*I (Promega), and cloned into the atpX vector (Goldschmidt-Clermont 1991), which had been similarly digested. Transformation of *E. coli* DH5$_\alpha$ and plasmid DNA analyses were done by standard methods. The *rluc* coding and upstream sequences were sequenced to ensure that no mutations had been introduced. Transformation of the chloroplast of *Chlamydomonas* H13 was performed as described previously (Deshpande et al. 1995) using DNA purified on CsCl gradients.

Bioluminescence assays

Bioluminescence assays were performed at room temperature with a scanning luminometer (MLX, Dynex Technologies) in "flash" mode with shaking enabled. For a standard measurement, an aliquot (20 µl) of cells in luciferase assay buffer [0.5 M NaCl, 0.1 M KH_2PO_4, 1 mM EDTA, pH 7.6 (modified from Matthews et al. 1977)] was pipetted into the well of a microplate. After loading, the microplate was acclimated for several minutes in the dark. Then, 100 µl of substrate was auto-injected into the well, and luminescence measured over a 20-s period, following a 10-s delay window. The substrate, either coelenterazine (Biosynth) or benzyl-coelenterazine (Prolume), was prepared immediately prior to use by diluting a 2.4 mM stock solution (prepared in methanol and stored in aliquots at −70° C) 500-fold into assay buffer. The luminescence data is reported as Relative Light Units (RLU) averaged over the read time. H13 transformed with the atpX vector alone was used as the control strain for most experiments; it did not show appreciable luminescence above background. Background readings with this system were in the range of 0.001–0.003 RLU, and were independent of cell number. Finally, wild-type *C. reinhardtii* (2137) showed no luminescence in this assay.

C. reinhardtii lysates were prepared by sonicating small volumes (less than 250 µl) of cells, resuspended in luciferase assay buffer, four times for 10 s each, with cooling on ice in between; this treatment disrupted the cells and chloroplasts. The sonicates were centrifuged for 5 min at 10,000 × *g* at 4° C, and the supernatants collected and assayed right away. To image luminescent colonies, the transformants were grown on thin agar plates of TAP medium in continuous light. Further manipulations are described in the legend to Fig. 3.

DNA isolation and analysis

Total DNA was isolated as described (Herrin and Worley 1990), except that ethidium bromide replaced bis-benzimide in the CsCl gradient; this change produced a single band of DNA. PCR with *Taq* polymerase, and Southern hybridization analyses were performed as described previously (Deshpande et al. 1995).

Protein electrophoresis and Western analysis

C. reinhardtii and *E. coli* were pelleted by centrifugation, resuspended in 250 mM TRIS-HCl pH 6.8, 10 mM EDTA, 5% SDS, 5% 2-mercaptoethanol, 40% glycerol, 0.125% bromophenol blue, and then heated for 3 min at 100° C. After centrifugation at 10,000 × *g* for 2 min, the supernatant was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels (Laemmli 1970). The gels were stained with Coomassie Brilliant Blue (Sigma) unless they were to be used for Western blotting, in which case, the proteins were transferred electrophoretically to a nitrocellulose membrane (0.45 µm, BioRad) (Memon et al. 1995). The primary antibody was rabbit anti-Rluc (provided by W. Lorenz, University of Georgia), and was used at a final dilution of 1:200. The secondary antibody, a goat anti-rabbit peroxidase conjugate, was used at 1:10,000. The antigen-antibody complexes were detected by chemiluminescence (ECL-Plus system, Amersham). The membrane blocking and washing steps were performed according to the manufacturer's protocols, and the labeled bands were visualized by exposing the blots to X-Ray film (BioMax MS, Kodak).

Results

The relevant part of the expression plasmid, atpX-rluc, including the sequence immediately upstream of *rluc* is shown in Fig. 1. Initially, DNA was isolated from six different transformants (randomly picked) and checked for the presence of *rluc* by PCR; all were positive. Subsequently, the DNAs were analyzed by digestion with *Pst*I and Southern hybridization, using *rluc* as the probe. The expected DNA band was observed in each, plus one additional band that was identical in all six transformants (data not shown). The identity of the latter band is uncertain, but it may have resulted from recombination at the *atpA* or *rbcL* locus (Goldschmidt-Clermont 1991). There was no evidence for integration into nuclear DNA, which normally occurs at random sites (Kindle 1998). All six transformants luminesced in the presence of substrate (coelenterazine), and the specific activities for four of them are given in Table 1. The luminescence

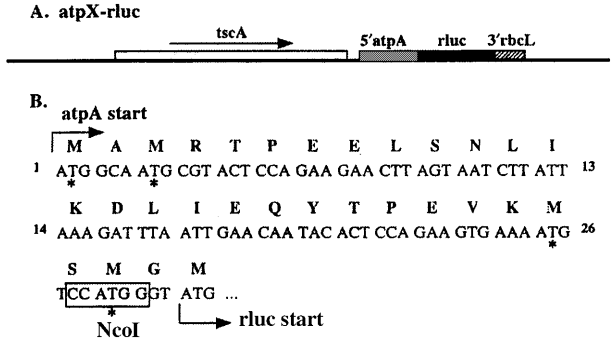

Fig. 1A, B Construct used to express *rluc* in the chloroplast. **A** Map of the relevant region of the atpX-rluc plasmid. The *open rectangle* is a fragment of chloroplast DNA that contains the *tscA* gene; the direction of transcription is indicated by the *arrow*. **B** Sequence upstream of the *rluc* start codon. In-frame start codons are indicated by *asterisks*

Table 1 Luminescence of atpX-rluc transformants

Clone	Luminescence[a]
1	12.2 ± 0.7
2	15.2 ± 1.1
3	12.4 ± 0.3
4	11.7 ± 1.0

[a] Luminescence was assayed with 1000 cells/well under standard conditions; the background was typically 0.0015 Relative Light Units (RLU). The values (±SEM) are expressed as RLU/10^6 cells

of the different transformants was similar, but not identical.

To optimize the in vivo luciferase assay, several parameters were varied. Light emission increased with increasing cell number up to 1×10^6 cells/well, and a further four-fold increase in cell number did not produce any additional light. Luminescence yield was reasonably linear with cell density up to 2.5×10^5 cells/well (data not shown). The luminescence obtained with equivalent amounts of cell lysate was also examined, and found to be similar to that of whole cells, indicating that the substrate readily penetrates the cells under these conditions (data not shown).

The effect of varying the substrate concentration (1–40 μM) on in vivo luminescence was examined using the natural substrate, coelenterazine, and its derivative, benzyl-coelenterazine. Maximal light emission was attained with both substrates in the 3–5 μM range; however, coelenterazine produced 65% more luminescence than did benzyl-coelenterazine. A similar result has been reported for the native enzyme (Inouye and Shimomura 1997). When TAP medium (pH 6.5 or 7.5) was used in place of the luciferase buffer the luminescence was significantly lower, although the reason for this is not clear.

To determine the stability of Rluc in the organelle, protein synthesis in the chloroplast was specifically inhibited with chloramphenicol (100 μg/ml). The luminescence (per 10^6 cells) decreased 18 ± 5% after 4 h incubation with the drug (based on three determinations). This result indicated that Rluc was reasonably stable, but not as stable as some proteins involved in photosynthesis (e.g. Iwanij et al. 1975; Herrin 1986).

Western analysis with an anti-Rluc antibody is shown in Fig. 2A. A single polypeptide of 38 kDa was specifically detected in the luminescent cells. The absence of a major band at this position in the stained gel (left panel) indicated that Rluc was not abundant. The size of the transgenic Rluc seemed to be 3 kDa larger than both the native enzyme and the polypeptide produced from pTZRluc-1, both of which migrate on SDS gels as 35-kDa proteins (Lorenz et al. 1991). To verify this difference, protein from an atpX-rluc transformant and an *E. coli* clone expressing pTZRluc-1 were fractionated on the same gel, blotted and probed with the anti-Rluc antibody. Although the antibody labeled some non-specific bands in the *E. coli* extract, there was only one band in the 30–40 kDa range and it is shown in Fig. 2B (lane 2). This polypeptide migrated at 34.5 kDa, and

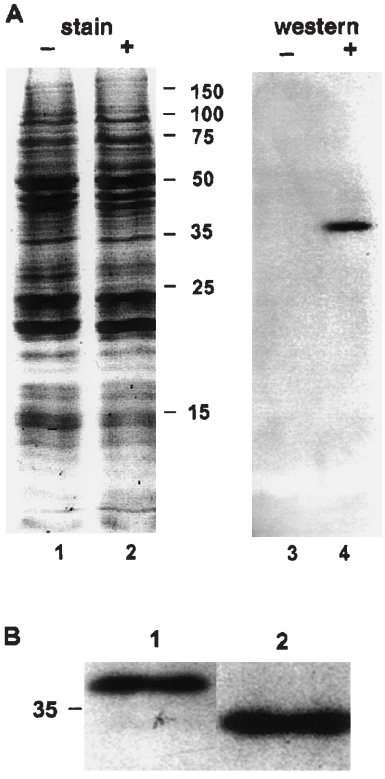

Fig. 2A, B Western analysis with an anti-Rluc antibody. **A** Total protein from the control strain (transformed with the atpX vector only) (–) and from atpX-rluc transformant #4 (+) was subjected to SDS-PAGE and Western blotting. The Coomassie pattern is shown (stain), and the positions and sizes of marker proteins are indicated. Western analysis of the same protein samples with an antibody to Rluc is shown on the right (western). **B** Comparison of Rluc produced in *C. reinhardtii* (lane 1) to that produced in *E. coli* (lane 2). Both samples were run on the same gel. Western analysis was performed as in **A**; lane 1 was exposed to film for 2 min and lane 2 for 20 s. The position of the 35-kDa marker is indicated

clearly had a higher mobility than the Rluc polypeptide produced in *C. reinhardtii* (lane 1).

Finally, it was of considerable interest to determine whether the luminescence of colonies growing on agar could be imaged with a CCD camera (Fig. 3A–C). The image in Fig. 3B was taken immediately after the lights were turned off and represents intrinsic fluorescence. Figure 3C shows the image obtained after 45 min in darkness, after incubating the right half of the agar block in substrate, and the left half in the same solution minus substrate. Signals were easily detected from the colonies incubated with substrate (Fig. 3C, right half), but not from those incubated in media alone. There are a few stray signals on the control exposure, but they did not co-localize with colonies.

Discussion

The Rluc polypeptide produced from the atpX-rluc construct in the chloroplast is 3 kDa larger than the protein produced from pTZRluc-1 in *E. coli*; the latter

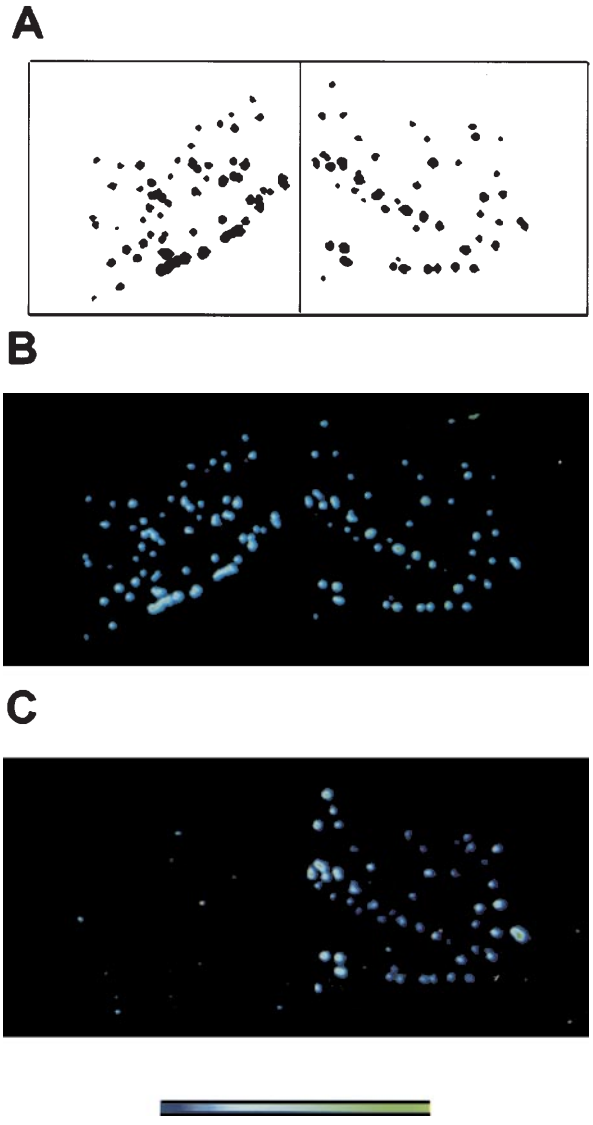

Fig. 3A–C Imaging of luminescent colonies on agar. Transformant #6 was grown on thin agar. Prior to imaging, two equal-sized blocks were excised – the one on the left was incubated in HSM medium (Harris 1989) and the one on the right in HSM plus coelenterazine (37 μM) for 20 min. Imaging was with a cooled CCD camera using a program written by Dr. T. Kondo (Liu et al. 1995). **A** Reproduction of the colonies as seen by eye; the blocks are delineated. **B** Camera image of autofluorescence. The (3-min) exposure was begun immediately upon placing cells in darkness. **C** Camera image of luminescence. After 45 min in darkness, the exposure was made for 15 min. The *colored bar* indicates the computer color representation of the light intensity detected

co-migrated with native Rluc on SDS gels (Lorenz et al. 1991). The most likely explanation for this difference is that synthesis of the transgenic Rluc begins primarily at the *atpA* start codon (Fiedler et al. 1995), which would add 29 amino acids to the primary translation product. Fiedler et al. (1995) also showed that the initiating methionine is removed from the endogenous ATP synthase subunit (product of *atpA*). If this also happens to the putative *atpA-rluc* fusion protein, that would still leave 28 extra amino acids, quite consistent with the estimated size difference. This hypothesis could, in principle, be confirmed by sequencing the N-terminus of the 38-kDa polypeptide; however, the relatively low level of this protein makes this task daunting.

One advantage of the *Renilla* luciferase, compared to the firefly and bacterial enzymes, is the absence of a co-factor requirement, except oxygen. Our data indicate that the substrate (coelenterazine) readily penetrates *C. reinhardtii*; thus, relatively low concentrations (4 μM) of substrate could be used in the assay. A potential disadvantage of *rluc* as a reporter gene, however, is the relative instability of the substrate in various media (Matthews et al. 1977; Inouye and Shimomura 1997; our unpublished results). Hence, long-term monitoring of gene expression in the same culture or colony may require re-application of the substrate before each measurement.

It was recently reported that plastids of higher plants transiently express GFP (Hibberd et al. 1998). We tried to express the wild-type and S65T forms of GFP (Helm et al. 1995) in the *Chlamydomonas* chloroplast. A fluorescence peak of the expected wavelength was observed with the S65T transformants, but it was relatively weak. We also attempted to express the firefly luciferase in the chloroplast, again using the atpX vector, but no luminescence was observed from the transformants. The same construct produced luminescent *E. coli* transformants, however, suggesting that the problem is specific to the chloroplast (I Minko and M Carter, unpublished results).

Acknowledgements We very much thank Dr. Walter Lorenz (University of Georgia) for the *rluc* cDNA and Rluc antibody. This research was supported by grants from USDA (NRICGP 96-35301-3420) and the Welch Foundation (F-1164) to DLH, a grant from the National Institute of Mental Health (R01 MH 43836) to CHJ, and a NSF-NATO Postdoctoral Fellowship (DGE-9617550) to IM. This work was performed in compliance with US laws concerning genetic engineering.

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