A codon-optimized luciferase from *Gaussia princeps* facilitates the in vivo monitoring of gene expression in the model alga *Chlamydomonas reinhardtii*

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**Abstract** The unicellular green alga *Chlamydomonas reinhardtii* has emerged as a superb model species in plant biology. Although the alga is easily transformable, the low efficiency of transgene expression from the *Chlamydomonas* nuclear genome has severely hampered functional genomics research. For example, poor transgene expression is held responsible for the lack of sensitive reporter genes to monitor gene expression in vivo, analyze subcellular protein localization or study protein–protein interactions. Here, we have tested the luciferase from the marine copepod *Gaussia princeps* (*G*-Luc) for its suitability as a sensitive bioluminescent reporter of gene expression in *Chlamydomonas*. We show that a *Gaussia* luciferase gene variant, engineered to match the codon usage in the *Chlamydomonas* nuclear genome, serves as a highly sensitive reporter of gene expression from both constitutive and inducible algal promoters. Its bioluminescence signal intensity greatly surpasses previously developed reporters for *Chlamydomonas* nuclear gene expression and reaches values high enough for utilizing the reporter as a tool to monitor responses to environmental stresses in vivo and to conduct high-throughput screenings for signaling mutants in *Chlamydomonas*.

**Keywords** Reporter gene · *Chlamydomonas reinhardtii* · Luciferase · *Gaussia princeps* · Heat-inducible expression · Bioluminescence

**Introduction**

The unicellular green alga *Chlamydomonas reinhardtii* has become an invaluable model organism for plant biology (Harris 2001; Gutman and Niyogi 2004; Pröschold et al. 2005). It represents one of the simplest photosynthetic eukaryotes, can be easily grown at large scale either photoautotrophically, mixotrophically or heterotrophically, and can be propagated sexually or asexually. Moreover, *Chlamydomonas* combines a powerful genetics with the availability of unique genetic and genomic resources: all three genomes are fully sequenced (nuclear, plastid, and mitochondrial; Merchant et al. 2007), large mutant collections have been established, and all three genomes are amenable to genetic manipulation by transformation (Hippler et al. 1998; Remacle et al. 2006). One of the few drawbacks of *Chlamydomonas* is that it has been notoriously difficult to express transgenes to reasonably high levels from the nuclear genome. Use of specialized promoters (Schroda et al. 2000; Fischer and Rochaix 2001) and adjustment of the transgene’s codon usage to that of the highly GC-rich nuclear genome of the alga (Fuhrmann et al. 1999, 2004) helped in some cases, but no general solution to the problem has been found to date. This is highly unfortunate, because a number of valuable tools available in higher plants currently cannot be used routinely in *Chlamydomonas*. These include all applications of in vivo reporters of gene expression, such as, promoter-YFP/GFP fusions for gene expression analyses and subcellular localization studies, as well as fluorescence resonance energy transfer (FRET) and biomolecular fluorescence complementation (BiFC) for monitoring protein–protein interactions.

Recently, codon-optimized reporter genes have been developed for nuclear and chloroplast expression in *C. reinhardtii*. Two such genes have been developed for nuclear
expression: a gfp gene encoding the green fluorescent protein from the jellyfish *Aequorea victoria* (Fuhrmann et al. 1999) and a luciferase gene (*R-Luc*) from the sea pansy *Renilla reniformis* (Fuhrmann et al. 2004). Synthetic luciferase and gfp genes were also designed for chloroplast transformation and successfully used to measure plastid gene expression (Minko et al. 1999; Mayfield and Schultz 2004; Barnes et al. 2005). However, the use of these reporter genes for nuclear transformation is still far from being routine. While both reporters allowed detection or quantitation of the expression of some (fusion) genes (Fuhrmann et al. 1999; Shao et al. 2007), their generally low sensitivity has precluded the universal use of these reporters.

To overcome these limitations, we have explored a recently discovered new luciferase for its suitability as a more sensitive reporter of gene expression in *Chlamydomonas*. The luciferase from the copepod marine organism *Gaussia princeps* represents one of the smallest and brightest bioluminescent proteins known to date (Tannous et al. 2005; Remy and Michnick 2006). In an ATP-independent reaction, it catalyzes the oxidation of the substrate coelenterazine resulting in light emission at a wavelength of 480 nm. The *Gaussia* luciferase gene (*G-Luc*) has been successfully used as a reporter of gene expression in mammalian cells (Tannous et al. 2005) and could also be split and used for the detection of protein–protein interactions by protein fragment complementation assays (Remy and Michnick 2006). It encodes a non-toxic, monomeric protein of only 185 amino acids and this small size may make it particularly suitable for expression in recalcitrant species.

We report here the development of the luciferase gene from *G. princeps* as a sensitive reporter gene for the in vivo monitoring of gene expression in *Chlamydomonas*. A codon-optimized version of the gene fused to *Chlamydomonas* expression signals generated more than 7-fold higher bioluminescence activity than the *R-Luc* gene from *R. reniformis*. Furthermore, *G-Luc* displayed drastically higher signal intensity than *R-Luc* in luminescence imaging (about 40-fold), thus facilitating the in vivo monitoring of responses to environmental stress stimuli in *C. reinhardtii*.

Nucleic acid manipulations

The coding region of the *G. princeps* luciferase gene (*G-Luc*) was synthesized de novo according to the nuclear codon usage of *C. reinhardtii* (GenScript, Piscataway, NJ). The synthetic *G-Luc* gene (GenBank accession number EU372000) was ligated as *NdeI/EcoRI* fragment into the similarly digested *PsaD* expression cassette (Fischer and Rochaix 2001). Analogously, a codon-optimized *R. reniformis* luciferase gene (*R-Luc*; Fuhrmann et al. 2004) was cloned as *NdeI/EcoRI* fragment into the *PsaD* cassette. For inducible expression, the *G-Luc* coding region was excised by digestion with *HincII* and *BamHI* and inserted into an inducible expression cassette driven by the *Hsp70A* promoter (Shao et al. 2007; Fig. 1b).

Nuclear transformation of *Chlamydomonas*

*Chlamydomonas* nuclear co-transformation was carried out using the glass bead method (Kindle 1990). Plasmid DNA used for transformation was purified by PEG precipitation. Prior to transformation, plasmid pCB412 containing the *C. reinhardtii ARG7* gene as selectable marker was linearized by digestion with *EcoRI*; all *Luc* constructs were linearized with *Scal*. Co-transformation and selection for arginine prototrophy were employed to introduce the *Luc* constructs into the *Chlamydomonas* nuclear genome. Arginine prototrophic clones were selected on TAP medium. Transformants harboring the *Luc* constructs were identified by luciferase assays.

Bioluminescence assays

Coelenterazine (P.J.K. GmbH; Kleinblittersdorf, Germany), the substrate for *G-Luc* and *R-Luc*, was dissolved in ethanol (1 mM stock solution). To assay luciferase activity, *C. reinhardtii* cultures were grown in liquid TAP medium under constant illumination (55 μE m⁻² s⁻¹) to a final cell density of 3–6 × 10⁶ cells ml⁻¹. After sampling, cells were spun down, resuspended in the same volume of sample buffer [1.5 mM Tris–HCl (pH 7.8), 1 mM EDTA], and frozen at −20°C for at least 20 min. After thawing, 20 μl samples were transferred to 96-well, white microtiter plates and 125 μl of the assay buffer [0.1 M K₂HPO₄ (pH 7.6), 0.5 M NaCl, 1 mM EDTA] was added to each well. Following incubation at room temperature for 15 min in the dark, bioluminescence was assayed using a luminometer (MicroBeta TriLux; PerkinElmer) by auto-injecting the substrate (coelenterazine 0.01 mM; 50 μl per well). The luminescence units are presented as luminescence counts per second (LCPS). The background was normalized by measuring wells containing only buffer or buffer with cells lacking the *Luc* gene.

Materials and methods

Algal strains and culture conditions

*Chlamydomonas reinhardtii* strain 325 (CW15 mt+, arg7–8) was used in this study (kindly provided by Dr. Christoph F. Beck, University of Freiburg, Germany). Cultures were grown mixotrophically in Tris–acetate phosphate (TAP) medium (Harris 1989) on a rotary shaker at 23°C under continuous irradiation with white light (55 μE m⁻² s⁻¹). The TAP medium was supplemented with 100 mg l⁻¹ of arginine when required.
normalized to the chlorophyll content of the cultures (Porra et al. 1989). The induction factor was calculated by comparison with untreated cells.

For in vivo luminescence imaging, *C. reinhardtii* cultures were grown in TAP medium under constant illumination (55 μE m⁻² s⁻¹) to a final density of 1–2 × 10⁶ cells ml⁻¹. A volume of 3 μl cell suspension was spotted onto TAP agar plates and incubated under constant illumination (55 μE m⁻² s⁻¹) for 4 days. Luminescence of the cells was visualized in the presence of the substrate (coelenterazine 0.05 mM) using an ultra-sensitive Photon Counting Camera (C2400-30H; Hamamatsu). Samples imaged in the absence of the substrate served as control.

The luminescence images were acquired and processed with the HPD-LIS software (Hamamatsu) using an integration time of 20 min and linear signal intensity.

RNA gel blot analyses

Total cellular RNA was extracted according to published protocols (von Gromoff et al. 1989). RNA samples (15 μg total RNA) were electrophoresed in formaldehyde-containing 1% agarose gels and blotted onto Hybond XL membranes (GE Healthcare). To produce a hybridization probe for detection of *G-Luc* transcripts, the coding region of the gene was excised from a plasmid clone. A *Hsp70A*-specific probe was prepared from a plasmid clone originally described as *hsp70-2* (von Gromoff et al. 1989). The probes were purified by agarose gel electrophoresis following extraction of the DNA fragments of interest from excised gel slices using the Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany) and then radiolabeled with ³²P-dCTP using the MegaPrime kit (GE Healthcare). Hybridizations were performed at 65°C in Church buffer (Church and Gilbert 1984).

Results

Design of the *Gaussia* luciferase as reporter gene for *Chlamydomonas*

Bioluminescent proteins are widely used as reporter genes to measure gene expression, determine subcellular protein localization, and study protein–protein interactions. Luciferases are nontoxic, bioluminescent reporter proteins...
suitable to monitor gene expression quantitatively. Unfortunately, the previously constructed Chlamydomonas-specific Renilla luciferase (Fuhrmann et al. 2004) suffers from low sensitivity, presumably due to low expression levels and/or low protein stability. As in mammalian cells, the luciferase from the marine copepod G. princeps proved to be a much more sensitive reporter than firefly and Renilla luciferases (Tannous et al. 2005); we set out to test this luciferase as a reporter gene in C. reinhardtii.

Previous work had shown that, in Chlamydomonas, adaptation of the codon usage of trangenes significantly improves expression levels (Fuhrmann et al. 1999, 2004). As the codon usage in the native luciferase from G. princeps deviated strongly from that in nuclear genes of C. reinhardtii, we adjusted all codons to the most preferred triplets in Chlamydomonas (according to the codon usage table for Chlamydomonas: http://www.kazusa.or.jp/codon; Fig. 1a). Following this codon optimization in silico, the gene was resynthesized and will be subsequently referred to as G-Luc standing for Gaussia luciferase gene (GenBank accession number EU372000). In all subsequent experiments, G-Luc was compared side-by-side to the Renilla luciferases (Fuhrmann et al. 2004), referred to as R-Luc.

Both luciferase genes were cloned into two different expression cassettes: (1) the PsAD cassette (Fischer and Rochaix 2001), whose promoter is constitutively active at least under photosynthetic conditions and (2) an inducible expression cassette driven by the Hsp70A promoter fused to the 5’ region of the Hsp70B gene (Shao et al. 2007; Fig. 1b). This heat shock gene promoter was shown previously to positively respond to a variety of inducing signals, including heat stress, light, retrograde signals from the plastid, and reactive oxygen species (von Gromoff et al. 1989; Kropat et al. 1997; Kropat and Beck 1998; Schroda et al. 2000; Shao et al. 2007). All gene constructs were introduced into arginine-auxotroph Chlamydomonas cells by glass bead-mediated co-transformation followed by selection for arginine prototrophy.

Expression and heat inducibility of the Gaussia luciferase reporter

To compare the sensitivity of the Chlamydomonas-specific G-Luc as a reporter gene with that of the previously designed R-Luc, 24 transgenic clones from each construct with the constitutive PsAD promoter were randomly chosen and the six best-expressing ones were assayed for their luciferase activities. While only three out of six best R-Luc clones had significant luciferase activity, all six G-Luc clones showed high activity (Fig. 2a). Moreover, when the activities were compared quantitatively, the G-Luc clones displayed, on average, more than 7-fold higher bioluminescence signal intensity than the R-Luc clones (Fig. 2a), indicating that the new G-Luc is more sensitive and more efficient than previously established reporter genes for Chlamydomonas. The very high luciferase activities measured indicate that G-Luc will also be suitable for reporting expression from promoters that are considerably weaker than the PsAD promoter.
Next we wanted to compare the two luciferase genes when expressed under the control of the inducible \textit{Hsp70A} promoter. To this end, inducibility was determined by measuring luciferase activities in the uninduced and induced states for the four best-expressing co-transformants from each construct (identified among 32 randomly picked clones). Transgene expression was induced by shifting the growth temperature of the algal culture from 23 to 40°C for 1 h. While background expression under non-inducing conditions was comparably low in \textit{R-Luc} and \textit{G-Luc} transformants, the \textit{G-Luc} transformants showed much higher bioluminescence under inducing conditions (on average more than 7-fold; Fig. 2b). This confirms the higher sensitivity of the \textit{G-Luc} reporter for another expression cassette (\textit{Hsp70A} promoter + \textit{RbcS} terminator) and, moreover, indicates that \textit{G-Luc} can be used as a highly sensitive reporter gene for measuring inducible gene expression in \textit{C. reinhardtii}.

Luciferase imaging and assessment of protein stability

One of the most powerful applications of luminescent reporter proteins is their use in genetic screens for mutants in cellular signal transduction pathways. This usually requires detection of the reporter gene activity by imaging techniques to facilitate high-throughput screening of mutagenized organisms. Unfortunately, due to the lack of sufficiently sensitive reporters, this has not been possible in \textit{Chlamydomonas} to date. We, therefore, were interested in testing whether the sensitivity of our new \textit{G-Luc} reporter gene was sufficiently high to allow visualization of gene expression by luciferase imaging.

To this end, we assayed luciferase activity from both the constitutive and the inducible expression constructs in vivo using a photon-counting camera. Even the best-expressing clone with \textit{R-Luc} controlled by the \textit{PsaD} expression cassette (Fig. 2a) did not show enough luminescence to be detectable by luciferase imaging (Fig. 3a). In contrast, \textit{G-Luc} activity was sufficiently strong to be readily detectable (Fig. 3a). Similar results were obtained for inducible expression from the \textit{Hsp70A} promoter. While \textit{R-Luc} activity was barely above the detection limit, inducibility of \textit{G-Luc} expression was detected with high sensitivity (Fig. 3b).

Different possible explanations can account for the much better performance of \textit{G-Luc} compared to \textit{R-Luc}: higher expression rates, higher enzymatic activity or higher stability of the \textit{Gaussia} enzyme. To distinguish between these possibilities, we performed stability assays by measuring luciferase activities in dependence on the temperature. To this end, algal cultures were subjected to 30 min of high temperature incubation followed by a 30 min recovery phase at room temperature prior to measurement of luciferase activity. If the \textit{Gaussia} enzyme were indeed more stable than the \textit{Renilla} enzyme, its activity should decline less sharply with temperature. This was indeed the case (Fig. 4a): while the \textit{Renilla} luciferase suffered a strong temperature-dependent decline in activity, the \textit{Gaussia} enzyme was much less affected, suggesting that higher enzyme stability contributes substantially to the superior performance of \textit{G-Luc}.

To explore the heat inducibility of \textit{G-Luc} under the control of the \textit{Hsp70A} promoter in somewhat greater detail, we...
sought to identify optimum experimental conditions for conducting genetic screens for signaling mutants. We, therefore, tested different combinations of temperatures of the heat shock and recovery times and also included a control construct, in which the heat-shock elements (HSE) were deleted from the Hsp70A promoter (Shao et al. 2007). As expected, this deletion completely abolished heat inducibility under all conditions tested (Fig. 4b and data not shown). Efficient heat induction of the G-Luc reporter was achieved in a wide temperature range, from 40 to 47°C (cp. Figs. 3b, 4b). However, heat shock at higher temperatures required longer recovery times at room temperature before luciferase activity could be visualized by imaging. Whereas following heat shock at 40°C, maximum bioluminescence was measured after 1 h recovery, a recovery phase of 3 h was required to obtain similarly high bioluminescence after a heat shock at 47°C (Fig. 4c).

Next we wanted to confirm that heat induction of luciferase activity parallels G-Luc mRNA accumulation. This was clearly the case upon both induction at 40°C and induction at 47°C (Fig. 4d). At both temperatures, mRNA levels peaked at about the same time as enzyme activities (cp. Fig. 4c, d). Moreover, the kinetics of G-Luc mRNA accumulation correlated, by and large, with heat induction of the endogenous Hsp70A gene (Fig. 4d), ultimately confirming that the luciferase reporter faithfully mirrors promoter activity.

Having established that G-Luc expression can be readily monitored by luminescence imaging, we finally wanted to provide a quantitative assessment of the superior performance of the G-Luc reporter by direct luciferase imaging of
primary transformants. To this end, Petri dishes with transformed *Chlamydomonas* colonies were exposed to the substrate and analyzed by luminescence imaging (Fig. 5). While transformation with *G-Luc* produced a high number of brightly luminescing colonies, *R-Luc* luminescence was much lower and barely detectable (Fig. 5). These data ultimately confirm the much higher sensitivity of the *G-Luc* reporter and its suitability for luminescence imaging.

**Discussion**

In this work, we have established the luciferase from the marine copepod *G. princeps* as a novel and highly sensitive bioluminescent reporter in the model alga *C. reinhardtii*. The *G-Luc* reporter outperforms previously developed reporter genes for *Chlamydomonas*, improves the monitoring of gene expression and, most importantly, represents the first nuclear reporter gene that is sufficiently sensitive to facilitate in vivo imaging in *Chlamydomonas*. This expands the toolbox available for *Chlamydomonas* genetics and cell biology and will make possible experimental approaches that heretofore could not be taken in *Chlamydomonas*. First and foremost, the possibility to conduct large-scale mutant screens by bioluminescence imaging of live algal colonies will facilitate powerful genetic strategies for the isolation of novel components of all those signal transduction cascades that modify gene expression by targeting specific promoters. For example, the inducible promoter used in this study (*Hsp70A*) is the target of several distinct signal transduction pathways in response to heat, retrograde signals from the chloroplast, and reactive oxygen species (Shao et al. 2007). Mutagenesis of our algal strains expressing *G-Luc* from this promoter (Fig. 4b, c), followed by selection for mutants incapable of inducing the luciferase gene in response to a specific stress stimulus, should allow the genetic dissection of the underlying signal transduction pathways. Our preliminary results indicate that reactive oxygen species, which are much weaker inducers of the promoter than heat stress (Shao et al. 2007), induce *G-Luc* expression sufficiently strongly to facilitate such a screen in a microtiter plate format, although screening on agar plates will require further optimization and improvement of the assay sensitivity.

In mammalian cells, a split version of the *Gaussia* luciferase was successfully used for the detection of protein–protein interactions in vivo by protein fragment complementation assays (Remy and Michnick 2006). As currently no method is available to identify protein–protein interactions in *Chlamydomonas* cells, the development of screens for protein interaction partners seems to be a particularly promising future application of the *G-Luc* reporter.

It should be noted that thus far, the *G-luc* reporter gene has been tested only in cell wall-deficient *Chlamydomonas* strains, which are easily transformable. It remains to be tested whether substrate uptake or luminescence imaging are influenced by the more rigid walls present in strains with wild type-like cell wall structure.
Although the Chlamydomonas-specific G-Luc described here provides a workable reporter of gene expression that is significantly more sensitive than previously established reporter genes, our data indicate that the superior performance of the Gaussia luciferase gene is not due to its better expression in Chlamydomonas reinhardtii, but rather due to its very high enzyme stability. Most probably, the adaptation of other widely used reporter genes to Chlamydomonas reinhardtii (like the genes for the fluorescent proteins GFP and YFP) will require a general solution to the transgene expression problem in Chlamydomonas. This could be achieved by either developing novel expression tools or generating dedicated expression strains in which the suspected epigenetic transgene silencing mechanism is inactivated.

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