

Complementarity to an miRNA seed region is sufficient to induce moderate repression of a target transcript in the unicellular green alga *Chlamydomonas reinhardtii*

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SUMMARY

MicroRNAs (miRNAs) are 20–24 nt non-coding RNAs that play important regulatory roles in a broad range of eukaryotes by pairing with mRNAs to direct post-transcriptional repression. The mechanistic details of miRNA-mediated post-transcriptional regulation have been well documented in multicellular model organisms. However, this process remains poorly studied in algae such as *Chlamydomonas reinhardtii*, and specific features of miRNA biogenesis, target mRNA recognition and subsequent silencing are not well understood. In this study, we report on the characterization of a *Chlamydomonas* miRNA, cre-miR1174.2, which is processed from a near-perfect hairpin RNA. Using *Gaussia* luciferase (*gluc*) reporter genes, we have demonstrated that cre-miR1174.2 is functional in *Chlamydomonas* and capable of triggering site-specific cleavage at the center of a perfectly complementary target sequence. A mismatch tolerance test assay, based on pools of transgenic strains, revealed that target hybridization to nucleotides of the seed region, at the 5' end of an miRNA, was sufficient to induce moderate repression of expression. In contrast, pairing to the 3' region of the miRNA was not critical for silencing. Our results suggest that the base-pairing requirements for small RNA-mediated repression in *C. reinhardtii* are more similar to those of metazoans compared with the extensive complementarity that is typical of land plants. Individual *Chlamydomonas* miRNAs may potentially modulate the expression of numerous endogenous targets as a result of these relaxed base-pairing requirements.

Keywords: *Chlamydomonas reinhardtii*, microRNA, RNA silencing, luciferase assay, transformation, seed sequence.

INTRODUCTION

MicroRNAs (miRNAs) are endogenous 20–24 nt non-coding RNAs that regulate gene expression by base pairing to mRNAs and direct their degradation or inhibit productive translation. To date, thousands of miRNAs have been found in a diverse range of eukaryotes and shown to have critical roles in diverse biological processes, including development, proliferation and stress responses (Huntzinger and Izaurralde, 2011; Pasquinelli, 2012). The canonical miRNA biogenesis pathway begins with the transcription of long RNAs that fold back to form distinctive hairpin structures. These transcripts are subsequently processed by protein complexes, which contain RNase III endonucleases and double-stranded RNA binding proteins, into miRNA/miRNA* duplexes with 2 nt 3' overhangs. One of

the strands of each miRNA/miRNA* duplex (proper miRNA) is loaded into the RNA-induced silencing complex (RISC) and serves as a guide to identify complementary mRNAs targeted for silencing (Voinnet, 2009; Axtell *et al.*, 2011; Yang and Lai, 2011). Plants and animals share several features of the miRNA biogenesis pathway, but there are also appreciable mechanistic differences between these organisms (Flynt and Lai, 2008; Voinnet, 2009; Axtell *et al.*, 2011; Yang and Lai, 2011).

The biogenesis of plant miRNAs has been studied primarily in *Arabidopsis thaliana*. Plant primary miRNAs (pri-miRNAs) are generally transcribed by RNA polymerase II and subjected to post-transcriptional processing, including 5' capping, intron splicing and 3' polyadenylation

(Bartel, 2004; Xie *et al.*, 2005). Local secondary structures of long pri-miRNAs are recognized by the DCL1/HYL1/Serrate protein complex, and are usually cleaved at the double-stranded bases to release hairpin structures as precursor miRNAs (pre-miRNAs), which in turn are cleaved again by DCL1 to generate miRNA/miRNA* duplexes (Han *et al.*, 2004; Kurihara and Watanabe, 2004; Kurihara *et al.*, 2006; Lobbes *et al.*, 2006). This sequential processing may occur in two directions: stem-to-loop or loop-to-base (Naqvi *et al.*, 2012). However, the processing of most plant pri-miRNAs appears to follow the stem-to-loop orientation, and only pri-miR159 and pri-miR319 are known to be processed in the reverse orientation (Bartel, 2004; Addo-Quaye *et al.*, 2009; Bologna *et al.*, 2009).

Most land plant miRNAs display perfect or near-perfect complementarity to their targets. Perfect pairing usually triggers endonucleolytic target cleavage (slicing) at the center of the miRNA/mRNA hybrid, and subsequent degradation of the target transcript (Llave *et al.*, 2002; Voinnet, 2009). In contrast, in animals, mature miRNAs generally form imperfect hybrids with target transcripts, leading to reduced protein expression through translation inhibition and/or accelerated exonucleolytic RNA decay. Most animal miRNAs display perfect pairing in the seed region, between nucleotides 2 and 8, and imperfect hybridization in the central region, between nucleotides 9 and 12 (Lewis *et al.*, 2005; Bartel, 2009). Because of this limited sequence complementarity, individual animal miRNAs appear to have multiple target transcripts (Bartel, 2009), whereas individual plant miRNAs are predicted to have a reduced number of target mRNAs given their requirement for more extensive sequence complementarity (Schwab *et al.*, 2005; Voinnet, 2009). Nonetheless, systematic mutagenesis studies of miRNA/target site hybrids have revealed that the miRNA seed region is more critical than the 3' region for target recognition in *A. thaliana* (Mallory *et al.*, 2004). Moreover, plant and algal small RNAs also induce translational repression of perfectly complementary target mRNAs without, or with only minimal, transcript destabilization, thereby adding to the mechanistic complexity of miRNA action (Brodersen *et al.*, 2008; Ma *et al.*, 2013).

Small RNA-mediated silencing remains poorly understood in the unicellular green alga *Chlamydomonas reinhardtii* (Ibrahim *et al.*, 2006, 2010; Molnar *et al.*, 2007; Zhao *et al.*, 2007; Casas-Mollano *et al.*, 2008; Cerutti *et al.*, 2011; Ma *et al.*, 2013). Considering the physiological and gene complement similarities between *C. reinhardtii* and *A. thaliana* (Merchant *et al.*, 2007), many features of the RNA interference (RNAi) pathway(s) are expected to be conserved between these organisms. However, the chlorophyten green algae lineage diverged from land plants over one billion years ago, which is similar to the time of divergence between yeast and mouse (Yoon *et al.*, 2004; Merchant *et al.*, 2007). Therefore, it is possible that differences in the

basic mechanisms of miRNA biogenesis and function do exist between green algae and higher plants. In this respect, the present study shows that release of an miRNA/miRNA* duplex in *C. reinhardtii* appears to occur from the loop of a near-perfect hairpin precursor (i.e. at least partial loop-to-base processing). In addition, pairing of the miRNA seed region alone was sufficient to cause moderate repression of a target transcript. These results suggest that certain aspects of miRNA biogenesis and mode of action in green algae differ somewhat from those in land plants and may resemble RNAi mechanisms operating in metazoans.

RESULTS

An miRNA, named cre-miR1174.2, is processed from the transcript of the *Cre10.g444300* gene

By deep sequencing of small RNAs in *C. reinhardtii* (Ibrahim *et al.*, 2010), we identified a 21 nt putative miRNA, which we named cre-miR1174.2. A homology search against the *C. reinhardtii* genome (<http://www.phytozome.net/chlamy.php>), using the cre-miR1174.2 sequence as the query, revealed a single precise match in a predicted gene, *Cre10.g444300*. The RNAfold secondary structure analysis tool (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) predicted that nucleotides 789–922 from the 5' end of the *Cre10.g444300* transcript form a hairpin with a 64 bp stem (including the cre-miR1174.2 sequence) and a 6 nt loop (Figures 1a and 2). Northern hybridization analyses showed that the *Cre10.g444300* transcript was not detected in the wild-type CC-124 strain, probably as a result of fast processing of the pri-miRNA to mature miRNA (Figure 1b). However, we observed a shorter RNA of approximately 0.7 kb in length (Figure 1b) that presumably corresponded to the 5' end of the pri-miRNA after Dicer processing. In contrast, a band of approximately 2.2 kb in size that corresponded to the predicted *Cre10.g444300* transcript was clearly detected in an RNAi-defective mutant, Mut-20, which was isolated from a library of insertional mutants in the Maa7-IR44s background (Ma *et al.*, 2013) (Figure 1b). The specific molecular alteration in Mut-20 remains uncharacterized; however, as the mutant showed greatly reduced levels of miRNA (Figure 1c), it is possible that a defect in miRNA biogenesis allowed detection of the full-length *Cre10.g444300* RNA.

The data obtained by deep sequencing of small RNAs also supported processing of cre-miR1174.2 from the *Cre10.g444300* hairpin because both miRNA and miRNA* matching sequences were identified; however, as expected, miRNA* reads were detected at a much lower abundance (Figure 2). Moreover, the *Cre10.g444300* hairpin appeared to code for two distinct miRNAs, one on each arm, which show 5' end homogeneity in over 98% of the reads and marked asymmetry in the accumulated reads (Figure 2, miRNA versus miRNA* reads). These are all

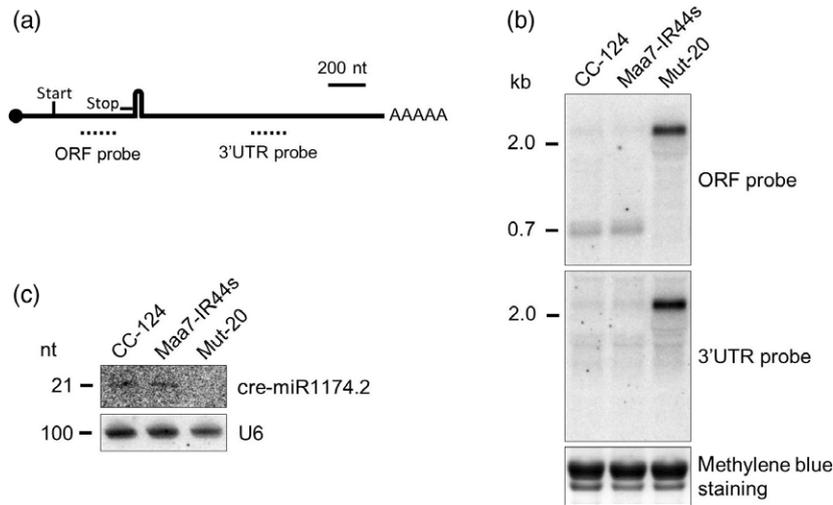


Figure 1. *Chlamydomonas* cre-miR1174.2 may derive from the *Cre10.g444300* transcript.

(a) Schematic diagram of the *Cre10.g444300* mRNA (*Cre10.g444300.t1.2*). A transcript of approximately 2.2 kb was predicted to encode a 189 amino acid protein with no significant similarity to any known polypeptide. Dotted lines indicate the position of probes used for Northern blot analyses.

(b) Northern blot analysis of the *Cre10.g444300* transcript in the indicated strains. The blot was subjected to hybridization with the ^{32}P -labeled probes indicated in (a). Ribosomal RNAs were visualized by methylene blue staining to check for equal lane loading. CC-124, wild-type strain; Maa7-IR44s, CC-124 containing an inverted repeat transgene targeting the 3' UTR of the *MAA7* gene for silencing; Mut-20, RNAi-defective mutant isolated by random insertional mutagenesis of Maa7-IR44s.

(c) Northern blot analysis of cre-miR1174.2 in the indicated strains. The same blot was re-probed with the U6 snRNA sequence as a control to check for equal lane loading.

expected features of canonical miRNAs (Tarver *et al.*, 2012). We also performed 5' RACE analyses on polyadenylated RNA from the wild-type CC-124 strain to identify cleavage sites within the *Cre10.g444300* hairpin structure. A *Cre10.g444300*-specific reverse primer was designed to anneal approximately 100 nt downstream of the 3' end of the hairpin, and PCR amplicons differing in size by approximately 40 bp were obtained. Cloning and subsequent sequencing of 27 amplicons revealed that the predominant sites of 5' termini truncation (putative cleavage sites) corresponded to the ends of the cre-miR1174.2 sequence in the 3' arm of the *Cre10.g444300* hairpin (Figure 2).

Chlamydomonas Cre10.g444300 is predicted to encode a 189 amino acid polypeptide of unknown function and lacks homology to proteins in any other organism, even closely related algal species (<http://www.phytozome.net/chlamy.php>). Thus, it is tempting to speculate that *Cre10.g444300* actually produces a non-coding RNA, probably transcribed by RNA polymerase II as for pri-miRNAs in other eukaryotes, that serves as a precursor for the biogenesis of miRNAs.

Reporter transcripts containing cre-miR1174.2 target sites are repressed in *Chlamydomonas*, probably by cre-miR1174.2 induced site-specific RNA cleavage

Currently, 19 526 protein-coding transcripts, including splice variants, are predicted to be encoded in the *Chlamydomonas* genome (<http://www.phytozome.net/chl>

amy.php). None of the transcript models, except for *Cre10.g444300*, contained a sequence that was perfectly complementary to cre-miR1174.2 however, several predicted mRNAs formed imperfect hybrids resembling the typical metazoan miRNA–target interaction. Thus, a series of four *Gaussia* luciferase (*gluc*) reporter genes (Ruecker *et al.*, 2008; Shao and Bock, 2008) were introduced into *C. reinhardtii* to demonstrate the ability of cre-miR1174.2 to induce target RNA repression. The 3' UTRs of these reporters showed no homology to cre-miR1174.2 or one, four or eight copies (1 ×, 4 × or 8 ×, respectively) of a sequence that was perfectly complementary to cre-miR1174.2 (Figure S1). Genomic PCR was performed to screen for transgenic strains that integrated the entire transforming DNA (Table S1), and individual transgenic lines were examined for *gluc* activity. Strains transformed with *gluc*-aph7 (no cre-miR1174.2 target site) showed variable luciferase activities, presumably as a result of integration site-dependent position effects (Figure S2). Nonetheless, approximately 83% of the strains tested exhibited adequate *gluc* activity, i.e. higher than 1000 relative light units (RLU) (Figure S2). In contrast, the majority of *Chlamydomonas* transformants containing reporter constructs with cre-miR1174.2 target sites showed low *gluc* activity (Figure S2). For instance, approximately 50% of the *gluc*(1 ×)-aph7-transformed strains exhibited *gluc* activity of <1000 RLU, and none of the *gluc*(4 ×)-aph7 or *gluc*(8 ×)-aph7-transformed strains exhibited luciferase activity above 1000 RLU (Figure S2).

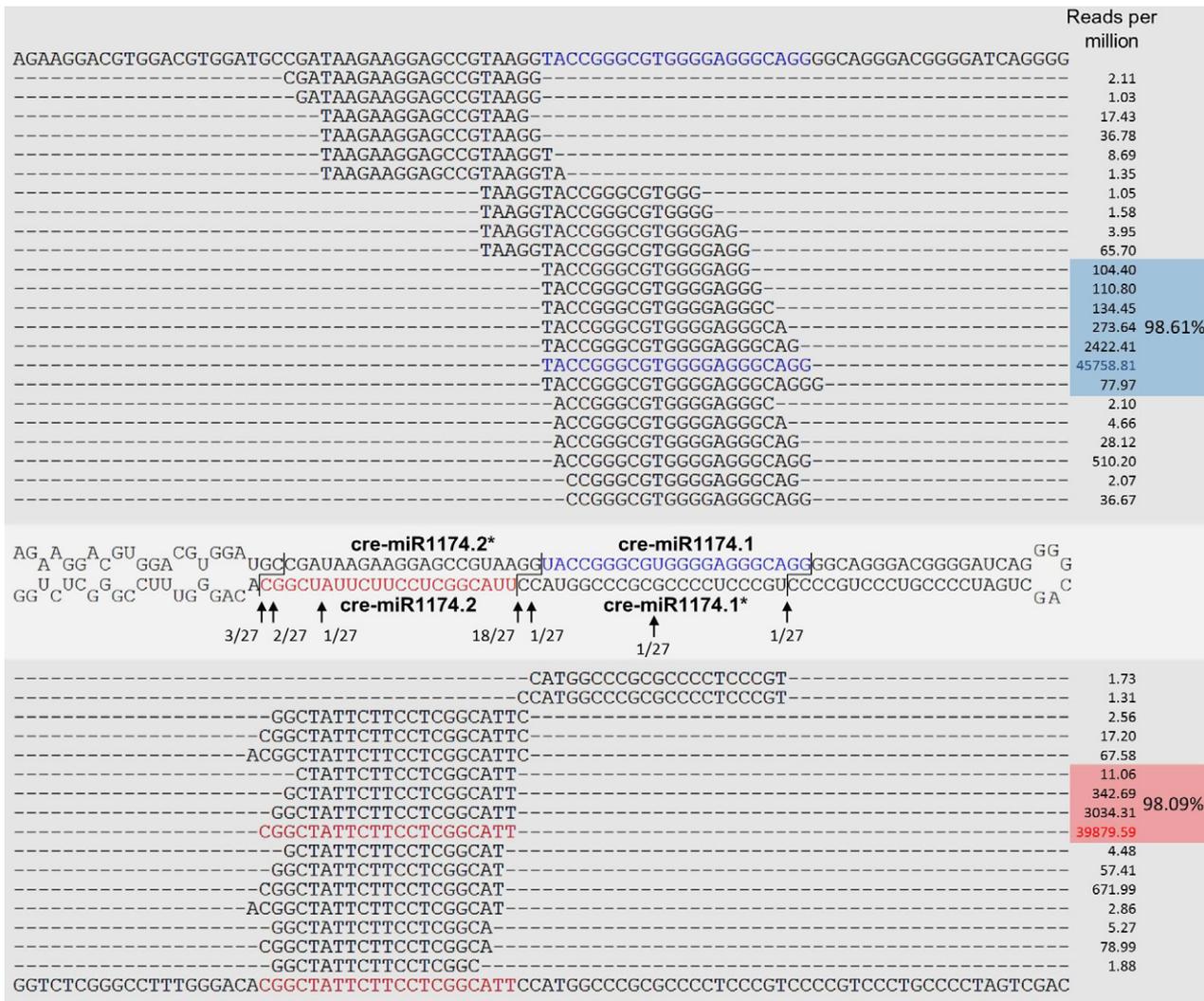


Figure 2. Secondary structure of the cre-miR1174.2 precursor in the *Cre10.g444300* transcript, and read data from a *Chlamydomonas* small RNA library. The sequence displayed corresponds to positions 770–941 from the 5' end of the *Cre10.g444300* transcript. The read counts for each small RNA sequence were normalized (reads per million). Note that the read counts are dominated (>98%) by a specific 5' end on each arm of the hairpin. Red letters indicate the cre-miR1174.2 sequence. Blue letters indicate the cre-miR1174.1 sequence. The arrows and numbers below the hairpin indicate the positions and frequencies of 5'-terminally truncated *Cre10.g444300* transcripts identified by 5' RACE.

These results suggested that *Chlamydomonas* cre-miR1174.2 may trigger silencing of *gluc* transgenes containing cre-miR1174.2 target sites in their transcripts.

To confirm that repression of the *gluc* reporters was indeed miRNA-mediated, we selected a single-copy *gluc*-aph7-transformed strain, termed *gluc*-10, and a single copy *gluc*(1 ×)-aph7-transformed strain, termed *gluc*(1 ×)-10, for further analyses. We then crossed these strains with the Mut-20 RNAi-defective mutant. Interestingly, *gluc* expression from the *gluc*-aph7 transgene in the Mut-20 background was comparable to that observed in the wild-type *gluc*-10 strain (Figure 3a–c). In contrast, *gluc*(1 ×) expression from the *gluc*(1 ×)-aph7 transgene was substantially increased in the Mut-20 background compared with that observed in the *gluc*(1 ×)-10 strain (Figure 3a–c). As

Mut-20 had virtually undetectable levels of cre-miR1174.2 (Figure 3c), these observations are consistent with direct involvement of this miRNA in repression of the *gluc*(1 ×)-aph7 transgene in the wild-type background, with a marked reduction of transcript (Figure 3c) and protein (Figure 3b) levels.

In both animals and land plants, RISC-mediated, site-specific target cleavage (slicing) occurs predominantly between the nucleotides pairing to positions 10 and 11 from the 5' end of a guide small RNA (Elbashir *et al.*, 2001; Llave *et al.*, 2002). Similar miRNA-mediated site-specific cleavage has been reported for some endogenous target transcripts in *Chlamydomonas* (Molnar *et al.*, 2007; Zhao *et al.*, 2007). Thus, we performed 5' RACE analyses to verify whether cre-miR1174.2 induced slicing of the *gluc*

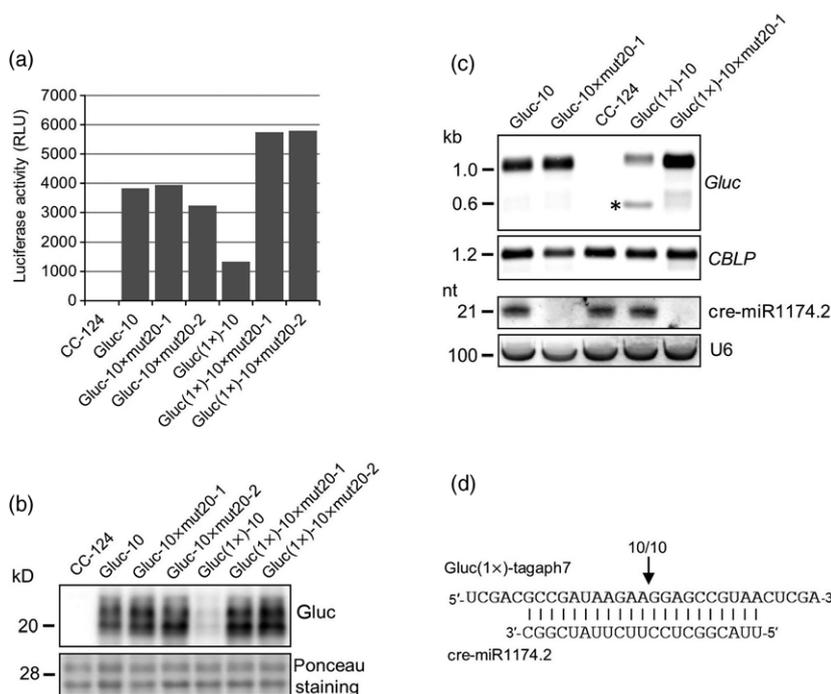


Figure 3. *Gaussia* luciferase (*gluc*) reporter genes containing a cre-miR1174.2 target site in their 3' UTRs were repressed in *Chlamydomonas*, presumably by cre-miR1174.2-triggered target RNA cleavage.

(a) Luminescence activity of *gluc* reporters with or without a single cre-miR1174.2 target site in their 3' UTRs. Values represent relative light units (RLU) per approximately 2.5×10^4 cells. *gluc-10*, a transgenic strain containing a single copy of *gluc-aph7*; *gluc-10* \times *mut20-1* and *-2*, progeny from a genetic cross between *gluc-10* and *Mut-20*, introducing the *gluc-aph7* transgene into the *Mut-20* background; *gluc(1x)-10*, a transgenic strain containing a single copy of *gluc(1x)-aph7*; *gluc(1x)-10* \times *mut20-1* and *-2*, progeny from a genetic cross between *gluc(1x)-10* and *Mut-20*, introducing the *gluc(1x)-aph7* transgene in the *Mut-20* background.

(b) Immunoblot analysis of the *gluc* protein in the indicated strains. The lower band (approximately 20 kDa) corresponds to the predicted size of the *gluc* protein. Ponceau S staining of the blots was performed to confirm similar loading of the protein samples before immunodetection.

(c) Northern blot analyses of the *gluc* mRNA and cre-miR1174.2 small RNA in the indicated strains. The asterisk indicates a truncated *gluc(1x)* transcript, probably generated by RISC-mediated slicing. The blots were re-probed using *CBLP* or U6 snRNA sequences as controls to check for equal lane loading.

(d) Assessment of the cleavage site(s) of the cre-miR1174.2-targeted *gluc(1x)* mRNA in the *gluc(1x)-10* strain by 5' RACE assays. The arrow and numbers indicate the position and frequency of the examined 5' truncated *gluc(1x)* transcripts. Hybridization of cre-miR1174.2 to the target site is shown.

($1x$) transcript. As expected for RISC-triggered RNA cleavage, the assays revealed that the *gluc(1x)* mRNA was frequently truncated at the center of the cre-miR1174.2 target site (Figure 3d). In addition, a truncated transcript, probably corresponding to the RISC-cleaved 5' end of the *gluc(1x)* mRNA, was detected in Northern hybridization analyses of the *gluc(1x)-10* strain (Figure 3c). Taken together, these results strongly suggest that cre-miR1174.2 is a functional miRNA in *Chlamydomonas*, and that it mediates site-specific cleavage of a perfectly complementary target transcript. Moreover, the *gluc* reporter system appeared to serve as a good indicator to estimate the potency of miRNA-mediated silencing.

The efficiency of cre-miR1174.2-mediated gene silencing may be examined using pools of transgenic strains in *Chlamydomonas*

Base pairing of the miRNA seed region (positions 2–8 from the 5' end of an miRNA) in animals is critical for target recognition and repression (Bartel, 2009; Pasquinelli,

2012). In contrast, most evidence indicates that miRNAs in land plants require more extensive pairing to their targets (Schwab *et al.*, 2005; Voinnet, 2009). Mismatch tolerance tests have revealed different requirements with regard to the degree of complementarity and length of base pairing among miRNA/mRNA hybrids in plants and animals (Kloosterman, 2004; Mallory *et al.*, 2004; Brennecke *et al.*, 2005). However, similar analyses have not been performed in algae. To address this, we first developed a reliable method to estimate the efficiency of miRNA-mediated repression using the *gluc* reporter genes. As already discussed, stable transgenic strains integrated luciferase genes in various genomic locations and displayed variable expression levels (Figure S2). However, as previously demonstrated (Lodha *et al.*, 2008), the position effects may be averaged by analyzing pools of *Chlamydomonas* transformants instead of individual transgenic lines.

For a comparative analysis of miRNA-mediated repression, pools of transgenic strains were generated by

transformation of *C. reinhardtii* with *gluc* reporter constructs linked on the same plasmid to a gp64 epitope-tagged *aph7* selectable marker (Figure S3). Transgenic pools were prepared by electroporating approximately 2.5×10^7 CC-3491 cells, followed by selection in liquid medium containing hygromycin B (see Experimental procedures). A single electroporation generated over 500 hygromycin B-resistant transformants and, according to previous estimations (Lodha *et al.*, 2008), the size of these transgenic pools is acceptable for comparative analyses of *gluc* expression. The *gluc* transgene *gluc*-tagaph7, which lacks an miRNA target site, was used to determine baseline levels of luciferase activity, protein abundance and mRNA quantity (Figure S4). We also used *gluc* transgenes containing reversed or randomized *cre*-miR1174.2 target sequences as controls; these exhibited *gluc* expression levels equivalent to those of *gluc*-tagaph7 (Figure S4). In contrast, expression of the *cre*-miR1174.2-targeted *gluc* transgenes *gluc*(1 ×)-tagaph7, *gluc*(4 ×)-tagaph7 and *gluc*(8 ×)-tagaph7 was substantially lower (Figure S4). Moreover, the degree of repression appeared to correlate positively with the number of repeated *cre*-miR1174.2 target sites in the *gluc* transcripts (Figure S4). Experimental controls revealed that gp64-tagged *aph7* was expressed equally in all transgenic pools (Figure S4), and the mean level of nuclear-integrated *gluc* transgenes was also comparable among the examined pools (Figure S4). Silencing of *gluc* reporter genes containing target sites for other *Chlamydomonas* miRNAs (miR910, miR912 and candidate 82; Molnar *et al.*, 2007; Zhao *et al.*, 2007) was also observed in pools of transgenic strains (Figure S5). Therefore, our results demonstrate that the degree of miRNA-mediated gene repression may be examined using transgenic pools in *C. reinhardtii*, thereby providing a useful tool to perform mismatch tolerance tests.

Base pairing of a target site to positions 2–12 from the 5' end of *cre*-miR1174.2 is important for gene repression

Ten dinucleotide substitutions were introduced into the *cre*-miR1174.2 target site in the *gluc* reporter gene to determine the possible negative effects of mismatches on *cre*-miR1174.2-mediated repression (Figure S6). Pairs of nucleotide mismatches within the region hybridizing to positions 1–12 of *cre*-miR1174.2 resulted in increased reporter expression levels. This was determined by both luciferase activity and immunoblot assays in comparison with a perfectly complementary control (Figure 4a,b). In particular, dinucleotide mismatches in the target site corresponding to positions 1–6 in *cre*-miR1174.2 led to noticeably higher expression levels relative to other mismatches (Figure 4a, b). In contrast, repression of the *gluc* reporter gene was not significantly affected by substitutions in the region pairing with the 3' end of the miRNA from nucleotide positions 13–20 (Figure 4a,b).

To examine reporter mRNA levels by real-time PCR, we designed a *gluc* primer set that amplifies a fragment located immediately upstream of the *cre*-miR1174.2 target site. However, this fragment may be amplified from both the full-length *gluc* transcript and from a truncated RNA that was presumably generated by slicing (Figure 3c), leading to erroneous quantification of the intact transcript. To avoid this complication, we used an oligo(dT)₁₈ primer for reverse transcription and generation of cDNA samples for PCR amplification, which prevented copying of the non-polyadenylated truncated RNA. Interestingly, the measured amounts of *gluc* mRNA (Figure 4c) broadly coincided with the relative levels of *gluc* activity for the various reporters, with the exception of *gluc*(9-10). In this transgenic pool, polyadenylated *gluc* mRNA accumulated to higher levels than expected relative to the observed *gluc* protein activity and amount (Figure 4a–c). Mismatches flanking positions 10 and 11 are known to have an inhibitory effect on RISC slicing activity (Brodersen and Voinnet, 2009); therefore, the increased *gluc* mRNA accumulation in *gluc*(9-10) was considered to result from a defect in *cre*-miR1174.2-mediated target cleavage, which may have been compensated for by some degree of translational repression. The gp64-tagged *aph7* control showed virtually identical expression in all transgenic pools (Figure 4b), and the mean level of the integrated *gluc* transgenes was also comparable (Figure 4d).

These observations suggest that hybridization of a target transcript to positions 2–12 of an miRNA is an important requirement for RNAi-mediated silencing in *C. reinhardtii*. The position 1 of the target sequence was also mutated, but complementarity at this position is probably inconsequential for repression because the 5' terminal nucleotide of miRNAs is bound in a pocket of the Argonaute protein and is unavailable for RNA pairing (Boland *et al.*, 2011). Our analysis also indicated that, as expected, mismatches flanking the cleavage site may be detrimental for slicing and transcript degradation, but target mRNAs may still be silenced by translational repression.

Seed matching alone is sufficient to induce moderate miRNA-mediated repression of target gene expression

In land plants, miRNA target selection only tolerates one mismatch in the region complementary to positions 2–12 of a given miRNA, provided that this does not occur at positions 10 and 11 (Schwab *et al.*, 2005). In addition, a total of up to four mismatches throughout the length of the miRNA, but no more than two mismatches in a row, are tolerated (Schwab *et al.*, 2005). Many miRNA targets have been successfully predicted in land plants by following these rules. However, there appeared to be some exceptions to these rules in terms of the detected activity of *cre*-miR1174.2 towards target reporters in the green alga *Chlamydomonas*.

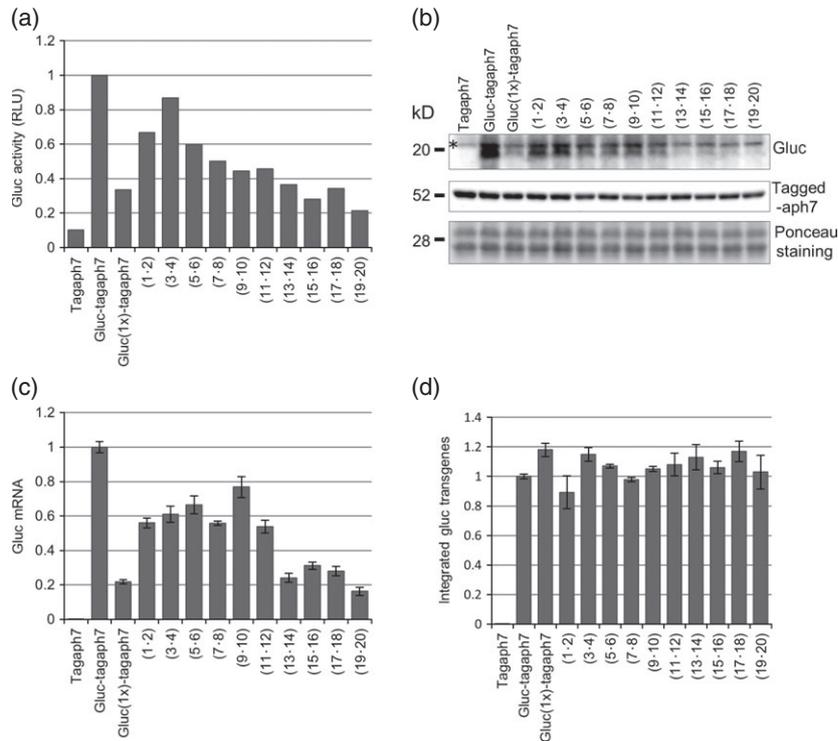


Figure 4. Mismatch tolerance test of cre-miR1174.2 and its target sequence in *GluSSia* luciferase (*gluc*) reporters.

(a) Relative luminescence activity of *gluc* in the indicated transgenic pools. Numbers in parentheses indicate the cre-miR1174.2 positions for which non-matching dinucleotide substitutions were introduced in the transgenic target sites (Figure S6). Pools of transgenic strains were generated by electroporation, and the luminescence activity (RLU) for a 30 μ l aliquot of each pool was directly measured. The RLU for each transgenic pool were normalized for cell numbers and then against the values for the *gluc*-tagaph7 pool (reporter lacking a miRNA target site). All experiments were performed in triplicate.

(b) Immunoblot analyses of the *gluc* and tagged *aph7* proteins in transgenic pools generated by transformation with the indicated constructs (Figure S6). The lower band (approximately 20 kDa) corresponded to the predicted size of the *gluc* protein. The asterisk indicates a non-specific signal from an antigen that cross-reacts with the antibody. Ponceau S staining of the blots was performed to confirm similar loading of the protein samples.

(c) Quantitative RT-PCR analysis of *gluc* mRNAs in the indicated transgenic pools. An oligo(dT)₁₈ primer was used for reverse transcription. *gluc* transcript levels were normalized to the mRNA level for the *CBLP* housekeeping gene, then the value corresponding to the *gluc*-tagaph7 transgenic pool was set to 1, and all other values were adjusted accordingly. Values are means \pm SD of three independent experiments.

(d) Quantitative genomic PCR analysis of the nuclear DNA-integrated *gluc* reporters in the indicated transgenic pools. The quantity of integrated *gluc* transgene was normalized to the level of the single-copy endogenous gene *CBLP*, then the value corresponding to the *gluc*-tagaph7 transgenic pool was set to 1, and all other values were adjusted accordingly. Values are means \pm SD of three independent experiments.

For instance, cre-miR1174.2 induced silencing when the miRNA/mRNA hybrid had dinucleotide mismatches in the region flanking the cleavage site (Figure 4). These observations imply that limited complementarity to a target site may be sufficient to induce cre-miR1174.2-mediated repression in *C. reinhardtii*. To address this question, the expression of a *gluc*-cre-miR1174.2 seed reporter containing a target site complementary to positions 1–8 of cre-miR1174.2 was tested in transgenic pools (Figure 5a). Moderately reduced expression levels were observed for the *gluc*-cre-miR1174.2 seed transgenic pool compared with the *gluc*-tagaph7 transgenic pool control that lacks a cre-miR1174.2 target site (Figure 5b–d), despite virtually identical amounts of integrated *gluc* transgenes in both transgenic pools. A similar result was obtained when analyzing the interaction of *Chlamydomonas* miR910 with its target site (Figure S7). These results suggested that pairing to the seed region alone may be sufficient to induce mild

cre-miR1174.2 and miR910-mediated repression in *C. reinhardtii*.

DISCUSSION

To date, 50 *Chlamydomonas* miRNA sequences have been deposited in miRBase (<http://www.mirbase.org>; Molnar *et al.*, 2007; Zhao *et al.*, 2007). However, a recent re-evaluation of these miRNAs suggested that many may actually correspond to endogenous small interfering RNAs (e.g. multiple products from the same long hairpin without corresponding star sequences and/or lacking stable 5' processing; Tarver *et al.*, 2012). Two reports indicated that only five (Nozawa *et al.*, 2012) or six (Tarver *et al.*, 2012) currently recognized miRNAs in *C. reinhardtii* may be genuine. In addition, no miRNA precursor has been experimentally characterized in this alga (Molnar *et al.*, 2007; Zhao *et al.*, 2007). In this study, we provide evidence that *Cre10.g444300* may actually encode a pri-miRNA transcript,

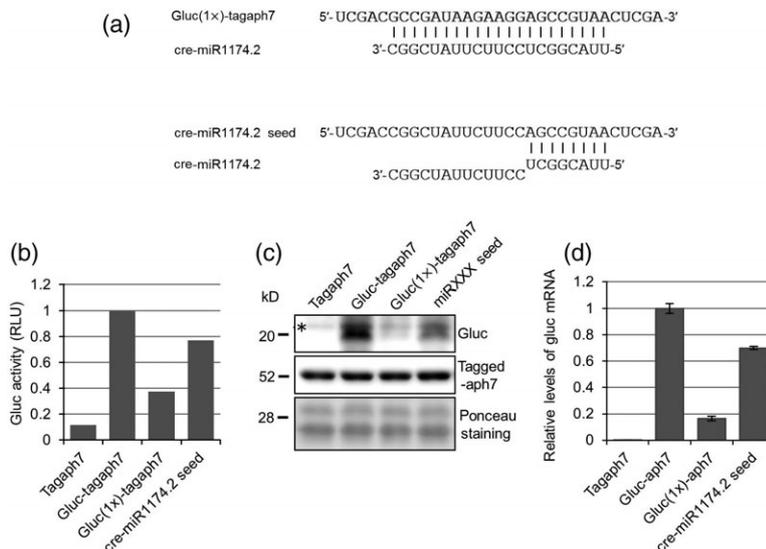


Figure 5. A *Gaussia* luciferase (*gluc*) reporter gene containing a target site matching only the cre-miR1174.2 seed region was moderately repressed in *Chlamydomonas*.

(a) Expected base pairing between cre-miR1174.2 and its target sequences in the indicated *gluc* reporter transcripts.

(b) Relative luminescence activity of *gluc* in the indicated transgenic pools. Pools of transgenic strains were generated by electroporation, and 30 μ l of each pool culture was directly used to measure luminescence activity (RLU). The RLU of each transgenic pool was normalized for cell numbers and then to the values of the *gluc*-tagaph7 pool (reporter lacking an miRNA target site). All experiments were performed in triplicate.

(c) Immunoblot analyses of the *gluc* and tagged *aph7* proteins in transgenic pools generated by transformation with the indicated constructs. The lower band (approximately 20 kDa) corresponded to the predicted size of the *gluc* protein. The asterisk indicates a non-specific signal from an antigen that cross-reacts with the antibody. Similar loading of the lanes was assessed by Ponceau S staining of the protein blots.

(d) Quantitative RT-PCR analysis of the *gluc* mRNAs in the indicated transgenic pools. An oligo(dT)₁₈ primer was used for reverse transcription. *gluc* transcript levels were normalized to the mRNA level for the *CBLP* housekeeping gene, then the value corresponding to the *gluc*-tagaph7 transgenic pool was set to 1, and all other values were adjusted accordingly. Values are means \pm SD of three independent experiments.

which is only detected in an RNAi-defective mutant (Figure 1). Based on established criteria for miRNA annotation, including evidence of expression of both miRNA and miRNA*, 2 nt 3' overhangs in the proposed miRNA/miRNA* duplex, and consistent 5' processing of candidate miRNAs (Bartel, 2004; Axtell *et al.*, 2011; Tarver *et al.*, 2012), the *Cre10.g444300* hairpin appears to serve as the precursor for two *Chlamydomonas* miRNAs: cre-miR1174.2 produced from the 3' arm and cre-miR1174.1 produced from the 5' arm (Figure 2). Moreover, cre-miR1174.2 was shown to function as a *bona fide* miRNA because reporter transgenes containing perfectly complementary cre-miR1174.2 target sequences in their 3' UTRs were efficiently silenced in *C. reinhardtii* (Figure 3 and Figure S4).

5' RACE analyses revealed predominant sites of 5' termini transcript truncation (putative cleavage sites) corresponding to the ends of candidate miRNA or miRNA* sequences in the *Cre10.g444300* hairpin 3' arm (Figure 2). Because 5' RACE products contain sequences downstream of the hairpin (approximately 100 bp to the annealing site for the reverse primer), a major PCR fragment truncated at the 3' end of the cre-miR1174.2 sequence (at the base of the double-stranded RNA stem) is expected if Dicer processing began from the base of the hairpin structure. In contrast, detection of several putative cleavage

intermediates on the 3' arm, including one of particular significance at the 5' end of the cre-miR1174.2 sequence (Figure 2), suggested that processing may start from the loop of the hairpin, as previously illustrated for biogenesis of plant miR319 and miR159 (Bologna *et al.*, 2009). However, we cannot rule out the possibility that processing of the hairpin structure may start from either end or in the middle of the double-stranded stem.

Dual luciferase reporter assays have frequently been used in cultured animal cells to study transcription factors, mRNA processing and miRNA-mediated repression (Dougherty and Sanders, 2005; Lynch *et al.*, 2007; Lee *et al.*, 2008). In this assay, the expression of two independent luciferase reporters that require different substrates is examined. Firefly luciferase and *Renilla* luciferase are typically used, with one reflecting the effect of a specific experimental condition and the other serving as a control for transfection efficiency (Dougherty and Sanders, 2005). To the best of our knowledge, a similar system is not currently available in green algae. In *C. reinhardtii*, two coelenterazine luciferases, from *Renilla reniformis* and *Gaussia princeps*, have been used for transgene expression analyses (Fuhrmann *et al.*, 2004; Ruecker *et al.*, 2008; Shao and Bock, 2008), but no luciferin-luciferase has been adapted for expression from the nuclear genome. Therefore, we

developed a semi-quantitative gene expression assay combining *Gaussia* luciferase as the experimental reporter and gp64-tagged *aph7* as the control, to test the pairing requirements for miRNA-mediated gene silencing in this alga. Gene expression was examined in pools of transgenic strains that were generated by electroporation and selected in liquid medium containing hygromycin B. In addition, the plasmid *gluc-tagaph7* (Figure S3) was designed for easy replacement of the *PsaD* promoter and terminator sequences by digestion at unique restriction enzyme sites to facilitate utilization of this strategy for other studies in *Chlamydomonas* such as promoter strength testing.

Numerous experimental and computational analyses in metazoans have demonstrated that miRNA target recognition is achieved predominantly through seed pairing (Bartel, 2009). In contrast, most land plant miRNAs pair with perfect or near-perfect complementarity to their targets (Schwab *et al.*, 2005; Voinnet, 2009). Because of these distinctive features regarding the extent of miRNA–target complementarity, approximately 30% of transcripts are predicted to contain miRNA-binding sites in mammals, whereas fewer than 1% of the transcripts in a land plant such as *Arabidopsis* are envisaged to be miRNA targets (Bartel, 2009; Axtell *et al.*, 2011). Given the close phylogenetic relationship between green algae and land plants, it was anticipated that *C. reinhardtii* miRNAs recognize their targets according to plant-like complementarity rules (Molnar *et al.*, 2007; Zhao *et al.*, 2007). However, our mismatch tolerance test using pools of transgenic strains revealed that *cre-miR1174.2* induced moderate silencing of *Gaussia* luciferase reporter genes when miRNA/target mRNA hybrids contained certain dinucleotide mismatches in the miRNA seed region or in regions flanking the cleavage site, and dinucleotide mismatches in the 3' miRNA region had no obvious detrimental effect on repression (Figure 4). Moreover, base pairing in the seed region alone (for both *cre-miR1174.2* and *miR910*) triggered mild silencing of target reporter genes (Figure 5 and Figure S7). Furthermore, G:U wobble pairing appeared to be well tolerated, as a reporter containing a *miR910* target site with one mismatch and five G:U matches, including one in the seed region and one flanking the cleavage site, was efficiently repressed in *Chlamydomonas* (Figure S5).

These results suggested that some aspects of miRNA biogenesis and function in green algae, at least for certain miRNAs, bear more resemblance to mechanisms operating in metazoans than those in land plants. In particular, relatively limited complementarity between miRNA and target site may be enough to trigger gene silencing in *Chlamydomonas*. Although the magnitude of repression in these cases may be modest, individual miRNAs may potentially target multiple endogenous transcripts, thereby fine-tuning gene expression levels, as proposed in metazoans (Bartel, 2009). Our observations also indicated that, in

Chlamydomonas, miRNAs may trigger RISC-mediated slicing of perfectly complementary target mRNAs (Figure 3). However, as in animal systems (Lewis *et al.*, 2005; Bartel, 2009), the silencing mechanism may involve translation repression when the target contains dinucleotide mismatches around the central miRNA region, flanking the predicted cleavage site (Figure 4). Therefore, as previously described for small interfering RNAs (Ma *et al.*, 2013), miRNAs may mediate silencing of target transcripts in *C. reinhardtii* by inducing RNA degradation and/or translation inhibition.

EXPERIMENTAL PROCEDURES

Culture conditions, transformation and genetic crosses

C. reinhardtii cells were grown photoheterotrophically in Tris/acetate/phosphate (TAP) medium as previously described (Yamasaki and Ohama, 2011). Nuclear transformation was performed by electroporation (Shimogawara *et al.*, 1998) by applying an exponential electric pulse of 0.7 kV at a capacitance of 50 μ F (BT600 Rev.G; BTX, <http://www.btxonline.com/>). The plasmids *gluc-aph7*, *gluc* (1 \times)-*aph7*, *gluc*(4 \times)-*aph7* and *gluc*(8 \times)-*aph7* (Figure S1) were digested using *KpnI*, and the transgene fragments were purified by agarose gel electrophoresis. Excised DNA (200 ng) was used for transformation of CC-124. Transgenic strains were selected on TAP/agar plates containing 15 μ g ml^{-1} hygromycin B (Wako, <http://www.wako-chem.co.jp/english/>; Berthold *et al.*, 2002). Approximately 2.5×10^7 cells of the CC-3491 strain (cell wall-deficient) in 250 μ l TAP medium supplemented with 50 mM sucrose (TAP/sucrose) were used for preparation of transgenic pools. Electroporation was performed using 300 ng of *KpnI*-linearized plasmids: *tagaph7*, *gluc-tagaph7*, *gluc*(1 \times)-*tagaph7*, *gluc*(no pair)-*tagaph7*, *gluc*(random1)-*tagaph7*, *gluc*(random2)-*tagaph7*, *gluc*(4 \times)-*tagaph7*, or *gluc*(8 \times)-*tagaph7* (Figure S3). Electroporated cells were incubated in 5 ml of fresh TAP/sucrose medium for 24 h. The cells were collected by centrifugation (1000 \times g, 5 min) at room temperature, washed once with TAP medium, and then inoculated into 100 ml TAP medium containing 6 μ g ml^{-1} hygromycin B. Hygromycin B-resistant transgenic strains were selectively grown in this medium for 5 days; subsequently, approximately 2×10^7 cells were collected by centrifugation (1000 \times g, 5 min) at room temperature. Pelleted cells were washed twice with TAP medium, and then grown in 100 ml of antibiotic-free TAP medium for 2 days. The cell density usually reached approximately 4×10^6 cells/ml after this period of cultivation, and this population of mixed strains was used as a transgenic pool. Control cells were electroporated in the absence of transforming DNA, and these cells did not survive the 5-day hygromycin B selection regime. Genetic crosses were performed as described by Harris (1989).

Construction of the *gluc* reporter genes

DNA fragments containing *cre-miR1174.2* target site(s) were constructed as previously described (Iwasaki *et al.*, 2009). In brief, a DNA fragment containing one *cre-miR1174.2* target site was amplified by PCR from the oligo-1 \times *cre-miR1174.2* oligonucleotide (5'-TGCGGCCGCTCGACCCGATAAGAAGGAGCCGTAACCTC GACCCTACTAGTGCC-3'; the target site is underlined), using primers *cre-miR1174.2-left-F* (5'-TGCGGCCGCTCGAC-3') and *cre-miR1174.2-right-R* (5'-GGCCACTAGTAGGGTTCGAG-3'), and cloned into the T-vector pMD20 (TaKaRa Bio, <http://www.takara-bio.com/>). A DNA fragment containing four *cre-miR1174.2* target sites was

digoxigenin-labeled probes or ³²P-labeled probes were performed as previously described (Rohr *et al.*, 2004; Yamasaki *et al.*, 2008). RNA blots were stained with methylene blue prior to hybridization to confirm equal lane loading (Herrin and Schmidt, 1988). DNA probes for hybridization were generated by PCR amplification with the following primers: au5.g528-t1-F1 (5'-GTGCCTACCACGTCACCAATC-3') and au5.g528-t1-R1 (5'-CTTCACGTCCACGTCCTTCTTC-3') for the *Cre10.g444300* ORF probe, au5.g528-t1-F4 (5'-GACGTGAACAATTGGCCATGACG-3') and au5.g528-t1-R4 (5'-CCACTGCACCTGCAGACTACAC-3') for the *Cre10.g444300* 3' UTR probe, gcluc-F2 (5'-ATCTGCCTGCCACATCAAGTG-3') and gcluc-R1 (5'-CGCCC TTAATCTTGTCCACCTGG-3') for the *gluc* probe, CBLP-F (5'-GAGTCCAACACTCGGCTACGCC-3') and CBLP-R (5'-CTGCCAATGGTGTACTTGACAC-3') for the *CBLP* probe, and U6snRNA-F (5'-GTGCTTCGGCACAACCTGTTAAAT-3') and U6snRNA-R (5'-AAAATTTGGAA CCATTTCTCGATTTATGC-3') for the U6 snRNA probe.

Quantitative PCR analyses

Purified total RNA was treated with DNase I (TaKaRa Bio) to remove contaminant DNA. First-strand cDNA was synthesized using an oligo(dT)₁₈ primer or random heptamers and PrimeScript reverse transcriptase (TaKaRa Bio) according to the manufacturer's instructions. Primer pairs for the quantitative PCR amplifications were as follows: gcluc-F2 and gcluc-R1 for the *gluc* probe, and CBLP-F and CBLP-R for *CBLP*. DNA fragments were amplified and quantified using SYBR Premix Ex Taq II (Tli RNaseH Plus; TaKaRa Bio) using the StepOne real-time PCR system (Life Technologies, <http://www.appliedbiosystems.com/>).

5' RACE assay

Putative cleavage sites of the *Cre10.g444300* transcript in strain CC-124 and putative slicing sites of the *gluc*(1 ×) transcript in the *gluc*(1 ×)-10 strain were examined using a GeneRacer kit (Life Technologies) as previously described (Molnar *et al.*, 2007). First and nested PCR amplifications were performed using the following primers: 444300-5' RACE-R (5'-GGCAGCCGTCAGATGACAGCAACTA-3') and 444300-5' RACE-nested-R (5'-CGGCCCATGCTTCTAGCAGCATACT-3') for *Cre10.g444300*, and *gluc*(*cre-miR1174.2*)-5' RACE-R (5'-CACTGGCTCACGCACGCTAACAT-3') and *gluc*(*cre-miR1174.2*)-5' RACE-nested-R (5'-TCCCCCTGATCTCTGTGGCTAAT-3') for *gluc*(1 ×).

Immunoblot analyses

Immunodetection was performed as described previously (Rohr *et al.*, 2004). *Gaussia* luciferase protein was detected using an anti-*gluc* antibody (E8023S, New England BioLabs, <https://www.neb.com/>). gp64-tagged *aph7* protein was detected using an anti-baculovirus envelope gp64 polypeptide antibody (14-6995, eBioscience, <http://www.ebioscience.com/>). Protein blots were stained using 0.5% w/v Ponceau S in 1% acetic acid.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Schematic diagrams of the *Gaussia* luciferase (*gluc*) reporter constructs used for *Chlamydomonas* transformation.

Figure S2. Expression of the *Gaussia* luciferase (*gluc*) reporter transgenes in *Chlamydomonas*, with or without *cre-miR1174.2* target sites in their 3' UTRs.

Figure S3. Schematic diagrams of the *Gaussia* luciferase (*gluc*)/tagged *aph7* reporter constructs used for *Chlamydomonas* transformation.

Figure S4. Monitoring of the potency of *cre-miR1174.2*-mediated silencing in pools of transgenic *Chlamydomonas* strains.

Figure S5. The *Gaussia* luciferase (*gluc*) reporter gene was silenced in *Chlamydomonas* to varying degrees when its transcript contained a single target site for miR912, miR910 or a putative miRNA (candidate 82; Zhao *et al.*, 2007).

Figure S6. Nucleotide substitutions in the *cre-miR1174.2* target site for the mismatch tolerance test.

Figure S7. A *Gaussia* luciferase (*gluc*) reporter containing a target site matching only the miR910 seed region was moderately repressed in *Chlamydomonas*.

Table S1. Frequency of hygromycin B-resistant transgenic strains in which the whole transforming DNA fragment is integrated.

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