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SHORT COMMUNICATION

Reduction in Carotenoid Levels in the Marine Diatom *Phaeodactylum tricornutum* by Artificial MicroRNAs Targeted Against the Endogenous *Phytoene Synthase* Gene

Simrat Kaur · Charles Spillane

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Abstract MicroRNAs (miRNAs) are key regulators of gene expression in eukaryotes where they can function to down-regulate expression levels or functioning of messenger RNAs (mRNAs) that are targeted by mature miRNAs displaying sequence homology. The 'active' mature miRNA forms are short RNAs which are processed from longer precursor miRNA molecules that have a stem-loop structure. While artificial miRNAs have been developed for gene knockdown experiments in a range of eukaryotes, it is not known whether artificial or endogenous miRNAs can functionally knock-down mRNA levels in the model marine diatom *Phaeodactylum tricornutum*. Here, we investigate the potential use of artificial microRNAs (amiRNAs) for targeted gene knockdowns in *P. tricornutum*, by generation of transformants harbouring a transgene cassette for the generation of amiRNAs designed to target the endogenous *phytoene synthase* (*PSY*) gene. In *P. tricornutum*, the amiRNA stem-loop precursor was processed to produce a mature amiRNA that successfully targeted the *PSY* mRNA and reduced *PSY* mRNA levels. As the *PSY* gene is a key component of the carotenoid biosynthetic pathway, the levels of carotenoids in the *P. tricornutum* amiRNA knockdown lines were reduced relative to untransformed control lines. This study demonstrates that artificial miRNAs can be successfully deployed for gene knockdown experiments in the model diatom *P. tricornutum*, providing a powerful tool for future metabolic engineering and synthetic biology experimentation in this model marine diatom.

S. Kaur · C. Spillane (✉)
Genetics and Biotechnology Laboratory, Plant and AgriBiosciences Research Centre (PABC), School of Natural Sciences, National University of Ireland Galway, University Road, Galway, Ireland
e-mail: charles.spillane@nuigalway.ie

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Introduction

MicroRNAs (miRNAs) are 21–24 nucleotide RNA molecules involved in post-transcriptional regulation (Molnar et al. 2007). miRNAs are processed from a precursor (pre-miRNA) molecule that has a characteristic hairpin secondary structure by an RNAase III-like endonuclease called Dicer (Mathelier and Carbone 2010). miRNAs bind to target RNA transcripts and can direct RNA cleavage and translational inhibition of messenger RNA (mRNA) as a means of regulating gene expression (Khraiwesh et al. 2008). miRNAs arose early in eukaryotic evolution and have played a key role in gene expression regulation since the emergence of multicellularity. In the plant kingdom, many miRNAs are evolutionarily conserved, typically begin with a U at the 5′ end of the RNA molecule and are transcribed from independent miRNA genes (Reinhart et al. 2002).

Phaeodactylum tricornutum is a model organism for pennate diatoms with a sequenced genome size of 27.4 Mb (Bowler et al. 2008). To improve the utility of model organisms for understanding gene function and biological processes, it is necessary to develop molecular tools for knockdown or overexpression of genes. Methods for genetic transformation of *P. tricornutum* have been developed which provide a basis for gene overexpression studies (Kroth 2007). However, because of the lack of sexual cycle and machinery for homologous recombination, there remains a need to develop new approaches for targeted gene knockdowns in *P. tricornutum*. RNA interference-based gene silencing in transgenic *P. tricornutum* has been achieved using antisense and inverted repeat-based approaches (De Riso et al. 2009; Allen et al. 2011).

It has been demonstrated that while the *P. tricornutum* genome contains protein-coding genes necessary for small interfering RNA (siRNA)-mediated gene silencing, the *P. tricornutum* genome may harbour an RNA-induced silencing complex (RISC) pathway that is distinct from other eukaryotes (De Riso et al. 2009). Indeed, evidence to date suggests that there are few miRNAs in the *P. tricornutum* genome (13 currently in miRBase) and the few miRNAs that have been detected have no identifiable homologues in other organisms (Huang et al. 2011). This raises some questions as to the functionality and extent of miRNA-based regulation in *P. tricornutum*.

Endogenous miRNA precursors can be redesigned to generate synthetic artificial miRNAs. The miRNA-miRNA* duplex molecule generated from miRNA precursors can be altered in sequence without changing the structural features of the precursor miRNA to generate mature artificial miRNAs that are targeted towards specific tracts of RNA sequence (Ossowski et al. 2008). Artificial miRNAs can hence be designed to target and downregulate any target gene expressing an RNA in the genome (Schwab et al. 2006). The first use of artificial microRNAs (amiRNAs) for gene knockdown was in human cell lines (Zeng et al. 2002) followed by the use of amiRNAs in the model plant *Arabidopsis thaliana* (Schwab et al. 2006).

Gene knockdown strategies based on RNA interference methods (namely, antisense or inverted repeat (IR)) can suffer from inherent problems due to their additional effects on transcriptional silencing. The siRNA produced by inverted repeats can mediate post-transcriptional and transcriptional silencing of both endogenously and exogenously introduced genes (Dunoyer et al. 2010). In the green microalga *Chlamydomonas reinhardtii*, the inverted repeat transgene constructs which transcribe long double-stranded RNAs (dsRNAs) have been shown to be prone to auto-silencing at the transcriptional level (Yamasaki et al. 2008; Rohr et al. 2004). In addition, production of many distinct siRNAs from Dicer-mediated cleavage of dsRNA increases the probability of off-target silencing effects which reduce the specificity of siRNA-mediated gene silencing (Xu et al. 2006). Such disadvantages can be overcome by the use of amiRNAs which are less prone to silencing for targeted gene knockdowns in both plants (Schwab et al. 2006) and the green microalga *C. reinhardtii* (Molnar et al. 2009).

Given the low number of miRNAs and the distinct protein machinery for RISC-based silencing in *P. tricornutum*, it is not known whether artificial miRNA techniques can be successfully deployed for targeted gene knockdowns in *P. tricornutum*. To address this question, we have made an artificial miRNA transgene construct that can express a double-stranded precursor artificial miRNA molecule under the control of the diatom-specific promoter fucoxanthin *chlorophyll a*/*c*-binding protein A (FcpA). We demonstrate that the precursor amiRNA is successfully processed into a mature artificial miRNA that downregulates the targeted *phytoene synthase* (*PSY*) gene in *P. tricornutum*. Our study demonstrates that artificial miRNAs can be successfully used for gene knockdown experiments in *P. tricornutum*.

Material and Methods

Vector Construction for Artificial MicroRNA

The artificial microRNA for the target *PSY* gene Phaeo 41878 (Bowler et al. 2008) was designed using the 'design' tool of Web MicroRNA Designer (WMD)2 platform at http://wmd3.weigelworld.org (Ossowski et al. 2008). The selected amiRNA showed high complementarity with the target *P. tricornutum PSY* gene and has a 50 % GC content. The artificial microRNA designer WMD identified four oligonucleotide sequences (i to iv), namely,

i. miR-s_2 5′ gaTTATTGCCACAAACGCGCCA Atctctctttgtattcc 3′
ii. miR-a_2 5′ gaTTGGCGCGTTTGTGGCAATA Atcaaagagaatcaatga 3′
iii. miR*s_2 5′ gaTTAGCGCGTTTGTCGCAATA Ttcacaggtcgtgatatg 3′
iv. miR*a_2 5′ gaATATTGCGACAAACGCGCTA Atctacatatatattcct 3′

The four oligonucleotide sequences (i to iv) were used to engineer the amiRNA into the endogenous miR319a precursor. The overlapping PCRs were performed using the four oligonucleotide sequences (i–iv) with pBluescript template plasmid RS300 (miR319a pBSK) containing a miRNA precursor (miR319a) from *A. thaliana* as a template (Ossowski et al. 2008). The amiRNA foldbacks generated in the template miR319a pBSK plasmid were cloned into pGEM-T easy vector after A tailing of PCR products, and sequencing was performed with oligonucleotide primers FA 5′ CTG CAA GGC GAT TAA GTT GGG TAA C 3′ and RB 5′ GCG GAT AAC AAT TTC ACA CAG GAA ACA G 3′ to confirm the sequence integrity and identity of the amiRNA transgene cassette. The amiRNA foldback cassette was subsequently cloned into the diatom expression vector pPhaT1-GFP, under the control of FcpA promoter to obtain the final amiRNA vector pPhaT1-GFP-amiRNA-PSY. The vector pPhaT1-GFP was kindly provided by Dr. Yusuke Matsuda (Kwansei Gakuin University, Japan). The expression vector pPhaT1-GFP-amiRNA-PSY was verified by restriction digestion and DNA sequencing with insert- and vector-specific primers prior to diatom transformation. The sequencing results indicated the presence of a 423-nucleotide-long fragment containing the stem-loop precursor sequence. The secondary structure of the artificial amiRNA precursor in vector pPhaT1-GFP-

amiRNA-PSY was predicted by RNA-fold (Vienna package) and drawn in VARNA (Darty et al. 2009).

Cell Culture and Genetic Transformation of *P. tricornutum*

The marine diatom *P. tricornutum* CCAP 1055/1 was grown in 50 % natural seawater media (F2-Si), at 17 °C under a 12:12-h light/dark photoperiod (40 μmol photons/m^2/s) until the culture appeared brownish. Standard protocol for biolistic transformation of diatoms (Kroth 2007) using gene gun (Bio-Rad, Hercules, CA, USA) was followed for transformation of *P. tricornutum* cells with the amiRNA vector pPhaT1-GFP-amiRNA-PSY. Upon bombardment of cells on 50 % F2 agar medium, the cells were grown for 24 h prior to selection with Zeocin. For selection of positive transformants, the cells were washed off the bombarded plates by repeated pipetting with 500–1000 μL of culture medium on the spot of cells and holding the plate in a tilted position. This algal suspension was spread on selection plates containing 100 μg/mL Zeocin. After 3–4 weeks, colonies were transferred onto fresh selection plates and further cultured.

Genomic DNA Extraction and Quantitative PCR for Transgene Copy Number

Genomic DNA was extracted from wild-type (WT) and amiRNA transformants by a cetyl trimethylammonium bromide (CTAB) DNA extraction method. Cells were pelleted by centrifugation at 4,000 rpm for 5 min and frozen at −80 °C. The frozen cell pellet was ground in liquid N_2, and powdered cells were incubated with 2× CTAB buffer (2 % *w*/*v* CTAB, 1.4 M NaCl, 100 mM Tris–HCl pH 8.0, 20 mM EDTA) at 65 °C for an hour. Upon cooling, an equal volume of chloroform was added and vortexed thoroughly, and layers were separated by centrifugation at 10,000 rpm for 10 min. The upper layer was transferred to a new Eppendorf tube; DNA was precipitated with chilled isopropanol and pelleted at 10,000 rpm for 10 min. The pellet was washed twice with 70 % (*v*/*v*) ethanol and air-dried prior to resuspension in sterile water. The integrity of DNA was checked on 0.8 % *w*/*v* agarose gel in TBE buffer. The absorbance (*A*) at 260 and 280 nm was checked with ImPlen NanoPhotometer.

For calculating copy number of the transgene in amiRNA transformant lines, oligonucleotide primers were designed using Primer3web software version 4.0.0 for sequences specific to the internal control gene *PSY* (forward 5′-AGTCGC AGCCTACTTGCGTT-3′, reverse 5′-CCAGGGAAGGAG CGTTGTCA-3′) and amiRNA transgene (forward 5′-GTGG ATCAAGCATGTTTTTGTGCAG-3′, reverse 5′-CCACAA ACGCGCCAATCTCTC-3′). Standard curves for six serial twofold dilutions were constructed for both sets of primer pairs to test for linearity and to ensure maximum specificity and efficiency for quantitative PCR. Quantitative PCR was performed using SensiMix SYBR No-ROX Kit (Bioline, UK) and the amplification reaction with standard thermal conditions (95 °C for 10 min followed by 40 cycles PCR, 95 °C for 15 s, 60 °C for 15 s for both annealing and extension) on a CFX96 machine (Bio-Rad, USA). Melt curve analysis was performed at the end of PCR amplification to check PCR product specificity and primer-dimer formation. The data for standard curves was analysed in CFX96 system software for calculation of efficiency, correlation coefficient and slope (Table 1). The copy number of the transgene (X_0) was derived from equation $X_0/R_0 = 10^{[(\mathrm{Ct},_X - I_X)/S_X] - [(\mathrm{Ct},_R - I_R)/S_R]}$ where I_X and I_R are intercepts of standard curves of transgene and internal control genes; S_X and S_R are slopes of standard curves of transgene and internal control genes, respectively; $\mathrm{Ct},_X$ and $\mathrm{Ct},_R$ are threshold cycles of amplification of the transgene and internal control to a tested sample; and R_0 is the copy number of internal control gene (Chen GaL 2010).

Measurement of Carotenoid Content in *PSY* Knockdown *P. tricornutum* Lines

The carotenoid content in wild-type and amiRNA transformants was measured according to Ryckebosch et al. (2011). In brief, 10-day-old diatom cultures were harvested and fresh weight was recorded. Carotenoids were extracted with methanol, and *A* was measured at 470, 665 and 652 nm using an ImPlen NanoPhotometer. The following equation was used to calculate the amount of carotenoids;

$$C_a = (15.65A_{665}) - (7.34A_{652})$$

$$X_{\text{carotenoids}} = \frac{(1,000A_{470}) - (2.86C_a)}{221} \times \mathrm{DF} \times V$$

where $X_{\text{carotenoids}}$ is the amount of carotenoids in micrograms, C_a is *chlorophyll a* content, DF is the dilution factor, and *V* is the volume of culture in millilitres.

Extraction of Total RNA and Quantitative Reverse Transcription PCR (RT-PCR) of *PSY* mRNA Levels

Total RNA was extracted from diatom cells using the TRI Reagent (Sigma-Aldrich) method (Molnar et al. 2007). *P. tricornutum* cells were harvested by centrifuging 50 mL of culture at 4,000 rpm for 5 min and flash freezing the pellet in liquid N_2 after discarding the supernatant. The pellet was powdered using a mortar and pestle in the presence of liquid N_2 and then suspended in 6 mL of TRI Reagent and vigorously mixed. The mixture was then centrifuged at 4,000 rpm for 15 min at 4 °C, and the upper phase was transferred into a new 15-mL tube. After incubation for 5 min at room temperature, 1.2 mL of

Table 1 Values derived from standard primer efficiency curves

Gene	Range of Ct mean values	Amplification efficiency	Slope	Correlation coefficient (R^2)	Intercept
amiRNA	15.12–19.68	1.13	−3.035	0.996	21.6
PSY	16.72–21.60	1.10	−3.097	0.994	23.5

Ct threshold cycle

chloroform was added and the solution was vigorously mixed for 15 s and subsequently incubated at room temperature for 5 min. The mixture was centrifuged at 4,000 rpm for 15 min at 4 °C, and the upper phase containing RNA was transferred to a fresh 15 mL tube on ice. The RNA was precipitated by adding an equal volume of isopropanol and centrifuged at 4,000 rpm for 15 min at 4 °C. The pellet was then washed with 80 % *v*/*v* ethanol twice and dried at room temperature. The pellet was resuspended in nuclease-free water. Genomic DNA contamination was avoided by treating the RNA with Amplification Grade DNase I (Sigma-Aldrich). Five hundred nanograms of RNA was reverse transcribed using a RevertAid H Minus Strand cDNA Synthesis Kit (Thermo Scientific). Reverse Transcription PCR (qRT-PCR) was performed using SensiMix SYBR No-ROX Kit (Bioline, UK), and the reaction was run onto a CFX96 machine (Bio-Rad, USA). mRNA levels of genes encoding TATA box-binding protein (TBP) and ribosomal protein small subunit 30S (RPS) were used as housekeeping controls. The quantification of relative expression was calculated using the standard $2^{-\Delta\Delta CT}$ method in CFX96 system software, and statistical significance was tested by one-way ANOVA using Minitab v. 16.

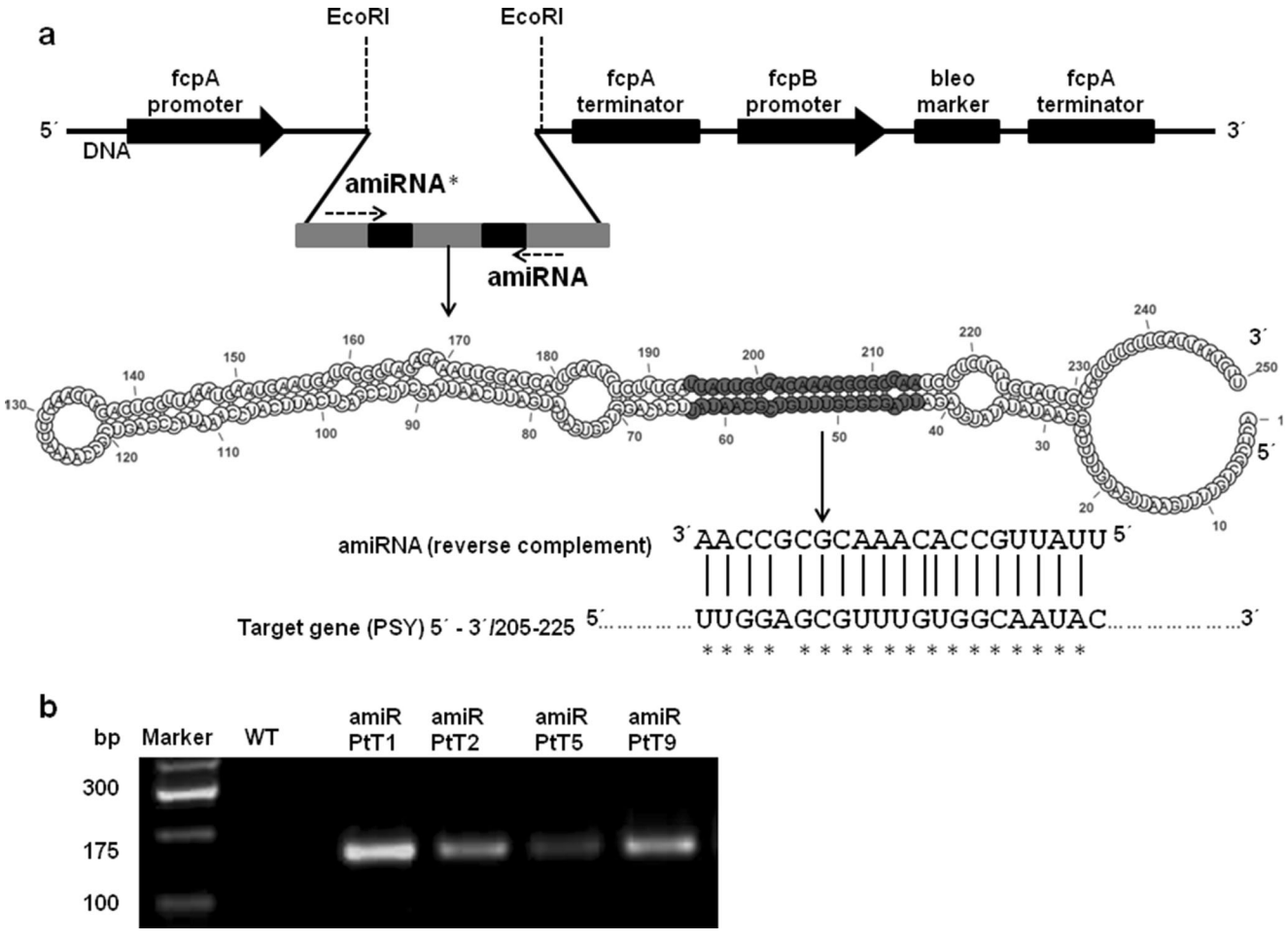

Fig. 1 **a** Diagram illustrating an amiRNA cloning cassette of the construct pPhaT1-GFP-amiRNA-PSY. *FcpA* fucoxanthin *chlorophyll a*/*c*-binding protein A, *FcpB* fucoxanthin *chlorophyll a*/*c*-binding protein B; secondary structure of amiRNA precursor and complementarity of the mature amiRNA to target site in PSY gene. **b** PCR verification of amiRNA-PSY insert in transformed lines using primers specific to the stem region as indicated by *dashed lines in the vector diagram*

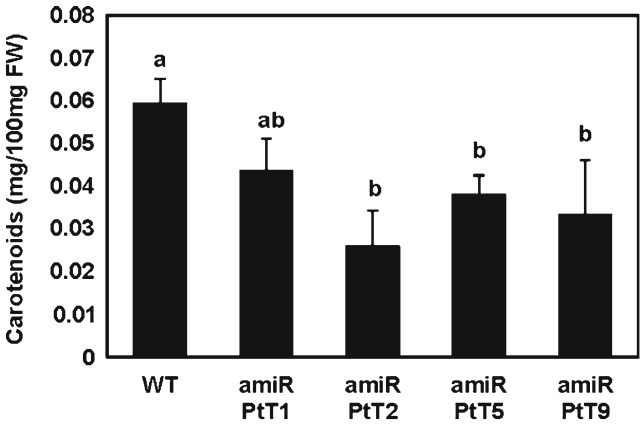

Fig. 2 Total carotenoid content in wild-type (*WT*) and transformed lines of *P. tricornutum*. Data is the mean of three biological replicates, and coefficient of variance was tested by performing Tukey's post hoc test by one-way ANOVA. Mean values indicated with *different letters* are significantly different

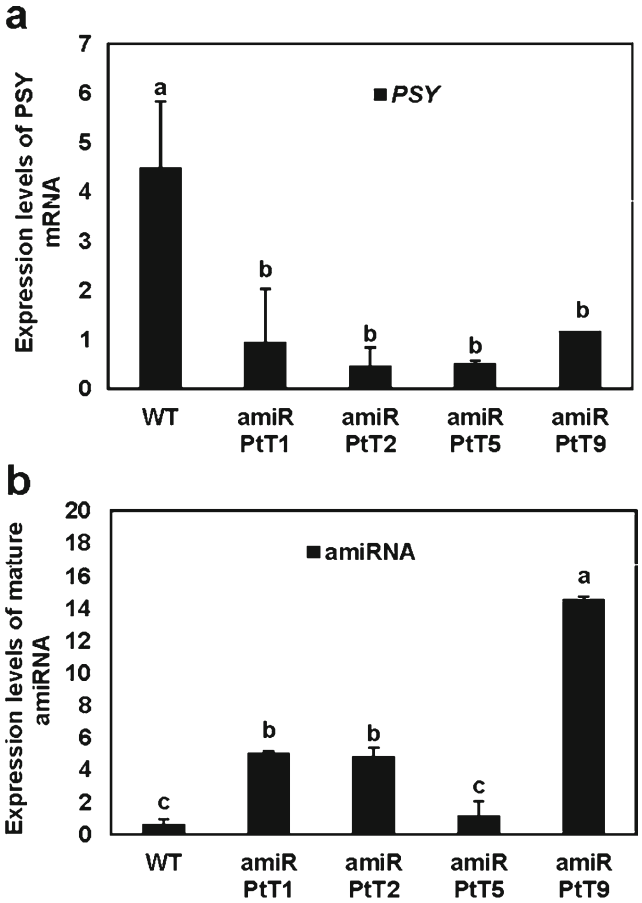

Fig. 3 Knockdown of PSY mRNA levels using amiRNA targeted against *PSY* mRNA sequence in transformed lines of *P. tricornutum*. **a** Expression analysis (qRT-PCR) of *PSY* mRNA levels, calculated using a standard $2^{-\Delta\Delta CT}$ method with TBP and RPS as internal controls. Statistical significance tested by one-way ANOVA using Minitab v. 16. **b** Expression of mature amiRNA (targeted against *PSY* sequence) calculated using a standard $2^{-\Delta\Delta CT}$ method with 5S rRNA as endogenous control and quantification performed by Taqman qRT-PCR

Analysis of Mature amiRNA Expression Levels

The cDNA for the mature amiRNA was prepared using a TaqMan MicroRNA Reverse Transcription Kit. In brief, 10 ng of RNA sample was reverse transcribed in a reaction mixture containing 1 mM dNTPs, 50 U of MultiScribe Reverse Transcriptase, 1× reverse transcription buffer, 20 U of RNase inhibitor, 1× reverse transcriptase stem-loop primer and nuclease-free water to make a total reaction volume of 15 μL. The reaction tube was subjected to thermal cycling conditions of 30 min at 16 °C, 30 min at 42 °C and 5 min hold at 85 °C. The quantitative PCR amplification was performed with TaqMan Universal PCR Master Mix II using TaqMan Small RNA Assay designed specifically for amiRNA and endogenous control as 5S ribosomal RNA (rRNA). The thermal cycling conditions for TaqMan qPCR were as follows: initial hold at 50 °C for 2 min, enzyme activation for 10 min at 95 °C followed by 40 cycles of denaturing at 95 °C for 15 s and both annealing and extension at 60 °C for 60 s.

Statistical Analysis

The data analysis was performed on Minitab statistical software package v. 16.2.4 (Minitab Inc.). The mean values were compared by one-way ANOVA (Tukey's test, $P<0.05$).

Results and Discussion

While there is now extensive genomic information regarding the model diatom *P. tricornutum*, there is a lack of experimental techniques for targeted gene knockdowns in *P. tricornutum*. To determine whether artificial miRNAs can be employed for targeted knockdown of mRNA levels of specific genes in the marine diatom *P. tricornutum*, we developed an amiRNA transgene cassette to target the mRNA of the endogenous *phytoene synthase* gene in *P. tricornutum*. *phytoene synthase* (*PSY*) catalyses the first step in the carotenoid biosynthesis pathway in both plants and microalgae. Disruption of *PSY* gene function has previously been shown to lead to the

Table 2 Transgene copy number detection in amiRNA transformant lines

Transformant lines	Ct (amiRNA)	Ct (*PSY*)	Transgene copy number
amiR-PtT1	14.31	16.13	2
amiR-PtT2	14.50	16.08	2
amiR-PtT5	14.12	16.34	1
amiR-PtT9	15.56	16.64	3

Ct threshold cycle

generation of white mutants in the green alga *C. reinhardtii* (McCarthy et al. 2004).

To generate the amiRNA transgene cassette for targeting *PSY*, the miR319a pBSK vector containing the miR319 stem-loop precursor derived from *A. thaliana* (Schwab et al. 2006) was used. The amiRNA oligonucleotides with sequence complementarity targeting *PSY* were cloned into the miR319a pBSK vector, via an exchange of endogenous miRNA and miRNA* of miR319a with amiRNA and amiRNA*. The mature amiRNA was designed to bind to the target *PSY* mRNA at positions 205–225 (Fig. 1a). The PSY amiRNA foldback cassette was subsequently cloned into the diatom expression vector pPhaT1-GFP, to generate the vector pPhaT1-GFP-amiRNA-PSY (Fig. 1a). The secondary structure of the artificial amiRNA precursor in vector pPhaT1-GFP-amiRNA-PSY was predicted by RNA-fold (Fig. 1a). Independent Zeocin-resistant *P. tricornutum* transformants obtained upon transformation with the vector pPhaT1-GFP-amiRNA-PSY indicated the presence of a 175-bp insert sequence amplified using primers specific to the stem region (Fig. 1b). The positive transformant colonies (amiR-PtT lines) grown under antibiotic (Zeocin) selection were transferred to liquid medium prior to analysis of carotenoid levels and PSY mRNA levels.

The analysis of carotenoid levels in a number of the amiR-PtT lines demonstrated a decrease in levels of carotenoids compared to wild-type (WT, untransformed) levels (Fig. 2). Four lines (amiR-PtT1, amiR-PtT2, amiR-PtT5 and amiR-PtT9) were randomly selected as candidate lines where reduction in the levels of carotenoids could be due to reduced PSY mRNA levels arising from the expression of the amiRNA targeted against *PSY*. The WT and amiR-PtT lines were analysed by qRT-PCR to determine the mRNA levels of *PSY*. Statistically significant reductions in the mRNA levels of *PSY* were observed in all four of the independent transformants compared to the *PSY* expression level in the WT line (Fig. 3a). The decrease in *PSY* mRNA levels in three of the four lines (i.e. amiR-PtT1, amiR-PtT2 and amiR-PtT9) was inversely associated with the expression of mature amiRNA levels when analysed by Taqman qRT-PCR (Fig. 3b).

Amongst the four transformants, there were significant differences in the expression levels of the mature amiRNAs between the WT and three of the transformant lines (Fig. 3b). The differences in transgene copy number obtained for the individual transformant lines may have contributed to these expression differences (Table 2). The copy number was positively associated with the expression level of the amiRNA in each of the four transformant lines (Table 2, Fig. 3b). For instance, the transformant amiR-PtT9 had the highest copy number (Table 2) and exhibited the highest levels of amiRNA expression (Fig. 3b).

The detection of the mature amiRNA in the transformed *P. tricornutum* cells indicates that *P. tricornutum* possesses the RNA silencing machinery to process the stem-loop precursors of amiRNA (or any miRNAs) resulting into single-stranded mature amiRNAs (Fig. 3b). Our data indicates that *P. tricornutum* harbours a functional miRNA processing pathway which can be harnessed to generate amiRNAs for functional gene knockdown experiments in this model diatom.

Overall, in this study, we demonstrate that artificial miRNAs can be successfully developed and deployed for gene knockdown experiments in *P. tricornutum* which is a necessary experimental approach for understanding gene function and biology in this model diatom.

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