Nourseothricin N-acetyl transferase (NAT), a new selectable marker for nuclear gene expression in *Chlamydomonas*

Xinjia Yang¹, Jialin Peng¹ and Junmin Pan¹,²*

**Abstract**

**Background:** *Chlamydomonas reinhardtii* is a unicellular green alga, which is a most commonly used model organism for basic research and biotechnological applications. Generation of transgenic strains, which usually requires selectable markers, is instrumental in such studies/applications. Compared to other organisms, the number of selectable markers is limited in this organism. Nourseothricin (NTC) N-acetyl transferase (NAT) has been reported as a selectable marker in a variety of organisms but not including *C. reinhardtii*. Thus, we investigated whether NAT was useful and effective for selection of transgenic strains in *C. reinhardtii*. The successful use of NAT would provide alternative choice for selectable markers in this organism and likely in other microalgae.

**Results:** *C. reinhardtii* was sensitive to NTC at concentrations as low as 5 µg/ml. There was no cross-resistance to nourseothricin in strains that had been transformed with hygromycin B and/or paromomycin resistance genes. A codon-optimized NAT from *Streptomyces noursei* was synthesized and assembled into different expression vectors followed by transformation into *Chlamydomonas*. Around 500 transformants could be obtained by using 50 ng DNA on selection with 10 µg/ml NTC. The transformants exhibited normal growth rate and were stable at least for 10 months on conditions even without selection. We successfully tested that NAT could be used as a selectable marker for ectopic expression of IFT54-HA in strains with paromomycin and hygromycin B resistance markers. We further showed that the selection rate for IFT54-HA positive clones was greatly increased by fusing IFT54-HA to NAT and processing with the FMDV 2A peptide.

**Conclusions:** This work represents the first demonstration of stable expression of NAT in the nuclear genome of *C. reinhardtii* and provides evidence that NAT can be used as an effective selectable marker for transgenic strains. It provides alternative choice for selectable markers in *C. reinhardtii*. NAT is compatible with paromomycin and hygromycin B resistance genes, which allows for multiple selections.

**Keywords:** *Chlamydomonas, Nourseothricin N-acetyl transferase, Transformation, Selectable marker, Genetic engineering*

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**Background**

*Chlamydomonas reinhardtii* (*C. reinhardtii*), a unicellular green alga, is a widely used model organism for basic scientific research as well as biotechnological applications [1]. Generation of transgenic strains plays a critical role in our deeper understanding of molecular mechanisms involved in various cellular processes and genetic engineering for producing valuable products [2, 3].

Because of low efficiency of transformation, a selectable marker is usually needed for selection of transgenic strains. Currently, there are three types of selections used in nuclear transformation of *C. reinhardtii*: auxotrophy rescue, herbicide resistance and antibiotic resistance [1–3]. Auxotrophy rescue involves using parental strains with mutations thus limiting its application. For example,
Results

Wild type C. reinhardtii strain is sensitive to nourseothricin

To explore the possibility to use NAT gene as a selectable marker for nuclear transformation, we first tested the sensitivities of C. reinhardtii to NTC. The selection concentrations for other organisms range from 20–400 μg/ml (https://www.jenabioscience.com/images/741d0cd7d0/NTC-Flyer.pdf). C. reinhardtii cells were placed on agar plates supplemented with different concentrations of NTC and grown for 4 days. The cells were sensitive to NTC at concentrations even as low as 2.5 μg/ml. At concentrations of 5 μg/ml and above, no viable cells were observed microscopically (Fig. 1) and even after 14 days (data not shown). Thus, we conclude that C. reinhardtii is sensitive to NTC, which paves the way for using NAT as a selectable maker.

NTC is compatible with paromomycin and hygromycin B selections

For studies involved with transgenic strains, multiple selections are usually required. For example, rescue of insertional mutants generated by using antibiotic resistance genes requires another antibiotic selectable marker. Commonly used antibiotics for selection of transgenic strains of C. reinhardtii include paromomycin and hygromycin B [1]. We wondered whether NTC is compatible with these two antibiotics for selection in C. reinhardtii. NTC, paromomycin and hygromycin B all inhibit protein synthesis, but their working mechanisms are different. Paromomycin inhibits initiation of translation or earlier steps of elongation while hygromycin B potently inhibits elongation [17]. NTC inhibits protein synthesis with miscoding activity [14]. It has been shown in mammalian cells and fungus that NTC is compatible with selections with hygromycin B [18]. Whether it is compatible with...
selection with paromomycin and/or hygromycin B in *Chlamydomonas* cells is not known.

Previously, our lab has generated strains with resistance to paromomycin, hygromycin B or both, which provided the needed resource for testing NTC compatibility with paromomycin and/or hygromycin B for selection. Wild type strain 21gr and strains with paromomycin, hygromycin B or both were grown on agar plate supplemented the antibiotics as indicated. As shown in Fig. 2, application of NTC killed wild type cells as well as cells with paromomycin, hygromycin B and both. Thus, there is no cross-resistance to NTC in strains that had been transformed with hygromycin B and/or paromomycin resistance genes, indicating that NTC can be used for multiple selection with paromomycin and/or hygromycin B.

### Expression of codon optimized NAT gene confers resistance to NTC of *C. reinhardtii*

The *Chlamydomonas* genome has a higher GC content and its gene expression exhibits bias of codon usage. Divergence of these properties in a foreign gene may adversely affect gene expression in *Chlamydomonas* likely due to changes in chromatin structure and abnormal low abundance of some tRNAs, respectively [19–22]. Though the GC content of NAT is similar to that of *Chlamydomonas*, some rarely used codons in *Chlamydomonas* are present in NAT. To test whether NAT can be used as a selectable marker, the NAT gene from *S. noursei* was synthesized with codon optimization (Fig. 3). The NAT gene encodes a protein of 190 aa. We generated a pHr-NAT-HA plasmid harboring codon optimized NAT (Fig. 4). The NAT ORF tagged with 3xHA was placed under the control of the *HSP70a/RBCS2* promoter containing one copy of *RBCS2* intron 1 and *RBCS2* 3′UTR (Fig. 4a). The construct was transformed by electroporation into wild type (WT) cells. The transformants were grown on agar plates with or without NTC (Fig. 4b). Transformants on agar plates without NTC grew like a lawn. In contrast, individual colonies were observed on agar plate with NTC, suggesting that these colonies are NTC resistant. The NTC resistance of these colonies may be caused by rare mutations or expressing NAT. To discern these possibilities, 7 colonies were randomly picked and subjected to analysis for NAT expression by immunoblotting. All the colonies showed expression of NAT (Fig. 4c). The protein mass of NAT was similar to calculated molecular weight. Thus, these data demonstrate that NAT can be successfully expressed in *C. reinhardtii* and its expression can confer transgenic strains with NTC resistance. The transformation efficiency was 528 cfu per 50 ng plasmid DNA for three independent transformations.

To examine the sensitivity of the transgenic strains with different levels of NAT expression, cells of strains with higher expression (strains #1 and #6) and lower expression (strain #2) were grown on agar plates supplemented with various concentrations of NTC (Fig. 4d). Consistent with results shown above, wild type cells were killed at 10 µg/ml NTC while the transformants grew normally. However, at higher concentrations of NTC, the transformants showed different extent of growth. For strain #2, which had lower expression of NAT, strong growth inhibition was observed at 50 µg/ml of NTC. In contrast, for strains #1 and #6, which had relatively higher expression of NAT, strong inhibition was observed at 100 µg/ml of NTC. These data further demonstrate that the tolerance to NTC is conferred by the expression of NAT and reveal that the extent of tolerance is correlated with the levels of NAT expression.

![Fig. 2](image_url)  
Fig. 2 No cross-resistance to NTC in strains with paromomycin and/or hygromycin B resistance. Wild type (WT) cells and transgenic strains with resistance to paromomycin, hygromycin B or both were grown for 4 days on TAP agar plates without antibiotics (a) or supplemented with paromomycin (b), hygromycin B (c) or NTC (d). paro, paromomycin resistant strains; hygro, hygromycin B resistant strains; paro/hygro, paromomycin and hygromycin B double resistance strains. Data shown are representative of three experiments.
**NAT can be used as a selectable marker for transgenic strains**

Given that NAT can be expressed and confer NTC resistance in *C. reinhardtii*, we decided to examine it as a selectable marker for transgenic strains. *ift54* is a mutant defective in *IFT54*, which was generated by insertional mutagenesis with paromomycin resistance gene *Aph-VIII* [23]. Loss of IFT54 blocks cilia formation (Fig. 5a). To prove our hypothesis, we generated a plasmid carrying NAT resistant gene as well as protein expression cassette of *IFT54* (Fig. 5b). The plasmid was transformed into *i54* and the transformants were selected on agar plates with 10 μg/ml NTC. 12 out of 192 (6.5%) colonies grown on NTC selection agar plates expressed *IFT54-HA* as examined by immunoblotting (Fig. 5c and data not shown). And all strains expressing *IFT54-HA* rescued the aflagellar phenotype of *i54* (Fig. 5a). These data demonstrate that NAT can be used as a selectable marker even in strains with paromomycin resistance.

Next, we examined whether NAT can be used as a selectable marker in strains with both paromomycin and hygromycin B resistance. The pPSAD-IFT54-HA(NAT™) was transformed into a strain with paromomycin and hygromycin B resistance. 7 out of 116 (6.03%) colonies grown on the NTC selection plates expressed *IFT54-HA* as examined by immunoblotting (Fig. 5d and data not shown). Taken together, we have shown that NAT is an efficient selectable marker, which is compatible with paromomycin and/or hygromycin B resistance genes.

**Fusion of a target gene IFT54-HA to NAT and processing with the FMDV 2A peptide increases gene expression efficiency**

It has been reported that the gene expression efficiency is much improved by fusion of a target gene to the FMDV 2A peptide [24]. Due to cleavage after gene translation at the 2A peptide sequence, the resulting protein is processed into two discrete proteins: a protein from the target gene and the selectable marker protein fused with short 2A peptide [25]. To demonstrate whether NAT can be used as such a selectable marker, we made a construct by fusing *IFT54-HA* to NAT and DNA sequence of FMDV 2A (Fig. 6a). The construct was transformed into *i54* and the transformants were selected on agar plates with 10 μg/ml NTC. 122 out of 204 (59.8%) colonies had flagella. Examination of a few transformants with flagella showed that they all expressed *IFT54-HA* (Fig. 6b). Thus, we predicted that all the transformants that had formed flagella should have expressed IFT54-HA. Compared to the construct that does not fuse IFT54-HA to NAT as shown in Fig. 5b, this construct led to a ninefold increase of selection efficiency (6.5% to 59.8%) for transgenic strains. However, by examination of the protein levels...
of IFT54-HA in transformants derived from these two constructs (Figs. 5c and 6b), we have not observed increased protein expression level by such a fusion construct as reported [24].

Discussion
The ability to generate transgenic cells is crucial for genetic engineering widely used in basic research as well as in biotechnological applications. As a model organism, C. reinhardtii is widely used for exploration of basic cellular processes such as cilia biogenesis and photosynthesis and for producing commercially valuable products as a cell factory [1–3]. Although a few selectable markers have been developed in this organism, few of them have been widely used. An ideal selectable marker may possess the following properties: (1) high stability, aqueous solubility and low dosage of the selection reagents;
(2) non-toxicity of the selection reagents in the presence of a selectable marker; (3) non-toxicity of the selectable markers; (4) high efficiency of transformation; (5) compatibility with other selectable markers and 6) no genotype requirement for the parental strains.

We have shown that NAT is an effective and stable selectable marker in C. reinhardtii that confers resistance to NTC. C. reinhardtii is very sensitive to NTC. No viable colonies were observed even in the presence of 5 μg/ml NTC though we have used 10 μg/ml for the selection. NTC is soluble in water and highly stable. The transformation efficiency of NAT is high. Around 500 cfu were routinely obtained by using 50 ng plasmid DNA for transformation. Expression of NAT was stable even in the absence of NTC. We have not observed any growth defects in NAT transgenic strains. As NTC is an antibiotic, it does not require strains with specific genotype. Thus, NAT provides an alternative choice for selectable markers in C. reinhardtii. Random insertion of foreign DNA into the genome of C. reinhardtii occurs during transformation and this property has been used to generate insertional mutants [26, 27]. Though we have not examined the patterns of integration of NAT into the genome of C. reinhardtii, NAT is expected to behave as other foreign DNA fragments. Thus, NAT may be used for generation of insertional mutants from which desired functional genes can be cloned.

We have tested using NAT as a selectable marker for transgenic expression of a target gene IFT54. We have used parental strains that had been previously transformed with paromomycin and/or hygromycin B resistant genes. Around 6.5% IFT54 transgenic strains were obtained from NTC resistant colonies, demonstrating that NAT can be used as a selectable marker. These data also indicate that NAT is compatible with hygromycin B and paromomycin resistant genes, which allow for multiple selections. We have developed a construct by fusing the target gene IFT54 to NAT and processing with FMDV 2A peptide. Compared to the non-fusion construct, the efficiency of expression of IFT54-HA has increased around ninefold. Thus, this fusion expression system can increase selection efficiency of transgenic strains. Because the NTC resistance is correlated with the expression levels of NAT, this system may also be used for obtaining strains with higher expression of target genes by selection at higher concentrations of NTC.

NAT as a selectable marker has been used in microalgae but so far only in marine diatoms, including Chaetoceros gracilis, P. tricornutum and T. pseudonana [16, 28, 29]. Our demonstration that NAT can be used a selectable marker in a fresh water green alga, opening a promising prospect in using NAT in other microalgae, especially in those algae with fewer choices for selectable markers. For example, Dunaliella, a saline green alga, is a popular model organism for the study of adaptation of eukaryotic cells to high salt concentrations and some Dunaliella species are of economic value for producing beta-carotene [30]. However, Dunaliella is resistant to paromomycin, hygromycin B, spectinomycin and kanamycin [3]. Thus, NTC resistance needs to be tested in Dunaliella before the NAT/NTC selection system can be used.

Conclusions

We have developed a new stable selectable marker for selection of transgenic strains in C. reinhardtii that confers resistance to NTC, which provides an alternative choice for selectable markers. In addition, NAT is compatible with paromomycin and hygromycin B resistance genes, two most commonly used selectable markers in C. reinhardtii, which allows combination of multiple selectable markers in transgenic studies.

Methods

Strains and culture

Chlamydomonas reinhardtii wild type strain 21gr (CC-1690, mt+) was from the Chlamydomonas Resource Center. ift54 (a paromomycin resistant strain) [23], if4::LF4-HA (a hygromycin B resistant strain) [31] and wdr92::WDR92-YFP (a paromomycin and hygromycin B double resistant strain) [32] were generated in our own lab. Unless otherwise stated, cells were grown at 23°C in M liquid medium in a 14/10 light/dark cycle [33]. Cells
used for transformation were grown at 23 °C in liquid TAP medium under continuous light [34].

Reagents
Paromomycin and hygromycin B were purchased from Merck Millipore, USA, while NTC was obtained from Jena biosciences, Germany. The antibiotics were solubilized in water and sterilized by filtration. The concentrations used for selection for paromomycin, hygromycin B and NTC were 10, 20 and 10 µg/ml, respectively.

Drug sensitivity assay
To determine the sensitivity of *C. reinhardtii* to NTC, 1 × 10⁶ of wild type cells were spotted on 1.5% TAP agar plates supplemented with various concentrations of NTC (0, 2.5, 5, 10 and 40 µg/ml) and incubated for 4 days at 23 °C in a 14/10 light/dark cycle. To test whether strains with paromomycin and/or hygromycin B resistant genes are sensitive to NTC, 1 × 10⁶ cells of wild type and strains with various resistant genes were grown on 1.5% TAP agar plates supplemented with different antibiotics as indicated in the text.

Construction of the transformation vectors
To generate a construct for expressing *NAT*, the coding region of *NAT* from *S. noursei* was codon optimized for *C. reinhardtii* and chemically synthesized (Genscript, China). Codon optimized *NAT* tagged with 3 × HA tag at the 3′ end driven by HSP70a/RBCS2 and terminated by RBCS2 terminator was cloned into ZT4-blunt vector. The HSP70a/RBCS2 promoter and RBCS2 terminator were cloned from pCB740 [35]. The 3 × HA tag was cloned from pKL-3XHA [36]. The final construct was termed pHR-NAT-HA. To enable expression of HA in IFT54- from pKL-3XHA [36]. The final construct was termed cloned from pCB740 [35]. The 3 × HA tag was cloned promoter and RBCS2 terminator were cloned into ZT4-blunt vector. The HSP70a/RBCS2 promoter and RBCS2 terminator were cloned from pCB740 [35]. The 3 × HA tag was cloned from pKL-3XHA [36]. The final construct was termed pHR-NAT-HA. To enable expression of HA in IFT54- from pKL-3XHA [36]. The final construct was termed cloned from pCB740 [35]. The 3 × HA tag was cloned

Electroporation transformation
Transformation of *Chlamydomonas* was performed by electroporation using BTX ECM630 (Harvard Apparatus Inc, USA) following a previously published protocol [38]. For each transformation, 5 × 10⁷ cells were mixed with 50 µg plasmid DNA linearized by *AclI*. After electroporation, the transformation mixture was diluted in 10 ml TAP + 50 mM sorbitol and kept away from light for 8 h. Transformants were selected on agar plates supplemented with 10 µg/ml NTC.

SDS-PAGE and immunoblotting
SDS-PAGE and immunoblotting analysis were performed as described previously [39]. Briefly, cells were lysed with Buffer A (50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 1 mM EDTA, and 1 mM DTT) containing protease inhibitor cocktail (Roche, Switzerland) and boiled for 10 min in 1 × SDS loading buffer. The proteins were separated in 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, USA) and probed with the indicated antibodies. The primary antibodies used include the following: rat anti-HA (Roche, Switzerland), 1:3000; mouse anti-α-tubulin (Sigma-Aldrich, USA), 1: 3000; rabbit anti-IFT54, 1:3000 [23] and rabbit anti-CDPK3, 1: 5000 [38].

Cell imaging
After cell fixation in 1% glutaraldehyde, DIC images were captured by Zeiss Axio Observer Z1 microscope (Carl Zeiss, Germany) equipped with a CCD camera (QuantEM512SC, Photometrics, USA). The images were processed in Photoshop and/or Illustrator (Adobe, USA).

Abbreviations
NAT: nourseothricin (NTC) N-acetyl transferase; NTC: nourseothricin; FMDV 2A: foot-and-mouth-disease-virus 2A; HR: hSP70a/RBCS2; IFT54: intraflagellar transport 54; CDPK3: calcium dependent kinase 3; DIC: differential interference contrast.

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Authors’ contributions
XJY and JLP performed the experiments. XJY and JMP analyzed the data. JMP and XJY wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article. Experimental materials generated during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
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