Influences of external nutrient conditions on the transcript levels of a nitrate transporter gene in *Skeletonema costatum*

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**Abstract**

To verify the feasibility of high-affinity nitrate transporter gene (Nrt2) as an indicator of nitrogen status, changes in the transcript levels of transcripts associated with phosphate starvation and different nitrate concentrations were studied using real-time quantitative reverse-transcription PCR (QRT-PCR) technology in batch cultures of *Skeletonema costatum*. The results show that compared with P-replete condition, P starvation could reduce the Nrt2 transcript levels apparently. Nrt2 transcript levels had a significant negative linear correlation with nitrate concentrations below 40 μmol/L. The results of 48 h short-term incubation experiment under different nitrate concentrations confirmed this correlation, and the following regression equation is built: \[ y = -3.305x + 98.95, \ R^2 = 0.998, \] where \( x \) represents nitrate concentrations (<40 μmol/L) and \( y \) represents the Nrt2 transcript levels.

**Key words:** high-affinity nitrate transporter gene, phosphate starvation, nitrate concentrations, real-time PCR, *Skeletonema costatum*

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1 Introduction

Nitrogen is an essential component of important biological macromolecules, such as proteins, and nucleic acids. Its availability is closely linked to biological activities. Phytoplankton serve as major contributors to the primary production in the marine food chain, but their growth is often limited by supplies of nitrogen (Howarth and Marino, 2006). Traditional methods used to detect N-limit conditions include nutrient concentration ratios, enrichment bioassays, and 15N tracer uptake, etc. (Dugdale and Wilkerson, 1988; Hecky and Kilham, 1988; Collos et al., 1993). With these techniques, we have discovered much about the relationships between N deficiency and phytoplankton responses. However, these techniques are typically designed for bulk measurements, but species-specific information is rarely known. It has been confirmed that different phytoplankton species respond differently to the same ambient nutrient environment (Dyhrman et al., 2002; Geider and La Roche, 2002). Therefore, it is important to assess the nutrient status of the organisms of interest from a cellular property that is unique to the nutrient stress in question and that enables assessment in a species-specific manner. The development of molecular probes for nutrient deficiencies is particularly suited for detecting phytoplankton responses to environmental stresses (La Roche et al., 1999).

Among all the nitrogen sources, nitrate is usually the predominant form available to phytoplankton in some ecosystems, although ammonium is usually considered to be the preferred N source. Nitrate uptake in phytoplankton is carried out by specific transporter proteins (Galvan and Fernandez, 2001). Nitrate transporters are classified into high-affinity transporter systems (HATS) and low-affinity transporter systems (LATS), depending on their affinity and transporting capacity for nitrate. Two gene families, NRT1 and NRT2, encode nitrate transporter systems (Brian, 2000; Galvan and Fernandez, 2001). NRT1 family encodes LATS, and NRT2 family encodes HATS. With regard to marine phytoplankton, the HATS (NRT2 type) are responsible for nitrate uptake because of the low nitrate concentrations in the marine environment. Nrt2 gene has several advantages that make it more suitable to be a marker of nitrogen status in comparison with other nitrogen utilization genes (Kang et al., 2009). First of all, the expression levels of Nrt2 genes are very sensitive and respond rapidly to the N status. In most of the studied eukaryotic algae, Nrt2 genes are highly expressed under N deficiency conditions, moderately expressed under nitrate sufficient conditions and significantly repressed under the presence of ammonium (Hildebrand and Dahlin, 2000; He et al., 2004; Kang et al., 2007; Song and Ward, 2007; Kang et al., 2009). The minimal and maximal Nrt2 mRNA expression levels can be brought about by ammonium addition and nitrogen deprivation, respectively. The degree of nitrogen deficiency thus can be determined by comparing original Nrt2 transcript levels with the minimal and maximal levels (Kang et al., 2009). Secondly, Nrt2 expression has no significant diel rhythm which facilitates the field sampling with no time restriction (Hildebrand and Dahlin, 2000). Finally, Nrt2 genes contain highly conserved transmembrane domains and
divergent loop domains (Brian, 2000). This structural feature is fit to design degenerate primers and species-specific primers, respectively.

Before a gene or protein can be used in field studies, its pattern of expression must be rigorously studied in relation to appropriate environmental factors under controlled laboratory conditions (La Roche et al., 1998; Scanlan and Wilson, 1999; Lindell and Post, 2001). In the past, the studies mainly focused on the relationship between Nrt2 mRNA expression levels and nitrogen forms. Although the formation of the Nrt2 gene family may reflect an evolutionary adaptation to ever-changing environmental conditions, it does require a thorough examination to identify the expression pattern relation to other nutrient conditions besides nitrogen forms. Phosphate availability can affect the uptake of nitrate in phytoplankton (Liu et al., 2010). As pointed out by Hildebrandt and Dahlin (2000), nitrate uptake may be controlled at the transcriptional level. Few reports studied how phosphate had effect on the transcript of Nrt2 gene. Therefore, in this study, we examined Nrt2 transcript levels in P starvation and different nitrate concentrations. The potential application of Nrt2 genes as indicators of N status in field studies is discussed. Our model organism for this study isSkeletonema costatum (Greville) Cleve, a dominant species in several estuarine systems.

2 Materials and methods

2.1 Algal cultures

Unialgal cultures of the marine diatom Skeletonema costatum (strain SC-C3) was isolated from the Changjiang River (Yangtze River) Estuary and grown in f/2 medium (Guillard, 1975) prepared with sterilized natural seawater. Cultures were maintained at 20°C and illuminated with cool white fluorescent tubes (90 μmol photon m⁻² s⁻¹) during cycles consisting of 12 h of light and 12 h of darkness. Aeration was not provided, but cultures were gently shaken by hand twice a day to increase gas exchange. In consideration of the functional extent of HAT5, S. costatum was cultured in low nitrate concentration (88.3 μmol/L) for at least five generations before experiments.

2.2 Experimental design

Batch cultures at mid-exponential phase were inoculated to 2 L of sterilized seawater in 3 L Erlenmeyer flasks. The initial cell density was on the average 44.25×10³ cells/ml. Two groups of experiments were designed:

(1) P starvation. The initial nitrate concentrations were designed at 88.3 μmol/L (10% f/2). For the P-starved treatment, the initial phosphate concentration was 3.63 μmol/L (10% f/2). The initial phosphate concentration 36.3 μmol/L (f/2) was the P-replete treatment and used as the control. Silicate, trace metals and vitamins were added in accordance with the f/2 medium. Samples were collected at different growth stages to analyze various parameters.

(2) Nitrate concentration series. A 48 h short term experiment was designed to study the effect of different nitrate concentrations on the transcript levels of Nrt2. Nitrate concentrations were 0 μmol/L, 44.15 μmol/L (5% f/2), 88.3 μmol/L (10% f/2) and 176.6 μmol/L (20% f/2). Phosphate, silicate and other nutrients were the same as in f/2 medium. Cultures were sampled at 6, 24 and 48 h after the addition of nitrate. All treatments were triplicate.

2.3 Growth parameters and nutrients

About 10 ml of algae suspension was removed from the culture and fixed with Lugol’s solution for determination of cell densities at 2 h after illumination started every morning. The specific growth rate (μ) was calculated following the equation:

\[
μ = \frac{\ln N_t - \ln N_0}{t_2 - t_1}
\]

where \(N_t\) and \(N_0\) represent cell numbers at \(t_0\) and \(t_2\), respectively. All nutrient samples were measured on a continuous flow analyzer (model: SAN++ system; SKALAR Inc., Netherlands) by colorimetric methods.

2.4 RNA extraction and first strand cDNA synthesis

Algal cells were harvested by centrifugation at 3 500 r/min (2 517 g) for 10 min and the cell pellets were collected in 1.5 ml freezing tubes. The cell pellets were flash frozen in liquid nitrogen immediately and transferred to −80°C and stored at this temperature until RNA extraction. RNA was extracted using an RNAprep pure Plant Kit (TIANGEN CO. LTD, China) according to the manufacturer’s instructions. Concentrations and purity of the extracted RNA were measured using a Nano Drop spectrophotometer (model: ND-1000; Thermo Fisher Scientific Inc., USA). The integrity of RNA was examined by electrophoresis on 1.5% agarose gels containing 0.5× Tris–boric acid–EDTA buffers. cDNA synthesis was carried out with 1 μg of total RNA using PrimeScript Reverse Transcriptase (TaKaRa Inc., Japan) that included an additional DNase I (Promega Inc., USA) treatment for 25 min.

2.5 Primer design

The sequences of 18S rRNA and Nrt2 genes were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). The accession numbers of 18S rRNA and Nrt2 genes are X85395.1 and AF078280.1, respectively. The PCR primer sets for real-time quantitative reverse-transcription polymerase chain reaction (QRT-PCR) were specifically designed with primer premier 5.0 (Premier). Primers were synthesized in Sangon Biotech, Shanghai, China (Table 1).

To verify the validity and specificity of these primers, the PCR products were sequenced (Sino Geno Max Co., Ltd.) and analyzed using National Center for Biotechnology Information (NCBI) Blast server (blastn).

Table 1. Primers for real-time quantitative PCR used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC18sRNF</td>
<td>AGGTCTGTAGATCCCTTAGTGT</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>SC18sRNFr</td>
<td>GTTGAATGACTCGGATATCAGGA</td>
<td></td>
</tr>
<tr>
<td>SCNrf</td>
<td>GAGATGGATGCTTCGTGCAAGG</td>
<td>Nrt2</td>
</tr>
<tr>
<td>SCNtr</td>
<td>TATGGAAACATTACGTAATGATC</td>
<td></td>
</tr>
</tbody>
</table>

2.6 QRT-PCR

QRT-PCR reactions were performed in PCR tube strips in a 20 μl reaction volume using the ABI prism 7300 Sequence Detection System (Applied Biosystems [ABI], USA). The reaction mix contained 10 μl SYBR® Green PCR Master Mix (TaKaRa Inc., Japan), 0.4 μl ROX reference dye (TaKaRa Inc., Japan), 100 ng of template and 0.4 μl of both forward and reverse primers (final primer concentration: 200 nmol/L). The QRT-PCR assays were performed in triplicate reactions for each of the biological triplicate cultures. Dissociation curve analysis of amplification products was performed following the amplification procedure. In all cases, the amplification product for each primer pair yielded one single dissociation peak with the expected melting temperature. This verifies that all primer pairs were gene specific and that there was no non-specific amplification or primer-dimer in
any case.

The results were analyzed using the ABI 7300 System Software (Version 1.3). To compare the expression of Ntr2 gene in different external nutrient conditions, we used the 2^(-ΔΔCT) method (Livak and Schmittgen, 2001), which is used to compare the expression of target gene relative to an endogenous reference gene under various environmental conditions. Here 18S rRNA was chosen as reference gene because its expression was presumed to be unaffected by environmental conditions (Nicot et al., 2005). The relative transcript level of Ntr2 genes with the cultures of 88.3 μmol/L nitrate and 36.3 μmol/L phosphate was used as the calibrator and defined as 1. Detailed calculation progress was as follows: ΔΔCT = ΔCT_target − ΔCT_reference = (CT_target − C(T18S rRNA))sample − (CT_target − C(T18S rRNA)reference, and then the relative amount of Ntr2 in the sample was 2^(-ΔΔCT). Biological triplicate cultures were analyzed separately, whereas triplicate CT values of the same sample were averaged before carrying out the 2^(-ΔΔCT) calculation.

2.7 Statistical analysis

The transcript levels of Ntr2 response to nitrate concentrations and experimental times were examined by Pearson correlation or linear regression analysis as appropriate. P values less than 0.05 were considered statistically significant. All statistical analyses were carried out using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 The amplification efficiencies of Ntr2 and 18S rRNA

The 2^(-ΔΔCT) method is valid only when all reactions have similar amplification efficiencies. In order to test the homogeneity of amplification efficiencies between primer pairs and over a range of template concentrations, standard curves were generated using serial dilutions of template cDNA over a 200-fold. The threshold cycles (CT) of two genes were linearly correlated to the logarithms of cDNA dilution. Slopes of the standard curves were −3.36 for Ntr2 and −3.34 for 18S RNA, respectively, and both were close to the theoretical value of −3.32. The slope of the curve plotted by ΔCT(Ntr2/18S rRNA) versus the logarithms of cDNA dilution was −0.02, which is close to zero. So the amplification efficiencies of Ntr2 and 18S RNA were similar, and the 2^(-ΔΔCT) method for the relative quantification of Ntr2 could be used (Livak and Schmittgen, 2001).

3.2 Effects of P starvation on the transcript levels of Ntr2 gene

3.2.1 Algal growth

When S. costatum was inoculated in P replete condition (NO_3^- = 88.3 μmol/L, PO_4^{3-} = 36.3 μmol/L), the cell density increased exponentially almost immediately. On Day 12, the cell density increased about 15 fold and reached 704.11×10^3 cells/ml. The exponential phase continued for 9 d and reached stationary phase on Day 9 (Fig. 1a). The specific growth rate got its maximum value on Day 1 which was about 0.77 d⁻¹ and declined rapidly to 0.15 d⁻¹ on Day 5. After that, the specific growth rate decreased slowly and was approximately 0 on Day 10 (Fig. 1b).

As for P starved condition (NO_3^- = 88.3 μmol/L, PO_4^{3-} = 3.63 μmol/L), S. costatum grew for 5 d and reached stationary phase on Day 6 (Fig. 1a). The maximum cell density was about 406.29×10^3 cells/ml, which was significantly lower than that in P-replete condition. However, the maximum specific growth rate of 0.71 d⁻¹ appeared on Day 1 (Fig. 1b) was similar to that observed in P replete condition.

3.2.2 Nutrient concentrations

During the batch cultivation of S. costatum, concentrations of nitrate (NO_3^-) and phosphate (PO_4^{3-}) in both treatments declined (Figs. 1c and d). Under P-replete condition, external nitrate provided with 88.3 μmol/L on Day 0 was almost exhausted by Day 4 (<1 μmol/L, Fig. 1c). But the nitrate in the P-starved condition was still 4.44 μmol/L on Day 7 (Fig. 1c). Maximum nitrate uptake rates in both treatments were reached in initial 2 d, which were 139.84×10^-9 μmol/(cell-d) on Day 1 for P-replete treatment and 216.72×10^-9 μmol/(cell-d) on Day 2 for P-starved one (Fig. 1e). By comparison, it can be found that the nitrate uptake rate in P-starved treatment was significantly higher than that in P-replete treatment on Day 2. After that, it decreased sharply to 42.76×10^-9 μmol/(cell-d) on Day 3, which was lower than 108.25×10^-9 μmol in P-replete treatment. This lower status lasted till Day 5 when the nitrate uptake rate in P-replete treatment was close to 0 μmol/(cell-d).

For the same period, the consumption of phosphate got started immediately after the inoculations (Fig. 1d). Maximum phosphate uptake rates appeared on Day 1 for both P-replete [108.84×10^-9 μmol/(cell-d)] and P-starved treatments [19.97×10^-9 μmol/(cell-d)]. The phosphate uptake rate was higher in P-replete treatment than in P-starved one from beginning to end (Fig. 1f). Phosphate in the P-replete medium was 0.29 μmol/L on Day 11 (>0.1 μmol/L), which indicates that phosphate did not limit the growth of S. costatum. However, phosphate in the P-starved medium was almost depleted on Day 2 and remained undetectable from Day 3 to Day 6 (<0.1 μmol/L). The low phosphate concentrations severely limited the algal growth.

3.2.3 Transcriptional responses of Ntr2 genes to P starvation

The transcript levels in P-replete and P-starved conditions are shown in Fig. 1g. For P-replete treatment, the transcript level of Ntr2 decreased from 18.49 fold on Day 1 to 14.98 fold on Day 2. With the depletion of nitrate in the medium, it increased rapidly to 84.51 fold on Day 4. The transcript level kept increasing and reached the highest value on Day 7, which was about 128.44 fold. On the following days, Ntr2 gene transcript levels maintained higher levels at around 120 fold. The variation trend of Ntr2 transcript levels in the P-starved treatment was similar to that in the P-replete treatment. The Ntr2 mRNA abundance declined from Day 1 to Day 2, and then increased slightly on Day 3. The maximum value was 75.67 fold on Day 5. The Ntr2 transcript levels were lower in P-starved treatment than in P-replete treatment all the time.

Correlation analysis indicates that the transcript levels of Ntr2 gene had a significantly negative correlation with nitrate (Pearson correlate, P <0.05, Fig. 2) but not phosphate concentrations (P >0.05). Furthermore, a strong negative linear relationship was found between Ntr2 transcript levels and nitrate concentrations when the nitrate in the medium was below 40 μmol/L.

3.3 Transcriptional responses of Ntr2 gene to various nitrate concentrations in 48 h

From the previous experiment, it can be inferred that Ntr2 gene expression may vary with the nitrate availabili-
Fig. 1. Various parameters of Skeletonema costatum grown in P-replete and P-starved conditions. a. Cell densities, b. growth rates, c. nitrate concentrations, d. phosphate concentrations, e. nitrate uptake rates, f. phosphate uptake rates, and g. transcript levels of Nrt2 gene. The solid circles (●) represent the P-replete treatment, and open circles (○) represent the P-starved treatment. Vertical bars indicate standard deviations.
ty in the medium. To further confirm this speculation, a short-term experiment was conducted with different nitrate concentrations and the influence of growth stages was eliminated. The results are shown in Fig. 3. The nitrate in the medium declined in all treatment groups except for the 0 μmol/L treatment in which the nitrate concentration maintained at 0 μmol/L throughout the experiment. Along with the depletion of nitrate in the medium, Nrt2 gene transcript levels increased in the three levels of N-added (44.15, 88.3 and 176.6 μmol/L) treatments. Among the four treatments, one with no N addition (0 μmol/L) exhibited higher Nrt2 gene transcript levels throughout the experiment, which were about 93.70–99.04 fold of the initial level. In general, the expression levels of Nrt2 in 0, 44.15 and 88.3 μmol/L were all up-regulated. However, the expression level in the 176.6 μmol/L treatment group was suppressed at 6 h with only 0.56 fold of initial level. The expression level in this treatment did rise throughout the rest of the experiment, albeit lower than the other three groups.

Linear regression analysis was adopted to analyze the relationship between nitrate concentrations in the medium and the relative abundances of Nrt2 genes. In accordance with the long-term experiment, a strong negative linear regression was also found between Nrt2 transcript levels and nitrate concentrations below 40 μmol/L (Fig. 4). The regression equation was $y = -3.305x + 98.95$, $R^2 = 0.988$, where $x$ represented nitrate concentrations and $y$ represented the Nrt2 transcript levels. But when the nitrate exceeds 60 μmol/L, the relative abundance of Nrt2 gene was very low and maintained below 7 fold.

4 Discussion

4.1 Nutrient uptake and growth characters of S. costatum

The diatom S. costatum is a dominant spring bloom species in several estuarine systems. These blooms may be related to factors such as turbulence in water column and higher nutrient levels; however, the higher nutrient assimilation rate may be the most important reason for the outbreak of algae blooms. In this study, the maximum nitrate and phosphate uptake rates initially appeared 2 d after nutrients were supplied (Figs 1e and 1f).

The diatom has luxury nutrient absorption phenomenon (Ketchum, 1939). Under N-limited conditions, algal cells tend to absorb more P; under P-limited conditions, algal cells tend to absorb more N (Liu et al., 2010). On the second day, phosphate was used up in P-starvation treatment, and it became P-limited for this treatment. So the cells showed luxury N absorption in this treatment, which lead to higher nitrate uptake rate in P-starved treatment than in P-replete one. The luxury nutrient absorption makes the algae cells store enough nutrients to overcome the external environment changes and get through the nutrient deficiency period (Andersen et al., 1991).

Phosphate uptake rate was higher in P-replete treatment than in P-starved one from beginning to end (Fig. 1f). The phenomenon may be explained from two aspects. Firstly, the algae cells were pre-cultured in the 1/2 phosphate medium before experiment and the cells were not P-deficit. When they were transferred to low phosphate medium, they may release phosphate from intracellular to the medium (Liu et al., 2010), which lead to low phosphate uptake rate. Secondly, in the P-replete treatment, the nitrate was 98.3 μmol/L, phosphate was 36.3 μmol/L. The N/P ratio in this treatment was 2.43, which was lower than the Redfield ratio. Therefore, it was relative N-limited in this treatment. The algae cells have luxury P absorption phenomenon under N-limited conditions as reported by other researchers previously (Liu et al., 2010). Consequently, phosphate uptake rate was higher in P-replete treatment than...
in P-starved one.

Accompanied with the rapid nutrient uptakes was the prosperous growth of S. costatum (Fig. 1a). The maximum growth rates under P-replete and P-starved conditions were not significantly different. But the maximum cell density under P-starved condition was lower than the P limitation.

4.2 Effect of nitrate on the transcript of Nrt2 gene

Higher nutrient uptake rates ought to be due to the effective transport systems. Several nitrate transporter genes have been discovered in marine phytoplankton (Hildebrand and Dahlin, 2000; Armbrust et al., 2004; Kang et al., 2007; Song and Ward, 2007). These genes are quite similar to those of the Nrt2 family that encode high-affinity nitrate transporters in higher plants. In most marine eukaryotic algae studied, Nrt2 transcript levels are repressed in the presence of ammonium and highly expressed under a nitrogen-deprived condition. NO$_3^-$ is an inducer for Nrt2 gene transcripts in a diatom, *Gloeocapsa muelleri*, whether the cells were in a N-sufficient or N-limiting condition (Song and Ward, 2007). In this study, Nrt2 mRNA expression levels were also sensitive to the degree of N stress (Figs 2 and 4). In the P-replete treatment, nitrate in the medium was almost depleted on Day 4 and the algal cells in this treatment were N-limited according to Nelson and Brzezinski (1990). As a response to the N limitation, Nrt2 transcript levels increased by 84.51 fold. Phytoplankton exhibit various responses to stress imposed by nutrient limitation, including down regulation of physiological rates, the synthesis of new proteins and development of more effective uptake systems (La Roche et al., 1999). Thus, the increasing induction of Nrt2 gene transcript in the cells under N limitation can be considered as an adaptive strategy, which enables them to synthesize more nitrate transporters to cope for limited nitrate. It is worth mentioning that although the nitrate in the medium had been depleted on Day 4 (Fig. 1c), Nrt2 gene transcript levels did not present the highest value immediately (Fig. 1g). Since diatoms can accumulate large internal pools (mmol/L) of dissolved NO$_3^-$ (Collos, 1982), the cells were not N-deprived in its true sense although there was no nitrate in the medium. This result demonstrates that molecular characterization of nitrate transport systems may assess the N status of phytoplankton more accurately.

Short-term incubation experiment shows that Nrt2 gene transcript levels are regulated by nitrate concentration in the medium to some extent (Fig. 3). Nrt2 gene transcript levels demonstrated a negative correlation with nitrate concentrations. It may be associated with the synthesis of intracellular inhibitors, such as NO$_3^-$, NH$_4^+$ and glutamine by nitrate assimilation (Galvan and Fernandez, 2001). Higher external nitrate concentration gives rise to higher intracellular nitrate levels to some extent, which can further improve the nitrate reductase activity (Lomas and Gilbert, 2000; Wang et al., 2008). The enhanced nitrate activity can rapidly oxidize nitrite to nitrate and its downstream metabolites, such as ammonium and glutamine. High levels of intracellular reduction forms of nitrate in turn inhibit the expression of Nrt2 gene. Thus, the Nrt2 gene transcripts remained higher under N depletion due to lack of intracellular inhibitors. In addition, the strong negative linear relationship between Nrt2 transcript levels and nitrate concentrations (<40 μmol/L) may be related to the functional extent of HATS. When extracellular nitrate concentrations are below 40 μmol/L, nitrate-uptake kinetics in diatoms initially saturated mediated by HATS (Lomas and Gilbert, 2000; Song and Ward, 2007). Nrt2 transcript levels decrease linearly with the uptake of nitrate in this process. Nitrate uptake at concentrations greater than 60 μmol/L is mediated by LATS. Therefore, the transcript of Nrt2 is depressed greatly, which is consistent with our results.

4.3 Effect of phosphate on the transcript of Nrt2 gene

Phosphorus is an essential mineral macronutrient for biological growth and development. It is an important element of intracellular biological organic molecules, such as nucleic acids, lecithin, and ATP. In this study, although the transcript levels of Nrt2 had no significant correlations with phosphate concentrations in the medium, the levels in P-starved treatment were lower than those in P-replete treatment (Fig. 1g). It may be nutrient-uptake-dependently. In the first two days, nitrate concentrations in the medium were more than 40 μmol/L for both P-replete and P-starved treatments, which were beyond the functional range of HATS. Thus, the nitrate uptake was mediated by LATS and the transcript levels of Nrt2 maintained at lower levels in both treatments. Furthermore, P-starved cultures showed lower Nrt2 transcript than P-replete. This may due to higher intracellular nitrate content in P-starved treatment. When nitrate is the sole nitrogen source in the medium, Nrt2 mRNA levels should be related to the overall nitrogen needs of the cell. Intracellular N status plays an important role in the expression of Nrt2. Higher nitrate assimilation under P-starved (Fig. 1e) raised the intracellular N content and then depressed the Nrt2 transcript levels. In addition, higher phosphate uptake rates in P-replete treatment provided sufficient phosphate to synthesize nucleoside, which was essential for Nrt2 mRNA synthesizing. From Day 3, when the nitrate in the medium fell into the functional range of HATS, the transcript levels of Nrt2 had a significant negative linear correlation with nitrate concentrations in the medium (Fig. 2). Higher nitrate concentration in the P-starved treatment resulted in lower Nrt2 transcript levels.

In conclusion, under these experimental conditions the expression levels of Nrt2 responded uniquely to the supply of nitrate but not phosphate in medium. Higher transcript levels of Nrt2 gene imply nitrate limitation, whereas lower transcript levels indicate sufficient nitrate. Nrt2 transcript levels may be a good indicator of nitrogen status of species of interest if nitrate

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**Fig. 4.** The linear relationship between Nrt2 transcript levels and nitrate concentrations within a 48 h period of cultivation of *Skeletomena costatum*. Spots in the ellipse are not included in the regression analysis. The linear regression equation is $y = -3.305x + 98.95$, $R^2 = 0.988$, where $x$ represents nitrate concentrations (<40 μmol/L) and $y$ represents the Nrt2 transcript levels.
is the sole nitrogen source. However, in practice, there are many other forms of nitrogen that can be utilized by phytoplankton in the marine. Among these nitrogen forms, the effects of NO$_3$—N and NH$_4$—N on Ntr2 transcript levels are relatively thorough stu-
died (Bruehl et al., 2010; Song and Ward, 2007; Hildebrand and Dahlin, 2000). But the effects of other forms of nitrogen, espe-
cially the organic nitrogen, remain unknown and need to be ex-
plored in the future. Moreover, the interpretation of a measured transcript level is also intricate. Kang et al. (2009) established the minimal and maximal transcript levels for Ntr2 genes to de-
tect nitrogen deficiency in the marine phytoplankton Isochry-
sis galbana and Thalassiosira pseudonana. The N-status of cul-
tures can be well estimated if the original transcript levels are on the verge of threshold values. But for the values falling be-
tween the minimum and maximum, they must be interpret-
ced with caution because there is more than one possibility. To avoid ambiguous conclusions, other nitrogen markers that are induced by various nitrogen forms specifically need to be mon-
tored simultaneously.

5 Conclusions

HATS play important roles in nitrate uptake in marine phytoplankton. The Ntr2 genes encoding HATS respond sensi-
tively to various forms of nitrogen in the extracellular environ-
ment. Our experimental data demonstrate that when nitrate is the sole nitrogen source, the Ntr2 gene transcript level depends on the overall nitrate needs of the algae cells and it has a linear relationship within the functional extent of HATS. The effects of phosphate on the Ntr2 gene transcript are produced not only by impacting nitrate uptake indirectly but also by the synthesis of mRNA directly. Ntr2 gene has tremendous potential to be a good indicator of nitrogen status of phytoplankton.

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