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Molecular characterization of nitrate reductase gene and its expression in the marine red alga *Gracilaria tenuistipitata* (Rhodophyta)

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Abstract The enzyme nitrate reductase (NR) responsible for the conversion of nitrate to nitrite is considered to be the rate-limiting step in nitrogen assimilation. The economically important marine macroalga *Gracilaria tenuistipitata* presents a circadian oscillation in NR protein content and activity. In order to identify if the regulation of NR in *G. tenuistipitata* happens at transcriptional levels, the NR cDNA and gene were sequenced and the NR mRNA expression was studied. Analysis of the sequenced gene revealed absence of introns which is unusual for NR genes. The transcriptional profiling revealed a circadian rhythm for NR; furthermore, a rhythm was observed in constant light condition, suggesting a possible regulation by the biological clock at the mRNA levels for NR in *G. tenuistipitata*.

Keywords Biological rhythm · Phylogeny · Gene expression · *Gracilaria tenuistipitata* · Nitrate reductase

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Introduction

The major source of nitrogen in the marine ecosystem is in the form of nitrate (Adams and Mortenson 1985), which must be reduced to ammonia or amine to be assimilated into the biosynthesis of nitrogen-containing compounds such as proteins and nucleic acids (Crawford and Campbell 1990; Solomonson and Barber 1990). The reduction of nitrate to nitrite is the first step in the reduction to ammonia; it is catalyzed by a NAD(P)H-dependent enzyme named nitrate reductase (NR; EC 1.6.6.1), and it is considered to be rate-limiting in the nitrate assimilation process (Lopes et al. 1997; Ramalho et al. 1995).

Nitrate reductase is a high-molecular-weight complex protein with three prosthetic groups, FAD, heme (cytochrome b_{557}), and Mo-pterin, which uses NADH or NADPH as the electron donor (Caboche and Rouzé 1990; Crawford 1995), and it is subject to tight controls at the levels of enzyme activity, synthesis, and degradation (Lillo et al. 2001). NR protein has been purified from several organisms, and it occurs in a wide variety of molecular weights as well as number of subunits. NR size ranges from 200 kDa in spinach, with two subunits (Hewitt 1975), to 500 kDa in the green alga *Ankistrodesmus braunii*, with eight subunits of 58 kDa (De la Rosa et al. 1981). In response to diurnal changes in photosynthesis, NR expression and activity vary between day and night (Stitt et al. 2002). Analysis of NR genes in plants showed that their expressions were primarily regulated at the transcriptional level (Campbell 1999). The expression of NR is a complex process regulated at the transcriptional level by different factors such as nitrate, CO_2, light, and

carbon skeletons. During a diurnal cycle, the NR mRNA level usually peaks at the end of the night or in the early part of the day, then declines and starts to increase toward the end of the night (Scheible et al. 1997; Geiger et al. 1998). NR mRNA circadian oscillations have been demonstrated for the plants *Zea mays* (Lillo and Ruoff 1989), *Nicotiana plumbaginifolia* (Deng et al. 1990), *Arabidopsis thaliana* (McClung and Kay 1994), and *Lycopersicon esculentum* (Galangau et al. 1988; Jones et al. 1998).

NR genes have been cloned from plants, fungi, and algae and their evolutionary relationships investigated (Stolz and Basu 2002). They have substantial similarity, particularly within functional domains, but present large variation in GC content at the third codon position and in the number of introns. NR genes from green algae have 18 introns in *Chlorella vulgaris* (Dawson et al. 1996), 15 in *Chlamydomonas reinhardtii* (Merchant et al. 2007), ten in *Volvox carteri* (Gruber et al. 1992), and also *Dunaliella tertiolecta* showed two introns in a partial region of the gene cloned (Song and Ward 2004). The intron positions are different between fungi and plants, but conserved within these groups (Zhou and Kleinhofs 1996).

The marine red alga *Gracilaria tenuistipitata* var. *liui* Zhang et Xia is economically important, being used for the production of agar, a phycocolloid which has many uses in the food, pharmaceutical, and biotechnology industries (Cardozo et al. 2007; Chiang and Lin 1989; Oliveira and Alveal 1990). This strain is extensively cultivated in ponds in southern China and Taiwan and exhibits a wide tolerance to environmental factors with a high growth rate and high agar yield, being very suitable for cultivation (Collén et al. 2003; Lee et al. 1999; Oliveira and Alveal 1990). The growth rate, quantity, and quality of agar extracted are limited by the availability and absorption rate of nitrogen, this being the major nutrient limiting macroalgal production (Macchiavello et al. 1999). *Gracilaria tenuistipitata* is obtained from mariculture in the sea, and therefore, the characterization of nutrition, growth, and regulation of the nitrogen-assimilating process of this alga is needed. Nitrate reductase from *G. tenuistipitata* showed to be NADH-specific with a molecular weight of 440 kDa (four subunits of 110 kDa); furthermore, a circadian variation was observed for NR activity and protein levels (Lopes et al. 1997, 2002). Nitrate reductase was also studied at protein levels in *Gracilaria chilensis* (Chow Ho et al. 2004; Chow Ho and Oliveira 2008). Although NR has been identified from several organisms, knowledge of NR at the molecular level in red algae is less detailed (Granbom et al. 2007), especially regarding mRNA expression levels. In this paper, we describe the sequence of NR cDNA and gene of *G. tenuistipitata* and the daily rhythm of NR mRNA.

Materials and methods

The tetrasporophytic phase of *G. tenuistipitata* var. *liui* Zhang et Xia was cultivated in seawater with a salinity of 20‰ enriched with 100% Von Stosch medium (Edwards 1970) and changed weekly under alternating periods of 12-h light (cool white fluorescent; 90 μmol photons m^{-2} s^{-1}) and 12-h dark (LD 12:12). The cultures were maintained at 20°C under constant air bubbling. The algae collected for RNA extractions were rapidly rinsed in freshwater, blotted dry, frozen in liquid N_2, and stored at −80°C. In the circadian rhythm assay, the algae were kept in a photoperiod of LD 12:12 (90 μmol photons m^{-2} s^{-1}) for 48 h, and then when the light went on starting the third cycle, the culture was transferred to a continuous light environment with 45 μmol photons m^{-2} s^{-1} for 36 h (the total light intensity during the 24 h was kept the same), and the first sample was just collected after 5 h from what would be the "dark period". Samples were collected every 4 h and in triplicate. The material utilized in our experiments is derived from a tetrasporophytic branch of a specimen collected in Haikou, Hainan Island, China, by E.C. Oliveira in 1993. This material has been kept in unialgal culture in the *Gracilaria* germoplasm bank at Institute of Biosciences of University of São Paulo (Lourenço and Vieira 2004). All the experiments were performed with apical fragments (approximately 1.5 cm).

Nitrate reductase cDNA and gene sequencing

The NR clones were identified and annotated from the *G. tenuistipitata* cDNA library (Pi et al., Inst. of Biosciences, Univ. of São Paulo, São Paulo, Brazil, personal communication). The phagemids were isolated from the bacteria using alkaline lysis minipreps. The concentration and presence of the insert was checked by electrophoresis on agarose gels. Sequencing of the 5′ end of cDNA clones was performed by single run using T3 primer and of the 3′ end was performed by single run using T7 primer (Gibco BRL). Purified phagemid DNA was sequenced using the Applied Biosystems (ABI) PRISM big dye terminator kit and an ABI 310 genetic analyzer. Using the primer walking technique, primers were constructed to obtain the whole NR cDNA sequence (Table 1). The NR gene sequence was obtained using as a template the product resultant from the PCR amplified with primers designed for start and stop codons (Table 1) from the total DNA extracted from *G. tenuistipitata* following a protocol described by Bellorin et al. (2002). The sequences were aligned and confirmed by BLASTX (Altschul et al. 1997).

Table 1 Primers for *G. tenuistipitata* NR gene sequencing

Primer	Sequence (5′ to 3′)	Orientation	Localization[a]
NR01	GCG.GCT.TGG.TCA.ACA	F→	469–483
NR02	GCC.ACT.ACT.ATA.CTC.CCA.TCC	R←	2,572–2,592
NR03	TCC.GAA.GTC.ATC.CGC.TC	F→	1,875–1,891
NR04	GGG.TCA.AGA.ACT.CCA.GC	R←	1,464–1,481
NR05	AGG.TTT.GGT.CGG.CGG	R←	1,519–1,533
NR06	TAT.CCT.TTG.GTT.GCG	R←	1,367–1,381
NR07	GCC.AAT.GGA.AAT.AGA.ATG.C	R←	453–471
NR08	GCT.CGG.CGT.CCA.CAT	R←	979–993
NR09	AAG.CCG.TCG.GAG.AAT.TAC.T	F→	927–945
NR10	TCA.AGC.CAC.TTC.TTC.GG	R←	1,975–1,991
NR11	AGA.AAG.GGC.GTT.TCA.TTG	F→	2,318–2,335
NR12	AGT.GGT.TGA.AGG.TAA.GGT.G	F→	1,662–1,680
NR13	CAG.CGT.GAA.GGT.CCC.AAG	R←	2,167–2,184
NR START	GGT.GAT.GAT.TTC.CGC.CAG	F→	8–25
NR STOP	GTG.AAA.ACC.ATG.ACG.AAA.CG	R←	2,770–2,789

[a] Position in the nucleotide sequence of the NR gene from *G. tenuistipitata*

Phylogenetic analyses

The amino acid sequence obtained from the in silico translation of NR gene from *G. tenuistipitata* was aligned and compared with other organisms' sequences, the red algae (*Porphyra* sp., sequence given by Y. Nakamura, Institute of Biological Sciences, University of Tsukuba, Japan) and *Cyanidioschyzon merolae*, green algae, plants, diatoms, and ascomycetes obtained from the GenBank. The alignment was performed using the ClustalW program in BioEdit (Hall 1999). From an initial alignment including the complete sequence of the proteins, divergent regions that could be aligned with ambiguity and indels were removed, generating a matrix with 18 sequences and 686 positions. From this matrix of homologous amino acid sequences, the trees were generated through different methods of phylogenetic reconstruction. All the phylogenetic analyses were carried in the PAUP 4.0b8 software (Swofford 2000). The trees were inferred using two distinct methods: distance and maximum parsimony. For the distance method was constructed a neighbor-joining (NJ) tree (Saitou and Nei 1987) with the substitution model of Tamura and Nei (1993). The maximum parsimony tree was inferred by heuristic search and by the stepwise addition algorithm, with addition of random sequences (10 replicates) and rearranged by the branch-swapping algorithm: tree bisection–reconnection. Bootstrap analyses (Felsenstein 1985) were carried out with 2,000 replicates for the methods described above. For all the analyses, the bootstrap values were considered low below 70%, moderate from 71% to 90%, and high above 90%. For the Bayesian analysis, we have performed two runs of four Markov chains over 4,000,000 generations sampling every 100 generations, with prior for the amino acid model to be mixed prior to analysis using MrBayes v.3.0 beta 4 (Huelsenbeck and Ronquist 2001).

RNA extraction and semiquantitative PCR

The algae were harvested, blotted dry and ground in liquid nitrogen, suspended in 6.5 M guanidine hydrochloride buffer followed by the TRIZOL protocol (Invitrogen), and at the homogenization step was also added 0.2 M KOAc to remove the polysaccharides (Falcão et al. 2008). The RNA concentration and quality was determined spectrophotometrically. The samples were treated with DNase prior to cDNA synthesis.

Samples were harvested every 4 h for 48 h of LD 12:12 or 36 h of continuous light, and the total RNA was extracted. Reverse transcription (RT) was performed for each sample (5 μg total RNA) with 1 μM NR-specific primer (NR04, Table 1); the first-strand cDNA was synthesized using the RT kit Improm-II™. The reaction stayed for 5 min at 25°C for primer annealing, 1 h at 42°C for cDNA synthesis, and 15 min at 70°C for inactivation of the enzyme. The amplification was performed with 0.5 μM of each primer (NR04 and NR09), and the reaction was stopped at intervals of five cycles. The amplified products were determined by the intensity of the bands analyzed with Imagequant (Molecular Dynamics, Sunnyvale, CA) on a 1.5% agarose gel. 18S RNA levels were assayed to determine equal loading. The SSU rRNA was amplified with the universal primers 18S5′ (5′-CAA.CCT.GGT.TGA.TCC.TGC.CAG.T-3′) and 536R (5′-GAA.TTA.CCG.CGG.CTG.CTG-3′) with the same PCR conditions. The relation between NR amplifications and SSU rRNA was performed in triplicates with three different cycles at exponential rate of product synthesis; both products have approximately 500 bp. Similar results were obtained in

three independent experiments. A control for DNA contamination was carried out using the total RNA extracted directly in the PCR, and no product was observed for any extraction.

Results

The EST library of *G. tenuistipitata* contained three clones annotated as NR (Pi et al. Inst. of Biosciences, Univ. of São Paulo, São Paulo, Brazil, personal communication). These clones were re-sequenced, one was a heme domain and one contained only part of the NR cDNA, and the larger clone, with 2932 bp, encoded an open reading frame (ORF) of 910 amino acids from 12 to 2,744 bp (Fig. 1). This sequence was deposited at the Genbank with accession number GQ866117. BLASTX of this ORF against the GenBank showed 49% identity (66% similarity) to NRs from *Nicotiana benthamiana*, *N.*

Fig. 1 Gene coding sequence (NR-nt) and inferred amino acid sequence (NR-aa) of the nitrate reductase of *G. tenuistipitata*

NR-nt	ATGATTTCCG	CCAGTCTCAG	CAGTTGGGAC	CCCAACAAGA	AGCCCGCACA	ATCGTCGTTA
NR-aa	-M--I--S--	A--S--L--S	--S--W--D-	-P--N--K--	K--P--A--Q	--S--S--L-
NR-nt	AAGAACACTC	CGGAAGCTAC	ACCCGAGTCC	TCTCTCCGGG	CCGGCCAGTT	TTACAAGAAG
NR-aa	-K--N--T--	P--E--A--T	--P--E--S-	-S--L--R--	A--G--Q--F	--Y--K--K-
NR-nt	ACGAAGAACA	ACCCCTCCAC	CGAGTTGCAC	CACGCAAAGG	CCGTCGCGTG	GACTCAATTC
NR-aa	-T--K--N--	N--P--S--T	--E--L--H-	-H--A--K--	A--V--A--W	--T--Q--F-
NR-nt	AAAAACGACC	TTGAAGCCGT	GTTCAACACT	CGACACCAGC	GTCTCGCCAA	GAACCACCAA
NR-aa	-K--N--D--	L--E--A--V	--F--N--T-	-R--H--Q--	R--L--A--K	--N--H--Q-
NR-nt	TGTACCGAAG	TCGACGTGCG	CGACAAAAAC	AGCCCGGACG	ATTGGATTCC	ACGCCATCCC
NR-aa	-C--T--E--	V--D--V--R	--D--K--N-	-S--P--D--	D--W--I--P	--R--H--P-
NR-nt	GACCTCGTTC	GCCTCACCGG	TAAACACCCG	TTTAACTGCG	AGCCGCCTAT	CGGTCAACTG
NR-aa	-D--L--V--	R--L--T--G	--K--H--P-	-F--N--C--	E--P--P--I	--G--Q--L-
NR-nt	TTGGACCAAG	GCTTCTTCAC	CCCTATTTCG	CTGCATTATG	TTCGAAATCA	TGGAAAAGCG
NR-aa	-L--D--Q--	G--F--F--T	--P--I--S-	-L--H--Y--	V--R--N--H	--G--K--A-
NR-nt	CCCAAGCTCG	ACTGGCACTC	GCATTCTATT	TCCATTGGCG	GCTTGGTCAA	CAAGCCGCTC
NR-aa	-P--K--L--	D--W--H--S	--H--S--I-	-S--I--G--	G--L--V--N	--K--P--L-
NR-nt	ACACTAAGCA	TGGAGGAATT	GGTACAACTT	CCAAGCGTCA	CTTTGCCCGT	CACGCTAGTT
NR-aa	-T--L--S--	M--E--E--L	--V--Q--L-	-P--S--V--	T--L--P--V	--T--L--V-
NR-nt	TGTGCGGGAA	ACAGACGCAA	AGAAGAGAAT	ATGGTCAAGC	AGACCATAGG	ATTCAGCTGG
NR-aa	-C--A--G--	N--R--R--K	--E--E--N-	-M--V--K--	Q--T--I--G	--F--S--W-
NR-nt	GGCTGTGCCG	CACATGCTTG	CAACTTGTGG	ACAGGCGTCA	GACTTTCTCA	CCTTTGGAA
NR-aa	-G--C--A--	A--H--A--C	--N--L--W-	-T--G--V--	R--L--S--H	--L--L--E-
NR-nt	CTGGCCGGTA	TCGACAAGAC	CCAAGCTCGG	CACGTGTGCT	TTTCAGGCGT	GGCAAAAGAG
NR-aa	-L--A--G--	I--D--K--T	--Q--A--R-	-H--V--C--	F--S--G--V	--A--K--E-
NR-nt	GGACTGCCGA	ATGGCACTTA	CGGCACTTCA	ATAGACATCG	CAACCGCCCT	CGATCCGTAC
NR-aa	-G--L--P--	N--G--T--Y	--G--T--S-	-I--D--I--	A--T--A--L	--D--P--Y-
NR-nt	GGAGAAGTTC	TCATCGCCTA	TGAACAGAAT	CATACTAAAC	TTCATCCCGA	CCATGGCTTC
NR-aa	-G--E--V--	L--I--A--Y	--E--Q--N-	-H--T--K--	L--H--P--D	--H--G--F-
NR-nt	CCTGTGCGCG	TGGTCATTCC	TGGTTGGATA	GGCGGTAGAA	TGGTGAAATG	GTTAGATTCC
NR-aa	-P--V--R--	V--V--I--P	--G--W--I-	-G--G--R--	M--V--K--W	--L--D--S-
NR-nt	GTTCTGGTGA	CTGATAAGCC	GTCGGAGAAT	TACTACCATT	ACTTTGACAA	CAGGATTCTA
NR-aa	-V--L--V--	T--D--K--P	--S--E--N-	-Y--Y--H--	Y--F--D--N	--R--I--L-
NR-nt	CCACCGCATG	TGGACGCCGA	GCTGGCCAAG	TCCGAAGGTT	GGTGGTACAA	GCCGGAATAC
NR-aa	-P--P--H--	V--D--A--E	--L--A--K-	-S--E--G--	W--W--Y--K	--P--E--Y-
NR-nt	CTCTTCAACC	AGCTCAACAT	CAATTCTGCC	ATTGTTTACC	CGGCGAACGG	CGAGCGTCTG
NR-aa	-L--F--N--	Q--L--N--I	--N--S--A-	-I--V--Y--	P--A--N--G	--E--R--L-
NR-nt	CAGCTCACTG	GCGCTGGTGT	ATACACCATT	AAGGGCTATG	CGTACTCCGG	TGGTGGGCGC
NR-aa	-Q--L--T--	G--A--G--V	--Y--T--I-	-K--G--Y--	A--Y--S--G	--G--G--R-
NR-nt	AAAGTTACTC	GTGTGGAGAT	ATCTCTGGAT	GGCGGAAAGA	CATGGCAACT	CTGCAAATTG
NR-aa	-K--V--T--	R--V--E--I	--S--L--D-	-G--G--K--	T--W--Q--L	--C--K--L-
NR-nt	GACTATCCGG	AAGAACGTTA	CAGTCATGCC	CCCAAGTTTG	GCAGGTACTA	CTGCTGGATG
NR-aa	-D--Y--P--	E--E--R--Y	--S--H--A-	-P--K--F--	G--R--Y--Y	--C--W--M-
NR-nt	TTTTGGGAGT	ACACCATTGA	CAATTTCGTG	TTTCTCAACG	TTGCTGCGGG	CGCCGGGGAG
NR-aa	-F--W--E--	Y--T--I--D	--N--F--V-	-F--L--N--	V--A--A--G	--A--G--E-
NR-nt	CTTCGATGCC	GTGCCTGGGA	TGAGGCCAGT	AACACGCAAC	CAAAGGATAT	TACATGGAAT
NR-aa	-L--R--C--	R--A--W--D	--E--A--S-	-N--T--Q--	P--K--D--I	--T--W--N-
NR-nt	CTTATGGGCA	TGGGGAATAA	CTGTCACTTC	ACAGTCAAAG	TCATTCCAAA	GCAAATATCA
NR-aa	-L--M--G--	M--G--N--N	--C--H--F-	-T--V--K--	V--I--P--K	--Q--I--S-
NR-nt	GGTGGCTTTG	CGCTGGAGTT	CTTGCACCCA	ACGGTGCCTG	GTCCGGCGAG	TGGGGGCTGG
NR-aa	-G--G--F--	A--L--E--F	--L--H--P-	-T--V--P--	G--P--A--S	--G--G--W-
NR-nt	ATGCTGCCGC	CGACCAAACC	TGTACCGAAT	GGGGTTGCGA	GCAGTGCTCC	GCCAGTCAAA
NR-aa	-M--L--P--	P--T--K--P	--V--P--N-	-G--V--A--	S--S--A--P	--P--V--K-
NR-nt	TCGGGAAGCG	CGCCATCATT	GTCCACTATG	ATCAAGTCTT	TCACAATGAA	GGACGTGGAG
NR-aa	-S--G--S--	A--P--S--L	--S--T--M-	-I--K--S--	F--T--M--K	--D--V--E-
NR-nt	AAGCAAAACT	CCGAGGACTC	CGCTTGGATA	GTGGTTGAAG	GTAAGGTGTA	TGACGCCACG
NR-aa	-K--Q--N--	S--E--D--S	--A--W--I-	-V--V--E--	G--K--V--Y	--D--A--T-
NR-nt	CCATATCTGG	AGGATCATCC	GGGAGGCAAG	GCATCCATTC	TCATGAATGC	TGGCCAGGAT

Fig. 1 (continued)

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NR-aa   -P--Y--L-- E--D--H--P --G--G--K- -A--S--I-- L--M--N--A --G--Q--D-
NR-nt   GCAACTGAAG AGTTCTTGGC AATTCATTCG GACAAAGCGA AGAAAATGCT AGAGGATTAT
NR-aa   -A--T--E-- E--F--L--A --I--H--S- -D--K--A-- K--K--M--L --E--D--Y-
NR-nt   TACATCGGTG AACTTGTAGC AGAAAAGACA ACTGCCAACG GTACTTCTCA TGCTATACAT
NR-aa   -Y--I--G-- E--L--V--A --E--K--T- -T--A--N-- G--T--S--H --A--I--H-
NR-nt   ATTTCGAAGT CATCCGCTCA GCTGATGAGG GATGATCTTC CGAATCAAAA CGTCGACACT
NR-aa   -I--S--K-- S--S--A--Q --L--M--R- -D--D--L-- P--N--Q--N --V--D--T-
NR-nt   ATGGATAAGA GCACACATCG CACTGGTCTT GTCGCTCTAA ACCCGAAGAA GTGGCTTGAG
NR-aa   -M--D--K-- S--T--H--R --T--G--L- -V--A--L-- N--P--K--K --W--L--E-
NR-nt   TTTGAACTTA TCGAGAAAAA AGAAGTTTCC CACGATACCA GACTATTCAA GTTCAAGTTG
NR-aa   -F--E--L-- I--E--K--K --E--V--S- -H--D--T-- R--L--F--K --F--K--L-
NR-nt   CCCACACCTG AGCATTGTCT TGGTCTTCCG GTTGGATACC ACATGTTCGT CAAATCTGTG
NR-aa   -P--T--P-- E--H--C--L --G--L--P- -V--G--Y-- H--M--F--V --K--S--V-
NR-nt   ATTGATGAAA ATCTTGTGAT GCGTGCGTAC ACACCTGTGT CCTCTGACGA CGATCTTGGG
NR-aa   -I--D--E-- N--L--V--M --R--A--Y- -T--P--V-- S--S--D--D --D--L--G-
NR-nt   ACCTTCACGC TGTGCATCAA GGTGTACTTT GCAGGGGTGC ACCCGAAATT CCCCGAGGGA
NR-aa   -T--F--T-- L--C--I--K --V--Y--F- -A--G--V-- H--P--K--F --P--E--G-
NR-nt   GGCAAAATGT CGCAACATAT GGAGGGAATG GAGATTGGTG ACATGCTCAA GGTGAAAGGC
NR-aa   -G--K--M-- S--Q--H--M --E--G--M- -E--I--G-- D--M--L--K --V--K--G-
NR-nt   CCTCTTGGTC ACTTTGAGTA CTTGGAGAAA GGGCGTTTCA TTGTGAAAGA TGTGGAAAGA
NR-aa   -P--L--G-- H--F--E--Y --L--E--K- -G--R--F-- I--V--K--D --V--E--R-
NR-nt   AAGGCGTCGA AGATCGGCCT TATTTGCGGG GGCACTGGAC TTACGCCCGC GTTCCAGGTG
NR-aa   -K--A--S-- K--I--G--L --I--C--G- -G--T--G-- L--T--P--A --F--Q--V-
NR-nt   ATGAAGGCTG TGTACAAGGA TCCAGAAGAT TACACGGAAA TATTCCTGTT GTATGCCAAC
NR-aa   -M--K--A-- V--Y--K--D --P--E--D- -Y--T--E-- I--F--L--L --Y--A--N-
NR-nt   CGAACGGAGC AGGACATTTT GATGCGCGAA GAACTGGAAA AGATGGCCGC GGAACGTGAA
NR-aa   -R--T--E-- Q--D--I--L --M--R--E- -E--L--E-- K--M--A--A --E--R--E-
NR-nt   AACATTCATG TTTGGTATAC ACTTGACAAA CCGGGTGACG GATGGGAGTA TAGTAGTGGC
NR-aa   -N--I--H-- V--W--Y--T --L--D--K- -P--G--D-- G--W--E--Y --S--S--G-
NR-nt   TTCATAAGCG AAGAGATGAT CCGCAGTCAT ATTCCTGCCC CTGGGGATGA TTGCTTCGTC
NR-aa   -F--I--S-- E--E--M--I --R--S--H- -I--P--A-- P--G--D--D --C--F--V-
NR-nt   GGAATGTGCG GTCCGCCACC TATGATCAAC TTTGCATGTA TCCCGAACCT CGAGAGGATC
NR-aa   -G--M--C-- G--P--P--P --M--I--N- -F--A--C-- I--P--N--L --E--R--I-
NR-nt   GGCTTCGAAG CCGACCACTA TATGCAGTTC TGA
NR-aa   -G--F--E-- A--D--H--Y --M--Q--F- -*.
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tomentosiformis, and *N. tabacum*. The three typical prosthetic groups found in NR protein were localized: MoCo 88–474 bp, Cyt-b5 533–606 bp, FAD 660–776 bp, and NAD 786–894 bp. The NR gene from *G. tenuistipitata* was fully PCR-amplified from total DNA and sequenced; the accession number in the Genbank is GQ866118. This NR gene does not present introns, being perfectly aligned to the cDNA sequence, presenting a G+C content of 50%.

Phylogenetic analysis for NR sequences showed that *G. tenuistipitata* NR was more closely related to the other rhodophyte sequences from *Porphyra* sp. and *C. merolae* (Fig. 2). The green algae (Chlorophyta), as well as land plants (Embryophyta), with the bryophyte *Physcomitrella patens* as a basal group, and the diatoms each formed monophyletic groupings with strong bootstrap support.

The effect of daily cycle on NR expression was tested with the algae growing on a 12:12-h light/dark cycle. The NR transcript was maintained at basal level in the light phase, but the levels of NR mRNA increased up to three times after 9 h of darkness. These results suggest a transcriptional regulation of NR gene in *G. tenuistipitata* (Fig. 2), with a maximum of expression during the last hours of the dark period. To verify if the observed rhythm was controlled by light, the alga was transferred to constant light conditions with 45 m^{-2} s^{-1} for 36 h.

The NR mRNA variation observed in light/dark cycle showed an interval of maximum expression of approximately 24 h time (−3 and 21 h, Fig. 3), supporting that NR expression has a circadian rhythm. This same 24-h interval was also observed for the short study of continuous light condition (time 5 and 29 h, Fig. 3), although the intensity of maximum expression is not significantly different, probably because the amount of irradiance in the period of 24 h was kept the same. However, there is a gap of 8 h between the maximum of NR expression observed in the regular 12: 12-h light/dark cycle and the constant light condition.

Discussion

Gracilaria tenuistipitata NR amino acid sequence is more similar to other red algae (Fig. 2), and unlike all the chlorophyte NR genes, it does not present any introns within the gene sequence. This is the first published description of a complete NR gene from a red macroalga (Rhodophyta) and also the first description of a NR gene without introns. NR genes described in the literature until

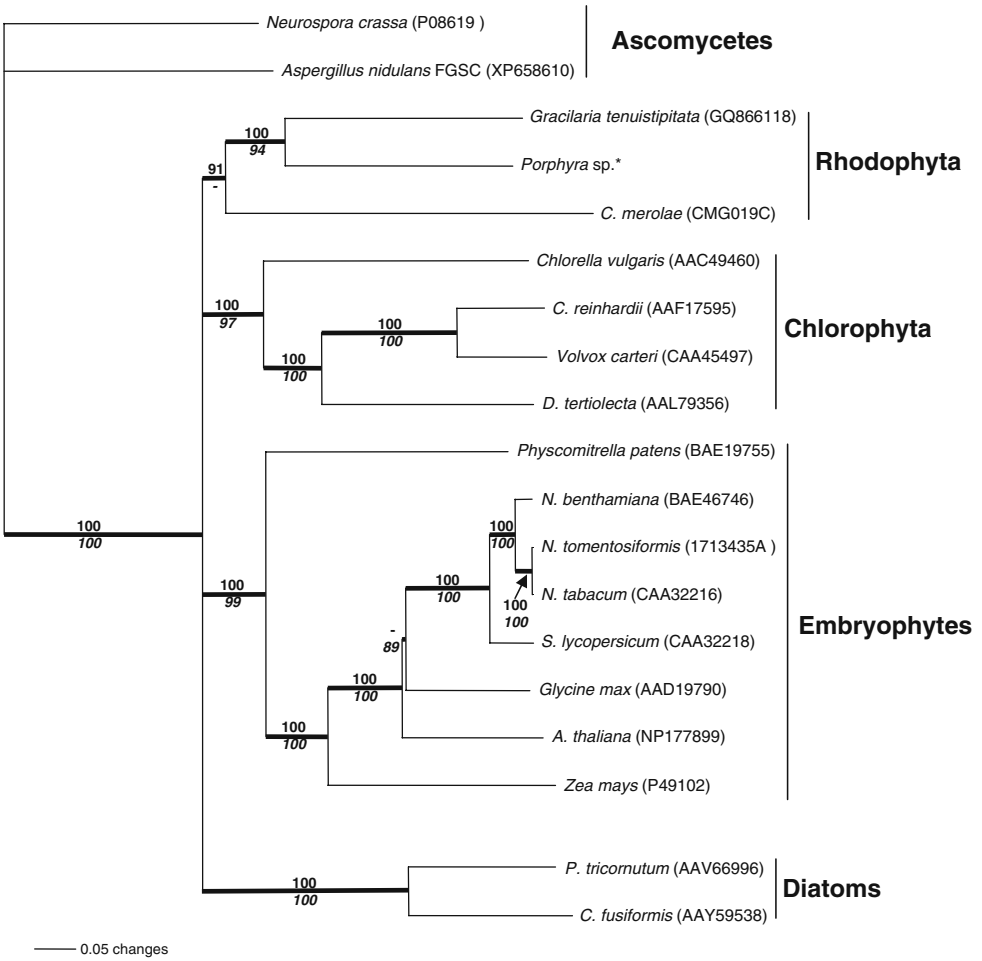

Fig. 2 Neighbor-joining (*NJ*) tree using the NR amino acids sequences matrix with 686 positions. The bootstrap values refer to 2,000 replicates and are indicated for NJ *over the branch* and for maximum parsimony (*MP*) *under the branch in italics*. The posterior probabilities (>99%) inferred from Bayesian analysis are shown as *thicker branches*. Species names are followed by GenBank accession number in brackets, except *C. merolae* that is at http://www.ddbj.nig.ac.jp/; *C. reinhardtii*; *D. tertiolecta*; *Nicotiana*; *S. lycopersicum* (*Solanum lycopersicum*); *A. thaliana*; *P. tricornutum* (*Phaeodactylum tricornutum*); *Cylindrotheca fusiformis*. **Porphyra* sp. (supplied by Y. Nakamura)

now always presented variable numbers of introns (Zhou and Kleinhofs 1996, Table 2). NRs from chlorophytes have a larger number of introns than other eukaryotes (10 to 18 in green algae vs. 1 to 3 in plants or 1 to 6 in fungi, Table 2). The higher number of introns was associated with posttranscriptional processing (Gruber et al. 1996). Introns in NR are conserved within the taxonomical groups and have been used for phylogenetic studies of closely related plant species (Howarth and Baum 2002). Although NR sequences are not utilized as molecular markers for

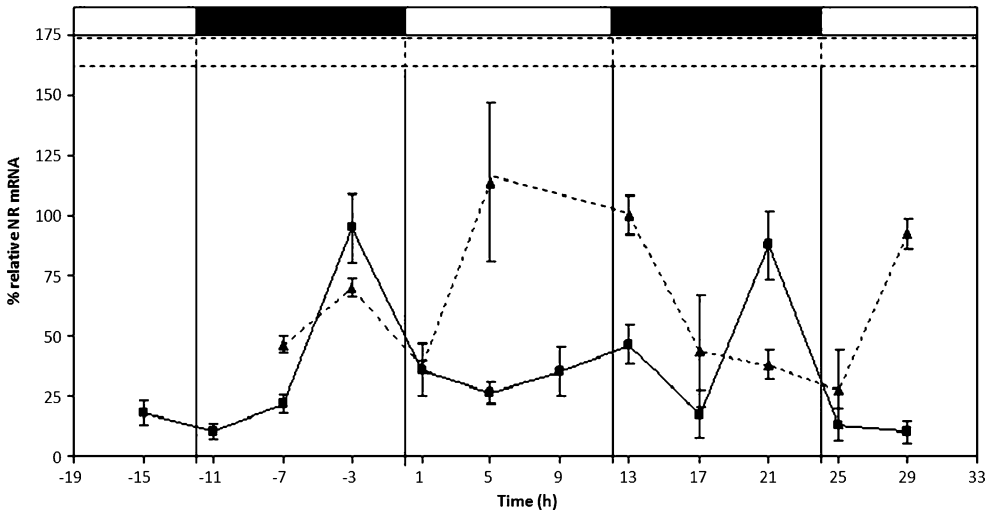

Fig. 3 Nitrate reductase mRNA expression of *G. tenuistipitata* for a period of 48 h in constant light (*triangle*) and in a regular light/dark cycle (*square*). The light period is represented by the *white bars*, the dark period by the *black bars*, and constant light by the *dotted white bars*

Table 2 Nitrate reductase coding region sequence, protein size, number of introns, and Genbank accession number of NR genes

	Species	NR CDS (bp)	NR protein (amino acids)	Number of introns	GenBank
Plants	*Arabidopsis thaliana*	3,363	917	2	NP_177899
	Hordeum vulgare	4,373	915	2	X57845
		2,970	891	1	X60173
	Lycopersicon esculentum	4,093	911	3	X14060
	Nicotiana tabacum	4,554	904	3	X14058
		5,395	904	3	X14059
	Oryza sativa	4,882	916	3	NP_001062006
		3,627	916	3	NP_001062009
		3,027	889	3	NP_001048253
	Phaseolus vulgaris	3,725	881	3	X53603
	Zea mays	4,695	910	3	AF153448
Algae	*Chlamydomonas reinhardtii*	6,312	882	15	XM_001696645
	Chorella vulgaris	7,061	877	18	CVU39931
	Gracilaria tenuistipitata	2,733	910	0	GQ866118
	Volvox carteri	5,871	864	10	X64136
Fungi	*Aspergillus niger*	2,954	867	6	AM270168
	Neurospora crassa	3,010	982	1	X61303

phylogenetic analyses, the partial alignment including the three conserved protein domains presented results coherent with what was established using other markers such as SSU rDNA (Lipscomb et al. 1998). Although NR does not display enough phylogenetic signals to recover ancient divergences, for example the monophyletic relationship of Chlorophyta and embryophytes, otherwise, NR alignment demonstrated the monophyly of well-established groups such as red algae (Rhodophyta), green algae (Chlorophyta), the diatoms (Bacillariophyta), and embryophytes with the bryophyte *P. patens* as a basal group of Angiosperms. *G. tenuistipitata* NR is more closely related to *Porphyra* than to divergent thermophylic microalgae *C. merolae* as expected based on other molecular data (Oliveira and Bhattacharya 2000; Yoon et al. 2006).

The NR gene sequence from *G. tenuistipitata* can be used as a tool for the study of other rhodophyte NRs. From these data, it was possible to design probes and primers to study the expression of NR gene in *G. tenuistipitata* which presented variations in the mRNA quantity throughout the day, being at a basal level during the light phase and increasing the transcription in the last hours of dark. Therefore, these indicate a rhythm for NR mRNA expression observed in a period of 24 h. This variation in the NR gene expression of *G. tenuistipitata* suggests a regulation at the transcription or posttranscriptional level, although it is more indicative of a transcription regulation since it does not have introns to be regulated for alternative splicings or 5′ and 3′UTR binding motifs observed within the cDNA sequenced. Nitrate reductase presents a complex regulation by various mechanisms involving controls at transcriptional and posttranscriptional levels. Nitrogen source and light are the main factors regulating NR synthesis and activity at different levels including gene transcription, mRNA stability, translation, and posttranslational modifications, but the molecular mechanisms of these various processes are not fully elucidated (Loppes et al. 1999). The control of NR gene expression in *C. reinhardtii* was demonstrated at transcriptional levels with the use of a reporter gene and NR promoter which was induced by ammonium deficiency and photosynthetic activity and repressed by nitrite and active nitrate reductase (Loppes et al. 1999). Therefore, a possible approach to specifically investigate the transcriptional regulation of NR in *G. tenuistipitata* would involve the identification of the 5′UTR of the NR gene to express a reporter gene under the control of the NR promoter from *G. tenuistipitata*. It is known that light activates photosynthesis, and therefore, the production of sugars, which stimulates the NR promoter and NR activity, on the contrary in the dark NR gene transcription decreases and NR protein is inactivated by phosphorylation (Lea et al. 2006).

There is a basal expression of the NR gene that could be related to two hypotheses: two NR genes are present, one being expressed constitutively and another regulated as described for *N. tabacum* (Vaucheret et al. 1989), or only one NR gene which is continuously transcribed, having, however, its expression regulated and enhanced at certain time of the day. Some higher plants contain only one NR type encoded by a single gene; other plants contain two or three different NR enzymes with different electron donor

specificity and/or different tissue specificity. The different enzymes seem to be encoded by different genes (Caboche and Rouzé 1990). In tomato, the NR is encoded by a single gene per haploid genome, while the amphidiploid tobacco has two NR genes per haploid genome (Daniel-Vedele et al. 1989; Vaucheret et al. 1989). The monocots maize, barley, and rice contain two NR (Kleinhofs et al. 1988; Redinbaugh and Campbell 1981). In *A. thaliana*, two different NR genes have been cloned and mapped to different loci (Cheng et al. 1988; Wilkinson and Crawford 1991). Both are nitrate-inducible. One is expressed at a much higher level than the other (Cheng et al. 1991). In beans, at least two NR genes exist. Three forms of NR have been identified in soy (Streit et al. 1987). The dominating NR is a nitrate-inducible NR, while the two others appear to be constitutive. The significance of the fact that many plants have several NR enzymes is not known (Hoff et al. 1992).

The rhythm observed for NR mRNA is parallel to the previously observed data for *G. tenuistipitata* NR protein levels and NR activity in the daily cycle, both presenting just one peak during the day. The amount of NR protein present in crude extracts was about 40-fold higher in cells extracted during the day phase, and the NR activity exhibited a circadian rhythm, peaking at midday phase when activity was 30-fold higher than at night. The NR activity was clearly enhanced by light; when algae were kept under continuous light, they presented a higher level of activity than *G. tenuistipitata* grown under a light/dark regime, and when kept in continuous darkness, only a basal activity was observed (Lopes et al. 2002). The maximum of expression of NR mRNA from *G. tenuistipitata* is similar to that observed in other photosynthetic organisms where the levels of NR transcription are higher at the end of the night or early morning, and then they decrease to a basal level during the day, increasing again after 24 h (Scheible et al. 1997; Geiger et al. 1998). This interval of maximum of expression during the dark period followed by the peaks of translation and activity of NR is observed in many plants (Matt et al. 2001). Galangau et al. (1988), for example, demonstrated similar variation in the levels of NR mRNA, NR protein, and NR activity for *N. tabacum* and *L. esculentum*.

The short constant light condition assay suggested that there is an endogenous regulator in the NR gene expression besides light once a rhythm of expression is observed; however, the peak seems broader, possibly suggesting that light may induce the regulation at a significant level of expression. The results indicate that there is a circadian rhythm in the NR gene expression from *G. tenuistipitata* because the interval of 24 h between the maximum levels of NR mRNA is kept. Similarly, the level of mRNA coding for NR in the leaves of *N. tabacum* plants increased throughout the night and then decreased until it was undetectable during the day. When plants were transferred to continuous light conditions for 32 h, similar variations in NR gene expression still took place. These results indicated the circadian rhythmicity and light dependence of NR expression (Deng et al. 1990). Different from what is commonly observed for most of the transcription regulation results from plants during constant light assays, which keep the maximum of expression around the same period between the supposed end of dark and beginning of the light cycle, in *G. tenuistipitata*, the rhythm is shifted, indicating possibly a stronger regulation at transcriptional levels by light than in plants. The expression of a number of plant genes is regulated by an endogenous circadian clock. *Arabidopsis* NR gene showed robust circadian oscillations in mRNA accumulation which persist for at least 5 days in plants that have been grown in a light/dark cycle and then transferred to continuous light (Pilgrim et al. 1993).

In conclusion, the NR gene and cDNA of *G. tenuistipitata* were completely sequenced, and an endogenous circadian rhythm of expression for the mRNA coding for NR was observed. This work has characterized the NR gene sequence for a red macroalgae which may help in the studies of other Rhodophyta.

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References

Adams MW, Mortenson LE (1985) Molybdenium enzymes. In: Spiro TG (ed) Mo reductases: nitrate reductase and formate dehydrogenase. Wiley, New York, pp 519–593

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Bellorin AM, Oliveira MC, Oliveira EC (2002) Phylogeny and systematic of Gracilariaceae (Rhodophyta) revisited: ribosomal gene sequencing. J Phycol 38:551–563

Caboche M, Rouzé P (1990) Nitrate reductase: a target for molecular and cellular studies in higher plants. Trends Genet 6:187–192

Campbell WH (1999) Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. Annu Rev Plant Physiol Plant Mol Biol 50:277–303

Cardozo KHM, Guaratini T, Barros MP, Falcão VR, Tonon AP, Lopes NP, Campos S, Torres MA, Souza AO, Colepicolo P, Pinto E (2007) Metabolites from algae with economical impact. Comp Biochem Phys C 146:60–78

Cheng C, Dewdney J, Nam H, den Boer BGW, Goodman HM (1988) A new locus (NIA1) in *Arabidopsis thaliana* encoding nitrate reductase. EMBO J 11:3309–3314

Cheng C, Acedo GN, Dewdney J, Goodman HM, Conkling MA (1991) Differential expression of the two *Arabidopsis* nitrate reductase genes. Plant Physiol 96:275–279

Chiang YM, Lin JL (1989) Nitrate uptake by nitrogen-starved plants of the red alga *Gracilaria tenuistipitata* var. *liui*. Jpn. J Phycol 37:187–193

Chow Ho F, Oliveira MC (2008) Rapid and slow modulation of nitrate reductase activity in the red macroalgae *Gracilaria chilensis* (Gracilariales, Rhodophyta): influence of different nitrogen sources. J Appl Phycol 20:775–782

Chow Ho F, Oliveira MC, Pedersén M (2004) In vitro assay and light regulation of nitrate reductase in red alga *Gracilaria chilensis*. J Plant Physiol 161:769–776

Collén J, Pinto E, Pedersén M, Colepicolo P (2003) Induction of oxidative stress in the red macroalga *Gracilaria tenuistipitata* by pollutant metals. Arch Environ Contam Toxicol 45:337–342

Crawford NM (1995) Nitrate: nutrient and signal for plant growth. Plant Cell 7:859–868

Crawford NM, Campbell WH (1990) Fertile fields. Plant Cell 2:829–835

Daniel-Vedele E, Dorbe M, Caboche M, Rouze P (1989) Cloning of the nitrate reductase-encoding gene: protein domain structure and amino acid homologies in higher plants. Gene 85:371–380

Dawson HN, Pendleton LC, Solomonson LP, Cannons AC (1996) Cloning and characterization of the nitrate reductase-encoding gene from *Chlorella vulgaris*: structure and identification of transcription start points and initiator sequences. Gene 171:139–145

De la Rosa MA, Vega JM, Zumft WG (1981) Composition and structure of assimilatory nitrate reductase from *Ankistrodesmus baunii*. J Biol Chem 256:5814–5819

Deng M, Moureaux T, Leydecker MT, Caboche M (1990) Nitrate-reductase expression is under the control of a circadian rhythm and is light inducible in *Nicotiana tabacum* leaves. Planta 180:257–261

Edwards P (1970) Illustrated guide to sea weeds and sea grasses, in the vicinity of Porto Aransas. Texas Contr Mar Sc 15:1–228

Falcão VR, Tonon AP, Oliveira MC, Colepicolo P (2008) RNA isolation method for polysaccharide rich algae: agar producing *Gracilaria tenuistipitata* (Rhodophyta). J Appl Phycol 20:9–12

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Galangau F, Danielvedele F, Moureaux T, Dorbe MF, Leydecker MT, Caboche M (1988) Expression of leaf nitrate reductase genes from tomato and tobacco in relation to light–dark regimes and nitrate supply. Plant Physiol 88:383–388

Geiger M, Walch-Liu P, Engels C, Harnecker J, Schulze ED, Ludewig F, Sonnewald U, Scheible WR, Stitt M (1998) Enhanced carbon dioxide leads to a modified diurnal rhythm of nitrate reductase activity in older plants, and a large stimulation of nitrate reductase activity and higher levels of amino acids in young tobacco plants. Plant Cell Environ 21:253–268

Granbom M, Lopes PF, Pedersén M, Colepicolo P (2007) Nitrate reductase in the marine macroalga *Kappaphycus alvarezii* (Rhodophyta): oscillation due to the protein level. Bot Mar 50:106–112

Gruber H, Goetinck SD, Kirk DL, Schmitt R (1992) The nitrate reductase-encoding gene of *Volvox carteri*: map location, sequence and induction kinetics. Gene 120:75–83

Gruber H, Kirzinger SH, Zchmitt R (1996) Expression of the *Volvox* gene encoding nitrate reductase: mutation-dependent activation of cryptic splice sites and intron-enhanced gene expression from a cDNA. Plant Mol Biol 31:1–12

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98

Hewitt EJ (1975) Assimilatory nitrate–nitrite reduction. Annu Rev Plant Physiol 26:73–100

Hoff T, Stummann BM, Henningsen KW (1992) Structure, function and regulation of nitrate reductase in higher plants. Physiol Plant 84:616–624

Howarth DG, Baum DA (2002) Phylogenetic utility of a nuclear intron from nitrate reductase for the study of closely related plant species. Mol Phylogenet Evol 23:525–528

Huelsenbeck JP, Ronquist FR (2001) MrBayes. Bayesian inference of phylogeny. Biometrics 17:754–755

Jones TL, Tucker DE, Ort DR (1998) Chilling delays circadian pattern of sucrose phosphate synthase and nitrate reductase activity in tomato. Plant Physiol 118:149–158

Kleinhofs A, Warner RL, Hamat HB, Juricek M, Huang C, Schnorr K (1988) Molecular genetics of barley and rice nitrate reductase. Curr Top Plant Biochem Physiol 7:35–42

Lea US, Leydecker M-T, Quilleré I, Meyer C, Lillo C (2006) Posttranslational regulation of nitrate reductase strongly affects levels of free amino acids and nitrate, whereas transcriptional regulation has only minor influence. Plant Physiol 140:1085–1094

Lee TM, Chang YC, Lin YH (1999) Differences in physiological responses between winter and summer *Gracilaria tenuistipitata* (Gigartinales, Rhodophyta) to varying temperature. Bot Bul Acad Sin 40(1):93–100

Lillo C, Ruoff P (1989) An unusually rapid light-induced nitrate reductase mRNA pulse and circadian oscillations. Naturwissenschaften 76:526–528

Lillo C, Meyer C, Ruoff P (2001) The nitrate reductase circadian system. The central clock dogma contra multiple oscillatory feedback loops. Physiol Plant 125:1554–1557

Lipscomb DL, Farris JS, Källersjö M, Tehler A (1998) Support, ribosomal sequences and the phylogeny of the eukaryotes. Cladistics 14:303–338

Lopes PF, Oliveira MC, Colepicolo P (1997) Diurnal fluctuation of nitrate reductase activity in the marine red alga *Gracilaria tenuistipitata* (Rhodophyta). J Phycol 33:225–231

Lopes PF, Oliveira MC, Colepicolo P (2002) Characterization and daily variation of nitrate reductase in *Gracilaria tenuistipitata* (Rhodophyta). Biochem Biophys Res Commun 295:50–54

Loppes R, Radoux M, Ohresser MCP, Matagne R (1999) Transcriptional regulation of *Nia1* gene encoding nitrate reductase in *Chlamydomonas reinhardtii*: effects of various environmental factors on the expression of a reporter gene under the control of the *Nia1* promoter. Plant Mol Biol 41:701–711

Lourenço SO, Vieira AAH (2004) Culture collection of microalgae in Brazil: progress and constraints. Nova Hedwigia 79:149–173

Macchiavello J, Saito R, Garofalo G, Oliveira EC (1999) A comparative analysis of agarans from commercial species of *Gracilaria* (Gracilariales, Rhodophyta) grown in vitro. Hydrobiologia 398(399):397–400

Matt P, Geiger M, Walch-Liu P, Engels C, Krapp A, Stitt M (2001) Elevated carbon dioxide increases nitrate uptake and nitrate reductase activity when tobacco is growing on nitrate, but increases ammonium uptake and inhibits nitrate reductase activity when tobacco is growing on ammonium nitrate. Plant Cell Environ 24:1119–1137

McClung CR, Kay SA (1994) *Arabidopsis*. In: Meyerowitz EM, Somerville CR (eds) Circadian rhythms in *Arabidopsis thaliana*. Cold Spring Harbor Laboratory Press, New York, pp 615–637

Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB et al (2007) The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science 318:245–250

Oliveira EC, Alveal K (1990) Introduction to applied phycology. In: Akatsuka I (ed) The mariculture of *Gracilaria* (Rhodophyta) for the production of agar. SPB Academic Publishing, The Hague, pp 553–564

Oliveira M, Bhattacharya D (2000) Phylogeny of the Bangiophycidae (Rhodophyta) and the secondary endosymbiotic origin of algal plastids. Am J Bot 87:482–492

Pilgrim ML, Caspar T, Quail PH, McClung R (1993) Circadian and light-regulated expression of nitrate reductase in *Arabidopsis*. Plant Mol Biol 23:349–364

Ramalho CB, Hastings JW, Colepicolo P (1995) Circadian expression of nitrate reductase in the unicellular algae *Gonyaulax polyedra* is due to changes in cellular protein levels. Plant Physiol 107:225–231

Redinbaugh MG, Campbell WH (1981) Purification and characterization of NAD(P)H:nitrate reductase and NADH:nitrate reductase from corn roots. Plant Physiol 68:115–120

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Scheible W-R, Gonzáles-Fontes A, Morcuende R, Lauerer M, Geiger M, Glaab J, Gojon A, Schulze E-D, Stitt M (1997) Tobacco mutants with a decreased number of functional nia genes compensate by modifying the diurnal regulation of transcription, post-translational modification and turnover of nitrate reductase. Planta 203:304–319

Solomonson LP, Barber MJ (1990) Assimilatory nitrate reductase: functional properties and regulation. Ann Rev Physiol Plant Mol Biol 41:225–253

Song B, Ward BB (2004) Molecular characterization of the assimilatory nitrate reductase gene and its expression in the marine green alga *Dunaliella tertiolecta* (Chlorophyceae). J Phycol 40:721–731

Stitt M, Muller C, Matt P, Gibon Y, Carillo P, Morcuende R, Scheible WR, Krapp A (2002) Steps towards an integrated view of nitrogen metabolism. J Exp Bot 53:959–970

Stolz JF, Basu P (2002) Evolution of nitrate reductase: molecular and structural variation on a common function. Chem Biol Chem 3:198–206

Streit L, Martin BA, Harper JE (1987) A method for the separation and the partial purification of the three forms of nitrate reductase present in wild-type soybean leaves. Plant Physiol 84:654–657

Swofford DL (2000) PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer Associates, Sunderland

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512–526

Vaucheret H, Kronenberger J, Rouze P, Caboche M (1989) Complete nucleotide sequence of the two homologous tobacco nitrate reductase genes. Plant Mol Biol 12:597–600

Wilkinson JQ, Crawford NM (1991) Identification of the *Arabidopsis* CHL3 gene as the nitrate reductase structural gene NIA2. Plant Cell 3:461–471

Yoon HS, Müller KM, Shealth RG, Ott DF, Bhattacharya D (2006) Defining the major lineages of red algae (Rhodophyta). J Phycol 42:482–492

Zhou J, Kleinhofs A (1996) Molecular evolution of nitrate reductase genes. J Mol Evol 42:432–442