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High-affinity nitrate/nitrite transporter genes (*Nrt2*) in *Tisochrysis lutea*: identification and expression analyses reveal some interesting specificities of Haptophyta microalgae

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Abstract :

Microalgae have a diversity of industrial applications such as feed, food ingredients, depuration processes and energy. However, microalgal production costs could be substantially improved by controlling nutrient intake. Accordingly, a better understanding of microalgal nitrogen metabolism is essential. Using in silico analysis from transcriptomic data concerning the microalgae *Tisochrysis lutea*, four genes encoding putative high-affinity nitrate/nitrite transporters (TlNrt2) were identified. Unlike most of the land plants and microalgae, cloning of genomic sequences and their alignment with complementary DNA (cDNA) sequences did not reveal the presence of introns in all *TlNrt2* genes. The deduced TlNRT2 protein sequences showed similarities to NRT2 proteins of other phyla such as land plants and green algae. However, some interesting specificities only known among Haptophyta were also revealed, especially an additional sequence of 100 amino acids forming an atypical extracellular loop located between transmembrane domains 9 and 10 and the function of which remains to be elucidated. Analyses of individual TlNrt2 gene expression with different nitrogen sources and concentrations were performed. *TlNrt2.1* and *TlNrt2.3* were strongly induced by low NO3− concentration and repressed by NH4+ substrate and were classified as inducible genes. *TlNrt2.2* was characterized by a constitutive pattern whatever the substrate. Finally, *TlNrt2.4* displayed an atypical response that was not reported earlier in literature. Interestingly, expression of *TlNrt2.4* was rather related to internal nitrogen quota level than external nitrogen concentration. This first study on nitrogen metabolism of *T. lutea* opens avenues for future investigations on the function of these genes and their implication for industrial applications.

Abbreviations

AA
: amino acids

AN
: ammonium nitrate

cDNA
: complementary DNA

cHATS
: constitutive high-affinity transport system

cLATS
: constitutive low-affinity transport system

CT
: threshold cycle

GAPDH
: glyceraldehyde 3-phosphate dehydrogenase

GS/GOGAT
: glutamine synthetase/glutamine oxoglutatate aminotransferase

HATS
: high-affinity transport system

iHATS
: inducible high-affinity transport system

iLATS
: inducible low-affinity transport system

LATS
: low-affinity transport system

MFS
: major facilitator superfamily

mRNA
: messenger RNA

NAR
: nitrite transporter

NiR
: nitrite reductase

NNP
: nitrate/nitrite porter

NR
: nitrate reductase

NRT
: nitrate transporter

PTR
: peptide transporter

Q-RT-PCR
: quantitative real-time polymerase chain reaction

Introduction

Nitrogen is an essential element for all living organisms. It participates in many crucial biological processes, including the formation of amino acids, proteins and chlorophyll. Nitrogen is preferentially absorbed in the ammonium form, however this form is scarce in the environment and nitrate is the most commonly used source of nitrogen (Forde, 2000).

Many reports have described the nitrate assimilation pathway in higher plants and certain microalgae (Crawford and Forde, 2002; Fernandez and Galvan, 2008, 2008; Ullrich, 1983). The first step of nitrate assimilation involves nitrate uptake across the plasma membrane by different types of nitrate transporters (NRT) and seems to be a major step in controlling nitrogen assimilation (Daniel-Vedele et al., 1998; Fernandez and Galvan, 2008). In cells, nitrate is reduced to nitrite in the cytoplasm by nitrate reductase (NR). Nitrite is transported into the chloroplast by a nitrite transporter (NAR1) for its reduction to ammonia by nitrite reductase (NiR). Ammonia is then incorporated into carbon skeletons by the glutamate dehydrogenase or the combined action of glutamine synthetase and glutamate synthase (Ahmad and Hellebust, 1984). In plants, nitrate can also be stored in vacuoles by the action of nitrate antiporters and nitrate transporters located on the tonoplast (De Angeli et al., 2006; Dechorgnat et al., 2012). In green microalgae, different storage structures (contractile vacuoles, intracellular granules) have been described but their origins and their functions remain unclear (Becker, 2007). Studies have shown that the transport capacities of the tonoplast membranes in green algae are similar to those of land plants (Becker, 2007) However, no nitrate transporters have yet been located on intracellular organelles of microalgae. Moreover, although many processes in nitrate assimilation are common to both algae and higher plants, Ullrich (1983) suggested that some differences could exist due to differential evolution, differences in the structural properties of the enzymes among these species and, most importantly, the large surface area of contact that algal cells have with external nutrients.

In recent years, the mechanisms that respond to nitrate availability have been the subject of much research. Previous studies indicate that NO_3^- uptake requires an active transport system (Forde, 2000; Galvan and Fernández, 2001). Molecular investigations in land plants have shown that four types of transport systems exist depending on the concentration of nitrate in the environment (Crawford and Glass, 1998). Two high-affinity transport systems (HATS) co-exist and can take-up NO_3^- at very low external concentrations (< 250 µM). The first system (iHATS) is inducible by NO_3^- and characterized by high values of K_m and V_{max} (for plants, typically 20–100 µM and 3–8 µmol.g.h^{-1}, respectively). The second system (cHATS) is constitutively expressed and does not depend on the nitrogen concentration, having low values of both K_m and V_{max} (for plants 6–20 µM and 0.3–0.82 µmol.g.h^{-1}, respectively) (Crawford and Glass, 1998). When the NO_3^- external concentration reaches values

around the 500 µM range, two other transport systems with low-affinity for nitrate (cLATS and iLATS) become involved in the absorption flux (Crawford and Glass, 1998; Galvan and Fernández, 2001; Glass et al., 1992; Siddiqi et al., 1990). The low-affinity transport system is unsaturable.

The Peptide Transporter (PTR) and the Nitrate/Nitrite Porter (NNP) gene families, belonging to the Major Facilitator Superfamily (MFS), have been identified as encoding putative nitrate transporters (Forde, 2000; Tsay et al., 2007). The MFS proteins have a typical membrane topology of 12 transmembrane domains arranged as two sets of six, connected by a cytosolic loop (Forde, 2000; Galvan and Fernández, 2001). The amino acid sequences share a conserved sequence motif [G-X_3-D-X_2-G-X-R] between transmembrane domains 2 and 3, and another motif [I-X_2-R-X_3-G-X_3-G] within transmembrane domain 4. The PTR family is a multigenic family found in a wide variety of plants and algae (Steiner et al., 1995). It includes several members characterized as low affinity nitrate transporters (*Npf* genes formely named *Nrt1* genes) (Crawford and Glass, 1998; Forde, 2000; Léran et al., 2014). One exception is observed for *Nrt1.1*, which possesses a double affinity for NO_3^- (HATS / LATS) (Liu et al., 1999; Wang et al., 1998). The NNP family includes high-affinity nitrate/nitrite transporters (*Nrt2* genes). The NRT2 proteins have between 500–600 AA and present a consensus motif [FYK]-X_3-[ILQRK]-X-[GA]-X-[VASK]-X-[GASN]-[LIVFQ]-$X_{1,2}$-G-X-G-[NIM]-X-G-[GTA] within the fifth putative transmembrane domain. This motif is proposed as the signature for the NNP family (Forde, 2000). A portion of this sequence [A-G-W/L-G-N-M-G] has been also suggested as the substrate recognition motif. The members of the *Nrt2* gene family show substrate specificity for nitrate and/or nitrite with different affinities. The first member of the NNP family to be cloned was the C*rnA* gene from *Aspergillus nidulans* (Brownlee and Arst, 1983; Unkles et al., 1991) and was identified to be a functional nitrate/nitrite transporter when expressed in Xenopus oocytes (Zhou et al, 2000). Then, *Nrt2* genes from *Chlamydomonas reinhardtii* were identified to mediate high affinity nitrate transport like C*rnaA gene*. After, many studies identified *Nrt2* genes in different species of land plants and microalgae (e.g. *Arabidopsis thaliana*, *Nicotiana tabacum*, *Oryza sativa* and *Dunaliella salina*) based on the sequence homology analysis (Cai et al., 2008; He et al., 2004; Krapp et al., 1998; Orsel et al., 2002). The number of high-affinity nitrate/nitrate transporters varies between species. Recent studies on the green microalga *C. reinhardtii* have provided information on the properties of NRT2 (Fernandez and Galvan, 2007; Galván et al., 1996; Quesada et al., 1994, 1998). In fact, *C. reinhardtii* have four NO_3^- or/and NO_2^- uptake systems, each with distinctive kinetic and regulatory properties, and some of these systems require the presence of an accessory protein (NAR2) to be functional (Galván et al., 1996; Rexach et al., 1999, 2002). Thereafter, other NAR2 proteins have since been identified in land plants (Orsel et al., 2006; Tong et al., 2005).

The high-affinity nitrate transporters in marine microalgae are of particular interest due to the low nitrate concentrations available in seawater (Collos et al., 2005; Song and Ward, 2007). Research has

been conducted on marine microalgae for many years for the economic development of its biomass and various molecules that have high added value such as antioxidants, pigments and polysaccharides. However, microalgae have significant nitrogen needs to develop and produce their biomass. In the future, the price of nitrogen fertilizer will tend to increase in relation to the price of energy. Therefore, it is important to improve our knowledge of nitrogen absorption and assimilation mechanisms in these species. Haptophyta represent a major phylum of marine eukaryotic phytoplankton, ecologically dominant in ocean euphotic zones. A culture strain originally isolated from Tahiti and named *Tisochrysis lutea* (formerly *Isochrysis* affinis *galbana*) has been extensively studied due to its widespread use in aquaculture as feed for shellfish and shrimps (Bougaran et al., 2003; Bendif et al. 2013). *T. lutea* remains very difficult to transform and genetic tools suitable for the study of the regulation of the N-metabolism pathway in this taxon are scarce. The present study used integrative physiological and molecular approaches to improve our understanding of nitrogen metabolism in *T. lutea*. In this paper, we present the first molecular characterization of individual genes encoding putative high-affinity nitrate/nitrite transporters (*Nrt2*) in the Haptophyta. Using transcriptomic data, we identified four *Nrt2* genes in *T. lutea*. Analyses of the *Tisochrysis* NRT2 sequences reveal some interesting specificities compared with NRT2 proteins of other phyla such as land plants and green algae. Moreover, several experimental investigations revealed gene expression profiles similar to land plants for three genes in *T. lutea* and, unexpectedly, one gene that presents a very atypical expression profile, never observed in any other phylum.

Material and Methods

Strain and strain maintenance

Wild-type *Tisochrysis lutea* was provided by the Culture Centre of Algae and Protozoa (CCAP 926/14). An axenic *T. lutea* strain was grown in a Conway medium (Walne, 1966) prepared with seawater, in a controlled environment with a constant irradiance of 150 µmol.m^{-2}.s^{-1} at 22°C. Population densities were estimated by cell counting and by spectrophotometry measuring the OD_{680} and OD_{800}.

Elementary analyses

Particulate Nitrogen (N) and Carbon (C) concentrations (µM) were estimated by filtering given volumes of cells on precombusted 25 mm GF/C filters (Whatman, 1.2 µm). The filters were then dried for 24 h at 70°C and further analysed using a CN Elemental Analyzer (Flash 2000, Thermoscientific). Particulate N/C ratios were calculated by dividing the particulate N concentration by the particulate C concentration.

Residual Nitrogen analysis

Residual NO_3, NO_2 and NH_4 concentration in 1.2 µm filtrates was measured using an AA3 HR autoanalyser (Serlabo technologies) according to an automated spectrophotometric method (Aminot and Kérouel, 2007).

Identification and cloning of nitrate/nitrite transporter genes in *Tisochrysis lutea*

Transcriptomic data (Carrier et al., 2014) were used to identify nitrate/nitrite transporter genes in *T. lutea*. The gene-specific primers were designed for each *TlNrt2* gene and used for PCR amplification (see Appendix S1 in Supporting Information). The amplified products were examined on agarose gels by electrophoresis and were purified using QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. The clean PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen) and the ligated plasmids were transformed into *Escherichia coli* (One Shot® TOP10 Chemically Competent *E. coli*). The transformed cells were spread on Luria-Bertani medium containing 50 $\mu g.mL^{-1}$ kanamycin with agar on plates. The full-length cDNA were re-sequenced using the SANGER method by GATC Biotech (Germany).

Sequence analyses

Several programs were used to analyse the *T. lutea* NRT2 sequences. The prediction of transmembrane domains was performed with TMPred software (Hofmann, 1993). WolfPsort was used to predict the subcellular localization of the proteins (Horton et al., 2007) and iPsort software was used as the subcellular localization site predictor for N-terminal sorting signals (signal peptide, mitochondrial targeting peptide, or chloroplast transit peptide) (Bannai et al., 2002). The NetphosK program was used to predict putative kinase phosphorylation sites in the amino acid sequences (Blom et al., 1999). The sequence alignments were performed with ClustalW or MUSCLE software. The homology analyses were performed by using the BLAST program on the NCBI database and *T. lutea* transcriptome.

Homology trees of NRT2 proteins

Reference amino acid sequences were obtained from the GenBank database: *Arabidopsis thaliana* (NP_190092 ; AEE28241), *Brassica napus* (CAC05338), *Glycine max* (NP_001236444), *Nicotiana plumbaginifolia* (CAA69387), *Oryza sativa* (BAA33382), *Solanum lycopersicum* (NP_001234127), *Triticum aestivum* (AAK19519), Zea mays (NP_001105780), *Chlorella sorokiniana* (AAK02066), *Chlorella variabilis* (EFN52690), *Cylindrotheca fusiformis* (AAD49572), *Dunaliella salina* (AAU87579), *Dunaliella_tertiolecta* (ABP01763), *Chlamydomonas reinhardtii* (CAD60538 ; CAA80925), *Emiliania huxleyi CCMP1516* (EOD39011), *Emiliania huxleyi* (ABP01765), *Tetraselmis chui* (ADU76799), *Thalassiosira pseudonana* (ACI64621), *Ostreococcus tauri* (XP_003091529), *Skeletonema costatum* (AAL85928), *Volvox carteri* (EFJ43757), *Aspergillus nidulans* (AAA76713),

Hebeloma cylindrosporum (CAB60009), *Neurospora crassa* (CAD71077), *Hansenula polymorpha* (CAA11229), *Agrobacterium sp. H13-3* (YP_004277958), *Burkholderia rhizoxinica* (YP_004022689), *Pseudomonas stutzeri* (WP_003298315), *Calothrix sp. PCC 6303* (AFZ00090), *Chroococcidiopsis thermalis PCC 7203* (YP_007092893), *Cyanobacterium stanieri PCC 7202* (AFZ48362). The amino acid sequences were aligned using MUSCLE software and the resulting alignment file was used to construct a homology tree using PhyML software through the phylogeny.fr website (Dereeper et al., 2008). The homology tree was drawn with Treedyn software.

Quantification of abundance transcripts by quantitative RT-PCR

RNA Extraction and Reverse Transcription

The samples were centrifuged (20 min, 5000 g, 4°C). The supernatant was discarded and the sea water was taken up to remove the salt. The pellets were resuspended in Trizol reagent (Invitrogen) and chloroform. After centrifugation, the upper phases were collected and 0.5 volume of absolute EtOH was added. The samples were transferred to a column of the RNeasy Plant mini kit (Qiagen) and the manufacturer's instructions followed thereafter. A DNase treatment (RQ1 DNase, Promega) was applied and total RNA purified using the RNeasy Plant Mini Kit with the RLT buffer and EtOH. The quality and concentration of RNA were determined with a spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE) at wavelengths of 260 nm and 280 nm. The PCR amplification of an RNA sample served as a check for genomic DNA contamination. Total RNA was stored at –80°C. Reverse transcription of RNA was performed using the High Capacity cDNA Reverse Transcription kit (Applied Technologies) following the manufacturer's instructions.

Real-Time PCR and SYBR Green Detection

Quantitative PCR reactions were initiated by adding the cDNA to the SYBR Green PCR master mix (Applied Biosystems) and 300 nM of forward and reverse primers. Ubiquitin and GAPDH genes were chosen as reference genes and remained stable in our experiments. These primer sets were specifically designed to amplify a 100- to 150-bp fragment from the genes *TlUbi*, *TlGAPDH*, *TlNrt2.1*, *TlNrt2.2*, *TlNrt2.3* and *TlNrt2.4* in *T. lutea* (see Appendix S2 in Supporting Information). The reactions were carried out on an Agilent Mx3000P QPCR System (Agilent technologies). PCR settings were 95°C for 10 min and then 95°C for 15 s, and 60°C for 1 min for 40 cycles. The fluorescence intensity from the complex formed by SYBR Green and the double-stranded PCR product was continuously monitored from cycles 1 to 40. The threshold cycle (CT) at which the fluorescence intensity became higher than a preset threshold was used to calculate the gene transcript level.

To obtain the gene expression data, the comparative CT method and standard curve method were combined to calculate an RNA molar ratio between a target gene and a reference gene, as previously described by Chung et al. (2005). Standard curves of each gene were generated to calibrate the PCR

efficiency, and the slopes of standard curves were –3.2 (*TlUbi* mRNA), –3.4 (*TlGAPDH* mRNA), –3.2 (*TlNrt2.1*), –3.28 (*TlNrt2.2*), and –3.33 (*TlNrt2.3*) and –3.353 (*TlNrt2.4*) respectively. The relative mRNA abundance (X0/R0, in mmol of the target gene (mol *TlUbi* mRNA)$^{-1}$), was calculated by the following equation: $\log (X_0/R_0) = \log (M_r/Mx) + (Ct_x/b_x) – (Ct_r/b_r)$, where X_0 and R_0 are the initial numbers of target and reference molecules, M_r and M_x are the molecular weights of the target and reference amplicons, Ct_x and Ct_r are the threshold cycle numbers of X and R, and b_x and b_r are the standard curve slopes of X and R, respectively. The specificity of the quantitative reverse-transcription PCR (Q-RT-PCR) products was confirmed by melting temperature analysis.

Experimental protocols

Expression studies of Tisochrysis Nrt2 genes during batch culture with $NaNO_3$

An axenic preculture of *T. lutea* was grown in a Conway medium with 1 mM $NaNO_3$. During exponential phase, which corresponds to a high nitrogen quota, cells were inoculated into a new modified Conway medium containing 200 µM $NaNO_3$. The initial cellular density in the new batch culture was about $2*10^6$ cells.mL^{-1}. The irradiance and temperature were set at 200 µmol.m^{-2}.s^{-1} and 22°C respectively. The cultures were aerated with 0.2-µm filtered air and pH was adjusted to 8.0 with CO_2 injections. Discrete sampling was realized and three parameters were monitored throughout the experiment: the nutritional status (particulate Nitrogen and Carbon), the composition of the medium (residual NO_3) and the abundance of *TlNrt2* transcripts.

Expression studies of Tisochrysis Nrt2 genes during batch culture with NH_4Cl

The axenic preculture of *T. lutea* was grown in a modified Conway medium with 200 µM NH_4Cl. In the stationary phase, corresponding to a low nitrogen quota, cells were inoculated into non-enriched seawater for 24 hours in order to lower the natural nitrogen content of the seawater. Then, a new modified Conway medium with 200 µM NH_4Cl was added. The initial cellular density in the new batch culture was about $1.5*10^6$ cells.mL^{-1}. The irradiance and temperature were fixed at 200 µmol.m^{-2}.s^{-1} and 22°C, respectively. The cultures were aerated with 0.2-µm filtered air and pH was adjusted to 8.0 with CO_2 injections. A discrete sampling was performed and three parameters were monitored throughout the experiment: the nutritional status (particulate Nitrogen and Carbon), the composition of the medium (residual NO_3) and the abundance of *TlNrt2* transcripts.

Concerning the *TlNrt2* relative mRNA abundance as a function of the N/C ratio (N status) in cells, N/C ratios were calculated by dividing the particulate N concentration by the particulate C concentration.

Regulation of TlNrt2 expression by different N-substrate conditions

To study the short-term regulation of *TlNrt2* expression by N metabolites, 3 L of axenic cultures growing in continuous culture with N-limiting conditions were transferred into sterilized bottles. The cultures were aerated with 0.2-µm filtered air and received a constant irradiance of 200 µmol m^{-2} s^{-1} and the pH was adjusted to 8.0 with CO_2 injections. Cells were left without any nutritional supply for the time necessary to clear their internal nitrogen quotas. It was considered that the cells had the lowest nitrogen quotas when the algal growth was at stationary phase. Different types of Conway media were prepared from different forms of nutrients ($NaNO_3$; $NaNO_2$ and (NH_4^+)(NO_3^-)), but all containing 1 mM of total nitrogen, and were added to the different cultures independently. Samples were collected and total RNA extractions were performed at 0, 15, 30, 60 and 120 min after N repletion. All experiments were performed in triplicate.

Statistics

Statistical analyses were performed with Matlab® (Mathworks, Natick, Massachusetts, United States) using Kruskal-Wallis test ($\alpha = 0.05$).

Results

Identification of *Tisochrysis lutea Nrt2* transcripts

In this study, four genes, encoding putative high-affinity nitrate/nitrite transporters, have been identified in *T. lutea* based on our transcriptomic data (Carrier et al., 2014) and were named *TlNrt2.1*, *TlNrt2.2*, *TlNrt2.3* and *TlNrt2.4*. The *TlNrt2* gene identification was performed by similarity searches, using several NRT2 sequences from different species (*Aspergillus nidulans, Hansenula polymorpha, Arabidopsis thaliana*, O*ryza sativa* and *Dunaliella salina*), against the transcriptome database of *T. lutea* (Fig. 1). The respective *TlNrt2* full-length cDNAs were cloned and re-sequenced to confirm sequence reliability.

In addition, *T. lutea* was checked for the presence of any NAR2 accessory protein. Nevertheless, despite numerous homology searches using various NAR2 sequences from several species [*A. thaliana (AED95911.1)*, *Cucumis savitus (ACV33078.2)*, *Micromonas sp. RCC299 (ACO68771.1)* and *C. reinhardtii (A8J4P7)*], no gene encoding a putative NAR2 protein was detected in *T. lutea*.

Intron-Exon Structure of the *Tisochrysis lutea Nrt2* genes

Amplifications of genomic sequences were performed using primers designed in the 5'UTR and 3'UTR regions (see Appendix S1 in Supporting Information). The amplicons were sequenced using the SANGER method (GATC, Germany). To report the genomic and cDNA sequences of the *TlNrt2* genes, a full alignment of the cDNA sequences and the genomic sequences was performed using

ClustalW software. No introns were detected in the genomic sequences using the alignments with the respective cDNA sequences.

Concerning the sequence comparison, the highest value of nucleotide sequence identity was found between *TlNrtT2.1* and *TlNrt2.3*, with a value up to 82.8% identity (See Appendix S3 in supporting information). However, strong identities were also observed between all of the *TlNrt2* nucleotide sequences (from 68.4% to 79.1 %).

Characteristics of deduced TlNRT2 amino acid sequences obtained using bioinformatic tools

The deduced TlNRT2.1, TlNRT2.2, TlNRT2.3 and TlNRT2.4 proteins have 633, 630, 634 and 661 amino acid residues, respectively. These protein sequences showed strong similarities, with identity scores ranging up to 92.9 % (See Appendix S3 in supporting information). The Wolf PSORT program (Horton et al., 2007) for prediction of protein subcellular localization placed the four *T. lutea* NRT2 on the plasma membrane. The iPSORT software (Bannai et al., 2002) did not detect the presence of any signal, mitochondrial targeting, or chloroplast transit peptides.

Hydropathic analysis was performed with the TMPred program and predicted the presence of 12 transmembrane domains. A first cytosolic loop was observed between transmembrane domains 6 and 7, and is specific to the MFS family. Contrary to the fungal and yeast NRT2 sequences, that have a large central cytosolic loop (90 AA), *Tisochrysis* NRT2 sequences show a short cytosolic loop (20-30 AA) as observed in land plants and green algae (Fig. 1). The specific domain (G-X_3-D-X_2-G-X-R) characteristic of the Major Facilitator Superfamily is identified between transmembrane domains 2 and 3 in *T. lutea* NRT2 sequences (Fig. 1). The signature motif for the Nitrate/Nitrite transPorters family (A-G-W/L-G-N-M-G), which is suggested as a recognition substrate motif, was also found in the fifth transmembrane domain.

A second loop is observed only in *Tisochrysis* NRT2 sequences and is located between transmembrane domains 9 and 10. This loop is extracellular and has about 100 AA, which is highly atypical compared with the NRT2 sequences from other species (Fig. 1). Homology researches were made on this atypical loop. The results of BLAST analysis showed that this additional sequence is not found in NRT2 sequences of other species, except in the NRT2 sequences of another haptophyte, *E. huxleyi*. Analysis of a shotgun proteomic experiment (not shown in this paper) confirmed the presence of these proteins and, more precisely, the additional amino acid sequence (see Appendix S5 in Supporting Information). Indeed, two peptides were identified in this additional loop of *Tisochrysis* NRT2 sequences. BLAST analyses then confirmed that these peptides did not match with other proteins from *Tisochrysis lutea* and were specific to TlNRT2 proteins.

The deduced TlNRT2 proteins show an extended N-terminal domain (30 AA) and an extended C-terminal domain as described in plants and green algae. However, the alignment in figure 1 shows that TlNRT1, TlNRT2.2 and TlNRT2.3 sequences harbour C-terminal domains smaller (40 AA) than all others (60-70 AA). Using NetPhosK program (Blom et al., 1999), several protein kinase C recognition

motifs are detected for only three TlNRT2 sequences (TlNRT2.1, TlNRT2.2 and TlNRT2.3), but at a different position to those found in other organisms. In contrast, no protein kinase C recognition motif was detected in TlNRT2.4. Furthermore, the conserved protein kinase C recognition motifs in land plants and green algae are not found for all TlNRT2 proteins excepted for TlNRT2.1, which harbours this motif type in the C-terminal domain (Fig. 1). Nevertheless, the same motif of phosphorylation by protein kinase C is predicted within the additional sequences in three TlNRT2 proteins (TlNRT2.1, TlNRT2.2 and TlNRT2.3). Contrary to the yeast and fungal NRT2 sequences, no protein kinase C recognition motifs were detected in the central cytosolic loop.

Similarity scores and homology tree

The amino acid sequences of TlNRT2 have fairly low homologies with NRT2 proteins of other species (See Appendix S4 in supporting information). Indeed, the identity scores obtained with AsCrnA (*Aspergillus nidulans*) and HpYNT1 (*Hansenula polymorpha*) were only about 26% and 23% respectively. Nevertheless, the identity scores seem to be greater with sequences of land plants and algae. For example, between 28.5 % and 34.3 % identity and up to 47.2 % similarity was found with AtNRT2.1 (*A. thaliana*). Moreover, it was also shown that the protein sequences of *T. lutea* NRT2 show up to 35 % identities with other NRT2 of algae (between 31 % and 35 % identities with *Chlamydomonas reinhardtii* NRT2.3).

In the same way, the homology tree highlights the fact that the *Nrt2* genes from this haptophyte cluster with the *Nrt2* genes from land plants and green algae (Fig. 2). This group, containing the *Nrt2* genes of land plants, green algae and haptophytes, differs completely from other phyla (diatoms, fungi, cyanobacteria and bacteria). However, inside this group, results showed that the *Nrt2* genes of green algae are closely related to the *Nrt2* genes of land plants, while the *Nrt2* genes of the Haptophyta are distinct to the green lineage. In addition, *T. lutea Nrt2* seems to form a single clade, separate from those of *E. huxleyi*.

Transcriptional responses of *TlNrt2* genes during nitrate batch culture

In order to observe the expression of individual *TlNrt2* genes during batch culture, cells with high nitrogen quotas (i.e. at the exponential phase) were inoculated in a modified Conway medium containing 200 µM nitrate as the sole source of nitrogen. Cell density exponentially increased for 2 days before reaching the stationary phase (Fig. 3A). Residual nitrate provided at 200 µM at day 0 was almost exhausted after 16 h, and totally depleted from 22 h until day 4. The transcript level of the nitrate transporter genes was monitored throughout the batch culture. The relative mRNA abundance was normalized using the reference gene (Ubiquitin).

The relative abundance of *TlNrt2.1* transcripts showed an average of 17.2 $mol.mol^{-1}$ *TlUbi* mRNA when external nitrate was still abundant (Fig. 3B). Thereafter, the residual concentration of nitrate began to drop and an increase of the *TlNrt2.1* gene expression (24.1 $mol.mol^{-1}$ *TlUbi* mRNA) was

observed when the external nitrate concentration was around 30 µM. However, the *TlNrt2.1* mRNA abundance expression peaked after the nitrate had been totally depleted from the medium (45.6 mol.mol^{-1} *TlUbi* mRNA, or 2.6-fold). This maximal transcript level of *TlNrt2.1* remained stable until the cells reached the stationary phase.

The transcript level of *TlNrt2.2* was relatively stable, with an average of 2.1 mmol.mol^{-1} *TlUbi* mRNA throughout the experimental period (Fig. 3C). The results suggested that *TlNrt2.2* was constitutively expressed in these culture conditions.

For *TlNrt2.3* gene, the transcript level remained stable until 28 h with an average of 1857 mmol.mol^{-1} *TlUbi* mRNA and declined at 40 h (1105 mmol.mol^{-1} *TlUbi* mRNA) when the residual nitrate was undetectable (Fig. 3D). The *TlNrt2.3* transcript level then reached 853 mmol.mol^{-1} *TlUbi* mRNA by the end of the experiment.

In contrast to other *TlNrt2* genes, *TlNrt2.4* transcript level was high (185 mmol.mol^{-1} *TlUbi* mRNA) when the external nitrate concentration was 200 µM (Fig. 3E). Its expression decreased during nitrate consumption by algal cells to an average of 82.2 mmol.mol^{-1} *TlUbi* mRNA. When the nitrate was totally depleted of the medium after 22 h, no gene expression was detected until the end of the experiment.

Transcriptional responses of *TlNrt2* genes during ammonium batch culture

To determine the expression profile of each *TlNrt2* gene when the nitrate in the medium is replaced by ammonium as the sole nitrogen source, cells were inoculated in non-enriched seawater for 24 hours in order to lower the natural nitrogen content of the seawater. In addition, to observe a strong repression of *TlNrt2* genes in the presence of ammonium, it was necessary that their expressions were high at the beginning of the experiment. For this reason, prior to their inoculation, cells with empty nitrogen quotas were preconditioned under N starvation. Once the initial physiological conditions were met, a modified Conway medium with 200 µM NH_4Cl was added. The growth pattern of the population showed that cell density increased exponentially for 3.8 days and then entered the stationary phase (Fig. 4A). The ammonium supplied was totally depleted after 20 h (day 0.8) and remained undetectable until day 4.8. The transcript level of the nitrate transporter genes was monitored throughout the batch culture.

In the nitrogen-starved cultures of *T. lutea*, the relative transcript levels of *TlNrt2.1* and *TlNrt2.3* were high, with average values of 64.8 mol.mol^{-1} *TlUbi* mRNA and 1808 mmol.mol^{-1} *TlUbi* mRNA, respectively (Fig. 4B and 4D).

When NH_4^+ was added to the N-starved cultures, the relative transcript levels of *TlNrt2.1* drastically declined to 80.5 mmol.mol^{-1} *TlUbi* mRNA (700-fold) observed 7 h after the addition of the NH_4^+ (Fig. 4B). Thereafter, a very strong induction to an average value of 4473 mmol.mol^{-1} *TlUbi* mRNA was observed when the ammonium was totally depleted. The increase of relative transcript abundance continued until the end of the experiment (46 500 mmol.mol^{-1} *TlUbi* mRNA) in the N-starved cells.

For *TlNrt2.3* expression, the relative transcript abundance declined very quickly 328-fold (5.5 mmol.mol^{-1} *TlUbi* mRNA) in 4 h after the addition of NH_4^+ (Fig. 4D). Thereafter, the transcript level increased when the external ammonium was completely depleted (309.9 mmol.mol^{-1} *TlUbi* mRNA). The increase of relative transcript abundance continued until the end of the experiment (1526.6 mmol.mol^{-1} *TlUbi* mRNA in day 2) in the N-starved cells.

The *TlNrt2.2* gene transcript level was low and remained relatively constant at an average of 2.7 mmol.mol^{-1} *TlUbi* mRNA throughout the experiment (Fig. 4C).

Finally, the *TlNrt2.4* expression profile was very atypical. In the nitrogen-starved cultures of *T. lutea*, the transcript level was low, with an average value of 1.5 mmol.mol^{-1} *TlUbi* mRNA (Fig. 4E). Following the addition of NH_4^+ to the N-starved cultures, the *TlNrt2.4* transcript level significantly increased by 6.6-fold after 4 h (10 mmol.mol^{-1} *TlUbi* mRNA) and remained approximately stable until day 1. Thereafter, the transcript level of *TlNrt2.4* fell dramatically in day 2 in the N-starved cells with an average of 1.76 mmol.mol^{-1} *TlUbi* mRNA.

Transcriptional responses of *TlNrt2* genes according to N/C cell ratio

N and C cell quotas have been analysed during the sampling of the ammonium batch culture in order to determine the N/C cell ratio. Thus the transcriptional responses of *TlNrt2* genes have been highlighted according to the N status of *T. lutea* cells (Fig. 5). In concordance with the results, three distinct expression profiles are observed as indicated in figures 5A, 5B and 5C respectively. *TlNrt2.1* and *TlNrt2.3* inducible genes responded with a same pattern, which is uncorrelated to the N/C cell ratio. For instance at the highest N/C values the expression of these two genes is only dependent of the residual nitrogen concentration (Fig. 5A). This non-correlation to the N/C cell ratio is signed by a “loop” pattern on the graph. As expected, the expression of *TlNrt2.2* constitutive gene was independent of N/C ratios as the residual nitrogen depletion (Fig. 5B). On the other hand, the *TlNrt2.4* gene was expressed regardless to the presence of ammonium substrate (Fig. 5C). A high expression of *TlNrt2.4* gene was observed for both residual nitrogen levels when the cell quota was full. In contrast, its gene expression was strongly repressed for empty cell quota (5-fold). Moreover a significant threshold near to N/C=0.11 ($P < 0.05$; $n = 21$) was observed.

Regulation of *Tisochrysis lutea Nrt2* genes by nitrate and different reduced nitrogen sources.

In our earlier experiment, we observed an expression of three *TlNrt2* genes in N-starvation conditions (Figs. 3 and 4). In order to investigate the impact of high concentrations of different nitrogen sources (1 mM of total nitrogen) on *TlNrt2* gene expression, a short-term incubation experiment (lasting two hours) using different sources of nitrogen was performed after N-starvation preconditioning.

Under nitrate-repletion, the mRNA abundance of *TlNrt2.1* decreased until reaching a value equal to 0.5-fold the value of the mRNA abundance observed under nitrogen-starvation (Fig. 6A). Interestingly, under nitrite repletion, the mRNA abundance of *TlNrt2.1* also decreased but quicker than

under nitrate-repletion (0.2-fold at 2 hours). Finally, under ammonium nitrate-repletion, the expression of *TlNrt2.1* gene was rapidly and strongly repressed after 30 min.

After 2 hours under nitrate repletion, the mRNA abundance of *TlNrt2.3* decreased very slightly to 0.7-fold compared with the mRNA abundance under nitrogen-free conditions. The slight decrease of *TlNrt2.3* gene was minor compared with the decrease in *TlNrt2.1* mRNA abundance. However, results also show that the *TlNrt2.3* expression profile was quite similar to that of *TlNrt2.1* in response to nitrite repletion and AN repletion (Fig. 6C). Indeed, after nitrite addition, the repression of the *TlNrt2.3* gene was very strong compared with nitrogen-free conditions (0.1-fold). The minimal transcript level of *TlNrt2.3* was observed 2 h after the addition of ammonium nitrate (no expression was observed after 2 hours).

The expression of *TlNrt2.2* gene was relatively constant throughout the experiment and seemed to be the same whatever the conditions of N repletion (Fig. 6B). Therefore, results confirmed that *TlNrt2.2* is constitutively expressed in these conditions.

Unexpectedly, the expression profile of *TlNrt2.4* gene under various N-substrate conditions was completely different from the other *TlNrt2* genes (Fig. 6D). Indeed, under nitrate- and nitrite-repletion, the mRNA abundance of *TlNrt2.4* decreased for at least 1 hour, with a ratio of 0.3-fold and 0.2-fold the level of mRNA observed under N-starvation conditions. Under AN repletion, results showed an induction of *TlNrt2.4* expression compared with the initial conditions (5.6-fold). Interestingly, at 2 hours, a peak of *TlNrt2.4* expression was observed whatever the nitrogen substrate, with ratios corresponding to 1.4-fold, 3-fold and 14-fold the mRNA abundance for the nitrate, nitrite and AN, respectively.

The addition of different nitrogen substrates involved in the nitrogen assimilation pathway allowed us to reveal the regulation kinetics of three *TlNrt2* genes (*TlNrt2.1*, *TlNrt2.3* and *TlNrt2.4*) and the absence of *TlNrt2.2* gene regulation in these conditions.

Discussion

To cope with the availability of nitrate in the environment, plants have transporters with different affinity for this substrate. A family of genes encoding high-affinity nitrate transporters (NRT2) is involved in nitrate uptake when the external nitrate concentrations are very low (µM). In recent years, several *Nrt2* genes have been identified in some land plants (Cai et al., 2008; Krapp et al., 1998; Orsel et al., 2002) and microalgae (He et al., 2004; Koltermann et al., 2003; Quesada et al., 1994). For instance, in the green algae *C. reinhardtii*, four members of the *Nrt2* gene family were identified (Galvan & Fernandez 2001). More recently, in the diatom *Thalassiosira pseudonana*, three putative nitrate transporter genes were reported (Song and Ward, 2007). However, most studies in microalgae only describe the global expression profile of the *Nrt2* gene family and not the individual *Nrt2* gene expression (Hildebrand and Dahlin, 2000; Kang et al., 2007; Song and Ward, 2007). Among these reports, only two studies have investigated the Haptophyta phylum. In this paper, we describe for the

first time the individual *Nrt2* gene expression pattern under specific physiological conditions in the haptophyte *T. lutea*.

In silico analyses reveal four genes encoding putative high-affinity nitrate transporters (*TlNrt2*) with original characteristics.

We identified four genes encoding putative high-affinity nitrate transporters in the marine microalgae *T. lutea*. The identification was performed from transcriptomic data (Carrier et al., 2014) using homology searches with NRT2 of other species. Interestingly, cloning of genomic sequences and their alignment with the cDNA sequences did not reveal the presence of introns in putative *TlNrt2* genes. Although the presence of introns in *Nrt2* genes appears to be very variable, the presence of at least two introns have been described in dicotyledonous plants and some microalgae (Bhadury et al., 2011; Orsel et al., 2002; Plett et al., 2010), whereas no intron was detected in grasses plants (Plett et al., 2010). In microalgae particularly, Song and Ward (2007) characterized *Nrt2* genes from several marine species and especially detected four introns in *Nrt2* gene sequence of another haptophyte *E. huxleyi*. Nevertheless, Bhadury *et al.* (2011) suggest that there are large variations in intron number and length in phytoplankton genomes.

In silico analyses showed that the four TlNRT2 deduced amino acid sequences are very similar to one another (95.7% similarity), but have fairly low similarities with NRT2 of other phyla (land plants, green algae, fungi and yeast) (See Appendix S4 in supporting information). This can be explained by the fact that these species are relatively distant in terms of evolution, which is supported by the homology tree (Fig. 2). The distances shown by the tree confirm that the *Nrt2* genes from haptophytes cluster with the *Nrt2* genes from land plants and green algae and differ completely from other phyla. Nevertheless, our results also reveal that the Haptophyta *Nrt2* genes are distinct to the green lineage and form an independent group.

The full bioinformatic analyses reveal that the deduced TlNRT2 amino acid sequences have all the features of High Affinity Nitrate Transporters described in literature (Forde, 2002): 1) the 12 membrane-spanning domains separated by a central cytosolic loop as described in Major Facilitator Superfamily members, 2) the MFS conserved domain and 3) the NRT2 conserved motif. These results confirm that the four putative TlNRT2 belong to the MFS family and encode high-affinity nitrate transporter proteins. The alignment also reveals that the central cytosolic loop in TlNRT2 proteins is reduced (20-30 AA), as in land plants and microalgae, whereas this loop is much extended in yeast and fungi (90 AA) (Fig. 1). Furthermore, TlNRT2 proteins present an extended N-terminal domain (20-30 AA) and a large C-terminal domain (60-70 AA), as described in NRT2 from green lineage species (Forde, 2000; Quesada et al., 1994; Trueman et al., 1996). In fact, previous studies suggested that the cytosolic loop and the C-terminal domain may have a regulatory function (Due et al., 1995;

Katagiri et al., 1992; Liu et al., 1995). Indeed, conserved protein kinase C recognition motifs (S/T-X-R/K) are present in the N- and C-terminal domains of plant and microalgal NRT2 and in the central loop of the fungal NRT2 (Fig. 1). The existence of these motifs could indicate that phosphorylation and dephosphorylation reactions play a part in the regulation of the activity of the NRT2 proteins. However, the analyses reveal that these kinase C specific phosphorylation motifs were not present in TlNRT2 sequences and the lack of these conserved phosphorylation sites in TlNRT2 proteins could imply that high-affinity nitrate transporters in *T. lutea* are regulated differently.

Interestingly, the presence of an atypical sequence of 100 amino acids length in TlNRT2 sequences reveals an original specificity of the Haptophyta. The BLAST analysis results of this additional part with the non-redundant protein sequences (NCBI) fitted only with NRT2 sequences of another haptophyte *E. huxleyi* (data not shown). This unusual sequence forms an extracellular loop located between transmembrane domains 9 and 10. Many hypotheses could be formulated about the function of this atypical loop, especially that this additional sequence could be implicated in substrate binding or have a sensor function. These hypotheses could be supported by the fact that *Tisochrysis* NRT2 transporters may not need any NAR2 accessory proteins to function correctly in nitrate uptake, unlike to the majority of NRT2 proteins from land plants and microalgae. Indeed, no genes encoding accessory proteins (NAR2) were identified *in T. lutea*, despite many searches. It seems, therefore, that these accessory proteins are absent, suggesting that the *Tisochrysis* NRT2 could be a "single component", as is the case in yeast and fungal species. This Haptophyta particularity will require further investigations to elucidate the effective role of this additional loop. To our knowledge, it is the first time that this feature has been described.

Genes encoding high-affinity transporters present different expression levels

In concordance with the literature, *TlNrt2* genes present different expression levels (Figs. 3 and 4). Indeed, high expression was detected for the *TlNrt2.1* gene, with a strong expression under N-starvation but also under nitrate-sufficient conditions. The *TlNrt2.3* is also strongly expressed in these conditions. Unlike the two latter genes, *TlNrt2.4* gene was poorly expressed and did not reach the levels of expression of two other genes. The lowest expression level for *TlNrt2* genes was observed for *TlNrt2.2*, which has a very low expression in all conditions tested (Figs. 3 and 4). This is in concordance with previous studies in plants and microalgae that described a specific role for each of the nitrate transporters of the same family (Galván et al., 1996; Orsel et al., 2002).

Experiments using NO_3^- or NH_4^+ as nitrogen sources reveal the presence of inducible and constitutive systems in *Tisochrysis lutea*.

Previous studies reported the total mRNA abundance of *Nrt2* genes in several microalgae species during batch cultures, but without distinguishing the *Nrt2* genes individually (Kang et al., 2007; Song

and Ward, 2007). In this study, we monitored, for the first time, the individual time-course of the expression of the four *Nrt2* genes in *T. lutea* depending on N source and N availability (Figs. 3 and 4). During the nitrate batch experiment, the transcript level of *TlNrt2.1* increased when the residual nitrate reached 30 µM and peaked when the nitrate became undetectable in the medium (Fig. 3B). These results showed that *TlNrt2.1* could be considered as inducible either by nitrate at low concentration or N starvation. This expression under N starvation can be considered as an adaptive strategy for facilitating the synthesis of NRT2 protein when nitrate is provided. Some microalgae have already demonstrated a high expression of *Nrt2* genes under N starvation (Kang et al., 2007; Song and Ward, 2007). Moreover, this behaviour is also observed in other transporter families that are strongly expressed in the absence of their substrates (Chung et al., 2003; Li et al., 2006; Matsuda and Colman, 1995). Differently, the *TlNrt2.3* expression was maximal at the beginning of the nitrate batch experiment, *i.e.* when the nitrate was sufficient in the medium (Fig. 3D). Later in this experiment, the mRNA abundance of *TlNrt2.3* was slightly decreased, although remaining high, when the cells were in N-starvation conditions (Fig. 3D). These results show that *TlNrt2.3* expression was induced in N-starved cells, but nitrate is required for its maximal expression. Although the expression pattern is not exactly the same, *TlNrt2.1* and *TlNrt2.3* genes belong to the inducible HATS system. In contrast to these observations, the abundance of *TlNrt2.2* gene transcripts was stable throughout the nitrate batch culture (Fig. 3C). This expression pattern suggests that *TlNrt2.2* belongs to the constitutive HATS component of the NRT2 family. Indeed, previous studies have shown that three genes encoding *Nrt2* in *A. thaliana* were characterized as constitutively expressed even under NO_3^--starved conditions and the expression levels did not change substantially during NO_3^- exposure (Okamoto et al., 2003).

Many studies have found that ammonium addition can inhibit the expression of *Nrt2* genes in plants and microalgae (He et al., 2004; Hildebrand and Dahlin, 2000; Koltermann et al., 2003; Krapp et al., 1998; Quesada et al., 1994; Vidmar et al., 2000). Therefore, to determine the ammonium-repressed transcript levels of *TlNrt2* genes in *Tisochrysis lutea*, the mRNA abundances were monitored during ammonium batch culture. As expected, the transcript levels of *TlNrt2.1* and *TlNrt2.3* genes decreased dramatically in presence of ammonium (Figs. 4B and 4D) and showed that these genes are actively repressed by this substrate. Once the ammonium became undetectable, very high induction was detected for *TlNrt2.1* and *TlNrt2.3* genes, as previously observed under N starvation. These expression profiles during ammonium batch culture are in concordance with a previous report that described a reduction of the total *Nrt2* mRNA abundance in the presence of ammonium, but also a strong increase when ammonium became undetectable in the medium in *I. galbana* (Kang et al., 2007). Considering the overall results, *TlNrt2.1* and *TlNrt2.3* genes present a typical inducibility by both nitrate and N starvation but also a down-regulation in the presence of ammonium.

TlNrt2.2 expression remained stable during the ammonium batch culture (Fig. 4C), confirming that this gene belongs to the constitutive HATS system. Indeed, in *C. reinhardtii*, studies showed the

existence of a nitrate transport system IV containing the *CrNrt2.4* gene, which is constitutively expressed and not repressed in the presence of ammonium (Rexach et al., 1999).
Our results on the *TlNrt2.4* gene expression profile from all of our experiments will be discussed in its own specific section below.

***TlNrt2* gene expression is differentially regulated by N metabolites**

Many reviews have reported the regulation of *Nrt2* gene expression by the accumulation of N metabolites in plant cells (Forde, 2000; Miller et al., 2008). So, a short-term experiment was performed by adding high concentrations of different metabolisable nitrogen sources (1 mM) to the medium in order to generate intracellular N-metabolite accumulation.
The results showed that *TlNrt2.1* and *TlNrt2.3* gene expression was down-regulated when nitrogen elements were provided at very high concentrations. A strong repression was observed after only 30 min of treatment with nitrate, nitrite and ammonium nitrate (Figs. 6A and 6C). This repression continued for 1 hour until reaching the maximal repression level. A variable kinetic of gene expression was also observed depending on the substrate (NH_4^+, NO_2^- and NO_3^-). In fact, the differential expression obtained with the different N substrates suggests that the rate of gene repression depends on the position of the substrate in the pathway of nitrate assimilation. These results support the idea that the intracellular N-metabolite accumulation coming from the nitrate assimilation pathway is involved in the regulation of *TlNrt2.1* and *TlNrt2.3* genes. In land plants, several studies have shown that the expression level of genes encoding NRT2 is quickly repressed by the metabolites generated through nitrate assimilation. In fact, ammonium and some amino acids supplied cause an inhibitory effect on nitrate transport. Krapp et al. (1998) showed a decrease of the *NpNrt2* gene expression level in *Nicotiana plumbaginifolia* in the presence of glutamine (Krapp et al., 1998). Similarly, in *A. thaliana*, the addition of arginine to the medium resulted in a strong inhibition of *AtNrt2.1* expression (Zhuo et al., 1999). In the green microalga *C. reinhardtii*, previous studies showed the repression of three *CrNrt2* genes in the presence of ammonium (Quesada et al., 1998). Furthermore, Kang *et al.* (2009) reported that the inhibition of the GS/GOGAT enzymatic system removed the repression of total *Nrt2* genes in *I. galbana* (haptophyte) and *T. pseudonana* (diatom). Our experiment reveals a regulation system of *TlNrt2.1 and TlNrt2.3* genes consistent with those already described in plants and some microalgae. As expected, expression of the constitutive gene *TlNrt2.2* is not affected by this regulation system (Fig. 6B).

***TlNrt2.4*: an atypical putative high-affinity nitrate transporter in *Tisochrysis lutea*.**

In silico analysis of the TlNRT2.4 protein revealed that this gene had some specificities that set it apart from other TlNRT2 proteins (Fig. 1). Only TlNRT2.4 harboured an atypical feature corresponding to the additional presence of 20 AA at the end of the C-terminal domain. Moreover, no protein kinase C recognition motif (conserved or not in NRT2 family) was predicted from its protein sequence.

Nevertheless, the highly conserved motif found in all NRT2 transporters (NRT2 and MFS motif) was clearly identified in the TlNRT2.4 protein and strengthens its status as belonging to the NRT2 family.

Very interestingly, the *TlNrt2.4* expression pattern was totally unexpected. In contrast to other inducible *TlNrt2* genes (*TlNrt2.1* and *TlNrt2.3*), the mRNA abundance of *TlNrt2.4* was very weak during nitrogen starvation (Figs. 3E and 4E). This result could indicate that the *TlNrt2.4* gene is not directly implicated in the N-starvation response, contrary to *TlNrt2.1* and *TlNrt2.3*.

TlNrt2.4 expression was induced under nitrate-sufficient conditions (200 µM), like *TlNrt2.1* and *TlNrt2.3* genes, but its expression levels remained weak compared with these other genes (Fig. 3E). In contrast, in a way surprisingly unlike *TlNrt2.1* and *TlNrt2.3*, the *TlNrt2.4* expression level was induced under AN-sufficient conditions (Fig. 6D). Furthermore, quantitative RT-PCR studies of the *TlNrt2.4* gene revealed a slight yet significant expression during the ammonium batch experiment (Fig. 4E). This pattern contrasts with the repression of *Nrt2* genes by ammonium, which is well described in many species (land plants and microalgae) (Forde, 2000; Koltermann et al., 2003; Krapp et al., 1998). Moreover, the *TlNrt2.4* profile expression was inversely correlated with *TlNrt2.1* and *TlNrt2.3* transcriptional gene responses for almost all experiments.

Considering the atypical expression profile of *TlNrt2.4* gene, a possible relation between its expression and the cell N status has been hypothesised. For this purpose, we observed the evolution of *TlNrt2* gene expression versus the cell N/C quota during the ammonium batch culture (Fig. 5). The results exhibited three distinct patterns depending on the gene. *TlNrt2.1* and *TlNrt2.3* inducible genes were down- and up-regulated according to the nitrogen depletion (total depletion occurred after the highest N/C ratio value) (Fig. 5A). As expected, the expression of the *TlNrt2.2* constitutive gene was independent to both N/C ratios and residual nitrogen (Fig. 5B).

In contrast, the *TlNrt2.4* gene was expressed irrespective of the presence of ammonium substrate (Fig. 5C). In fact, for both of these residual nitrogen levels, high *TlNrt2.4* expression was observed when the cell quota was full. Conversely, expression of this gene was strongly repressed for an empty cell quota. This could mean that *TlNrt2.4* expression would be linked to the cell N status with a significant threshold effect close to N/C = 0.11 ($P < 0.05$; $n = 21$).

These results suggest that *TlNrt2.4* has a different role in the nitrogen transport and/or metabolism pathway. Among several hypotheses, TlNRT2.4 could be involved in storage processes. This hypothesis would be supported by the presence of putative signal sorting in the C-terminal domain leading to a possibly different subcellular localization. Indeed, one study has even reported a storage function for the *AtNrt2.7* gene in *Arabidopsis thaliana* seeds (Chopin et al., 2007). However, to date, none of the NRT2 proteins have been clearly localized in the intracellular compartment of microalgae. Alternatively, given its converse expression pattern, the *TlNrt2.4* gene could be involved in a regulation system. Lastly, the *TlNrt2.4* gene could be a vestigial gene stimulated by N metabolism. Indeed, haptophytes, like other microalgae groups, are issued from secondary endosymbiosis, which implicates genomic rearrangement (McFadden, 2001).

To our knowledge, this is the first time that this original expression profile for *Ntr2* genes has been described. Today, the function of *TlNrt2.4* gene remains unclear and further investigations will be necessary for to improve our understanding of its involvement in the nitrogen metabolism of *T. lutea*.

Conclusion

Due to its natural nutritional properties, the haptophyte *T. lutea* is commonly used as a feed in aquaculture. Recently, it has also been the subject of domestication for various applications (Cadoret et al., 2012). Beyond the obvious economic interest, there is also a fundamental interest in the Haptophyta lineage, which is highly diverse and ecologically dominant in ocean euphotic zones. To date, our understanding of the nitrogen metabolism of *T. lutea* is limited. Accordingly, it would be of great interest to understand the biology of *T. lutea* in relation to nitrogen availability. This first study is an attempt to further knowledge in this direction.

Four genes, encoding putative high-affinity nitrate/nitrite transporters, were identified, cloned and sequenced in *T. lutea*. *In silico* analysis revealed that the *TlNrt2* sequences did not contain introns, but had a unusual sequence of 100 AA forming an extracellular loop, whose function remains to be determined. This original additional sequence seems to be a particularity of the Haptophyta. The monitoring of individual *TlNrt2* gene expression under different nitrogen sources highlights a differential expression profile that leads us to conclude that there are two inducible genes (*TlNrt2.1* and *TlNrt2.3*), one constitutive gene (*TlNrt2.2*) and another gene characterized by an atypical expression profile (*TlNrt2.4*). The expression of the *TlNrt2.4* gene appears to be independent of the substrate used, while that of the other *TlNrt2* genes seems to be related to the residual nitrogen. From this preliminary study, it is hazardous to speculate on the role of each gene in sensing and/or uptake of nitrogen substrates. Further investigations need to be done to reveal the true function of high-affinity nitrate/nitrite transporters in *T. lutea*. As for most microalgae of interest, the lack of genetic tools prevents us from using mutants to characterize the true function and localization (using GFP-chimeras) of these *TlNrt2* genes in the cell.

Finally, this study contributes to an important topic of current research in plant nutrition via the analysis of nutrient transporter proteins, in our case those involved in nitrogen metabolism of microalgae. The understanding of microalgal nitrogen metabolism will provide us with fundamental knowledge but will also make a major contribution to the establishment of sustainable nitrogen management during biomass production with respect to the well-being of human populations.

Author contributions

AC wrote the manuscript. All cell culture experiments and sampling were performed by AC, JBB, FF AFC, CR and BSJ. Gene expression analyses were made by AC and FF. MG and JBB helped for homology tree analysis and figure design, respectively. GC helped with bioinformatic analyses. Molecular analyses (cloning, sequencing, etc.) were performed by AC and NS. Elementary analyses

(N, C) were done by EL. GB, AC, JBB, JPC and BSJ conceived the study and the project was coordinated by GB and BSJ. All authors corrected and approved the final manuscript.

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Supporting Information

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

Appendix S1. List of primers used for *TlNrt2* gene cloning.

Appendix S2. List of RT-Q-PCR primers used for *TlNrt2* gene expression.

Appendix S3. Comparative analyses of *TlNrt2* sequences.

Appendix S4. Comparison of the amino acid sequences of TlNRT2 with NRT2 sequences from *Aspergillus nidulans*, *Hansenula polymorpha*, *Arabidopsis thaliana*, *Oriza sativa*, *Chlamydomonas reinhardtii* and *Dunaliella salina*.

Appendix S5. Proteomic identification of the atypical sequence in *Tisochrysis lutea*.

Figure legends:

Fig. 1. Alignment of NRT2 protein sequences from *Aspergillus nidulans*, *Hansenula polymorpha*, *Arabidopsis thaliana*, *Oriza sativa*, *Dunaliella salina* and *Tisochrysis lutea*. Alignment was performed with ClustalW. Sequence identities are indicated by a black background and homologies by a grey background.

Fig. 2. Homology tree of high-affinity nitrate transporters (NRT2) composed of 37 NRT2 sequences from bacteria, yeast, fungi, algae and land plants. Accession numbers are given in the Material and Methods section. The amino acid sequences were aligned using MUSCLE software and the phylogeny tree built in PhyML software (Dereeper et al., 2008).

Fig. 3. Various parameters of *Tisochrysis lutea* cells grown in nitrate batch culture. Axenic preculture of *Tisochrysis lutea* was grown in a Conway medium with 1 mM $NaNO_3$. During exponential phase, cells were inoculated into a new modified Conway medium containing 200 µM $NaNO_3$. (A) concentrations of particular carbon (measured using a CN elemental analyzer) and residual nitrate (measured using an automated spectrophotometric method) (µM) during the batch culture (B) relative mRNA abundance of *TlNrt2.1* (C) relative mRNA abundance of *TlNrt2.2* (D) relative mRNA abundance of *TlNrt2.3* (E) relative mRNA abundance of *TlNrt2.4*. Each value represents the mean ± SE of three independent experiments.

Fig. 4. Various parameters of *Tisochrysis lutea* cells grown in ammonium batch culture. Axenic preculture was grown in a modified Conway medium with 200 µM NH_4Cl. In stationary phase, cells with an empty quota were inoculated into non-enriched seawater for 24 hours in order to lower the natural nitrogen content of the seawater. Then, a new modified Conway medium with 200 µM NH_4Cl was added (arrow on the temporal axis). (A) Concentration of particular carbon and residual nitrate (µM) during the batch culture (B) relative mRNA abundance of *TlNrt2.1* (C) relative mRNA abundance of *TlNrt2.2* (D) relative mRNA abundance of *TlNrt2.3* (E) relative mRNA abundance of *TlNrt2.4*. Each value represents the mean ± SE of three independent experiments.

Fig. 5. Relative mRNA abundance of *TlNrt2* genes versus the N/C cellular ratio (N-status) in the ammonium batch culture of *Tisochrysis lutea*. The initial condition (N-starved) is figured by a filled symbol and the timeline of the culture is initiated by an arrow. Culture was enriched with 200 µM ammonium immediately after the first sampling. The numbers represent the residual ammonium concentrations (µM). N/C ratios were calculated by dividing the particulate N concentration by the particulate C concentration. (A) Relative mRNA abundance of *TlNrt2.1 and TlNrt2.3* genes (B) *TlNrt2.2* gene and (C) *TlNrt2.4* gene versus the N/C cellular ratio.

Fig. 6. Effect of different N-substrates on transcript levels of *TlNrt2* genes. After N starvation, *Tisochrysis lutea* cells were inoculated with different forms of nitrogenous nutrients (1 mM of total nitrogen). RNA extractions were performed at 0, 15, 30, 60, 120 min. Graphs represent the expression profiles of each *TlNrt2* genes under the different N-substrate conditions. Each value represents the mean ± SE of three independent experiments.

Appendix S1. List of primers used for *TlNrt2* gene cloning.

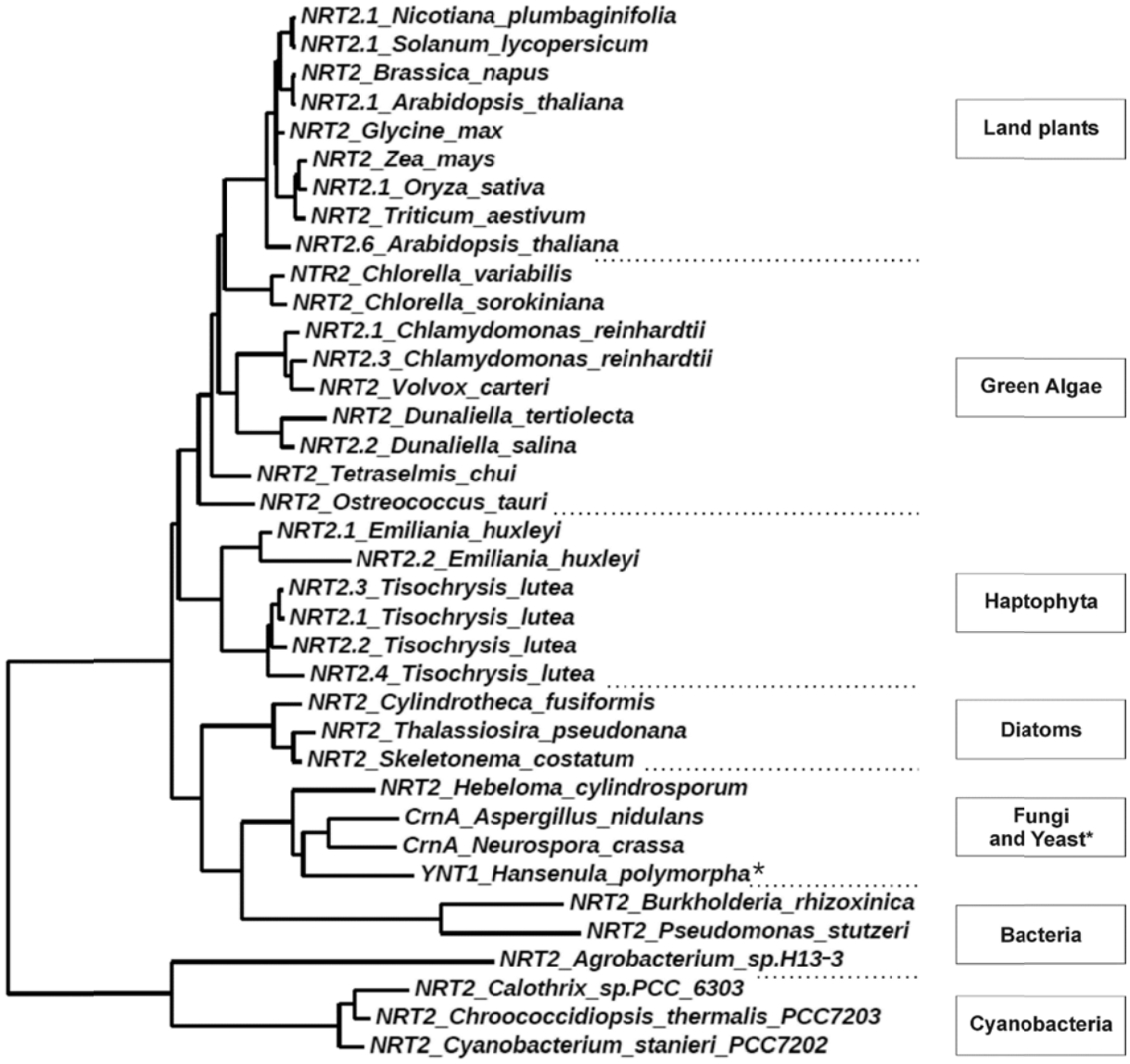
Appendix S2. List of RT-Q-PCR primers used for *TlNrt2* gene expression.

Appendix S3. Comparative analyses of *TlNrt2* sequences. (A) Identities of nucleotide sequences of *TlNrt2* genes (B) Identity and similarity analysis of amino acid sequences. Id: identities and Sim: similarities; all values are indicated in percentages.

Appendix S4. Comparison of the amino acid sequences of TlNRT2 with NRT2 sequences from *Aspergillus nidulans*, *Hansenula polymorpha*, *Arabidopsis thaliana*, *Oriza sativa*, *Chlamydomonas reinhardtii* and *Dunaliella salina*. Id: identities and Sim: similarities; all values are indicated in percentages.

Appendix S5. Proteomic identification of the atypical sequence in *Tisochrysis lutea*. Alignment of TlNRT2.1 protein with some peptides identified by shotgun proteomic analysis performed on *Tisochrysis lutea*. The atypical sequence harboured by TlNRT2 proteins was boxed. Two peptides (peptides 6 and 7) were identified on the atypical sequence of all TlNRT2 proteins.


MFS domain
NRT2 domain
1
2
3
4
5
6
7
8
9
10
11
12
Transmembrane domain
Extended N-terminal sequence for plants and microalgae
Extended C-terminal sequence for plants and microalgae
Large cytosolic loop for fungi and yeast
Atypical loop for TINRT2
Conserved protein kinase C recognition motif
Protein kinase C recognition motif in TINRT2
Protein kinase C recognition motif for fungi and yeast


NRT2.1_Nicotiana_plumbaginifolia
NRT2.1_Solanum_lycopersicum
NRT2_Brassica_napus
NRT2.1_Arabidopsis_thaliana
NRT2_Glycine_max
NRT2_Zea_mays
NRT2.1_Oryza_sativa
NRT2_Triticum_aestivum
NRT2.6_Arabidopsis_thaliana
NTR2_Chlorella_variabilis
NRT2_Chlorella_sorokiniana
NRT2.1_Chlamydomonas_reinhardtii
NRT2.3_Chlamydomonas_reinhardtii
NRT2_Volvox_carteri
NRT2_Dunaliella_tertiolecta
NRT2.2_Dunaliella_salina
NRT2_Tetraselmis_chui
NRT2_Ostreococcus_tauri
NRT2.1_Emiliania_huxleyi
NRT2.2_Emiliania_huxleyi
NRT2.3_Tisochrysis_lutea
NRT2.1_Tisochrysis_lutea
NRT2.2_Tisochrysis_lutea
NRT2.4_Tisochrysis_lutea
NRT2_Cylindrotheca_fusiformis
NRT2_Thalassiosira_pseudonana
NRT2_Skeletonema_costatum
NRT2_Hebeloma_cylindrosporum
CrnA_Aspergillus_nidulans
CrnA_Neurospora_crassa
YNT1_Hansenula_polymorpha*
NRT2_Burkholderia_rhizoxinica
NRT2_Pseudomonas_stutzeri
NRT2_Agrobacterium_sp.H13-3
NRT2_Calothrix_sp.PCC_6303
NRT2_Chroococcidiopsis_thermalis_PCC7203
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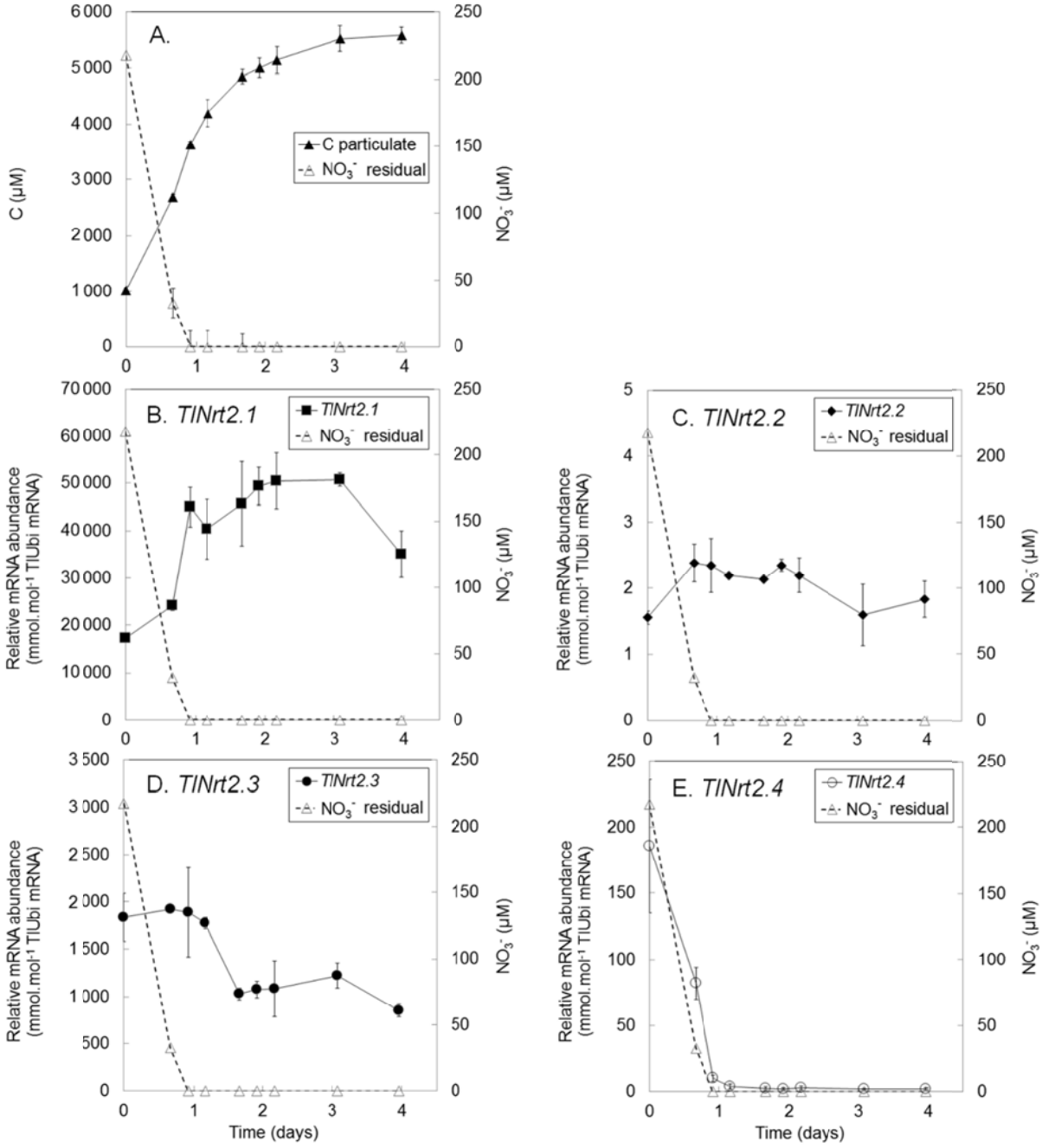
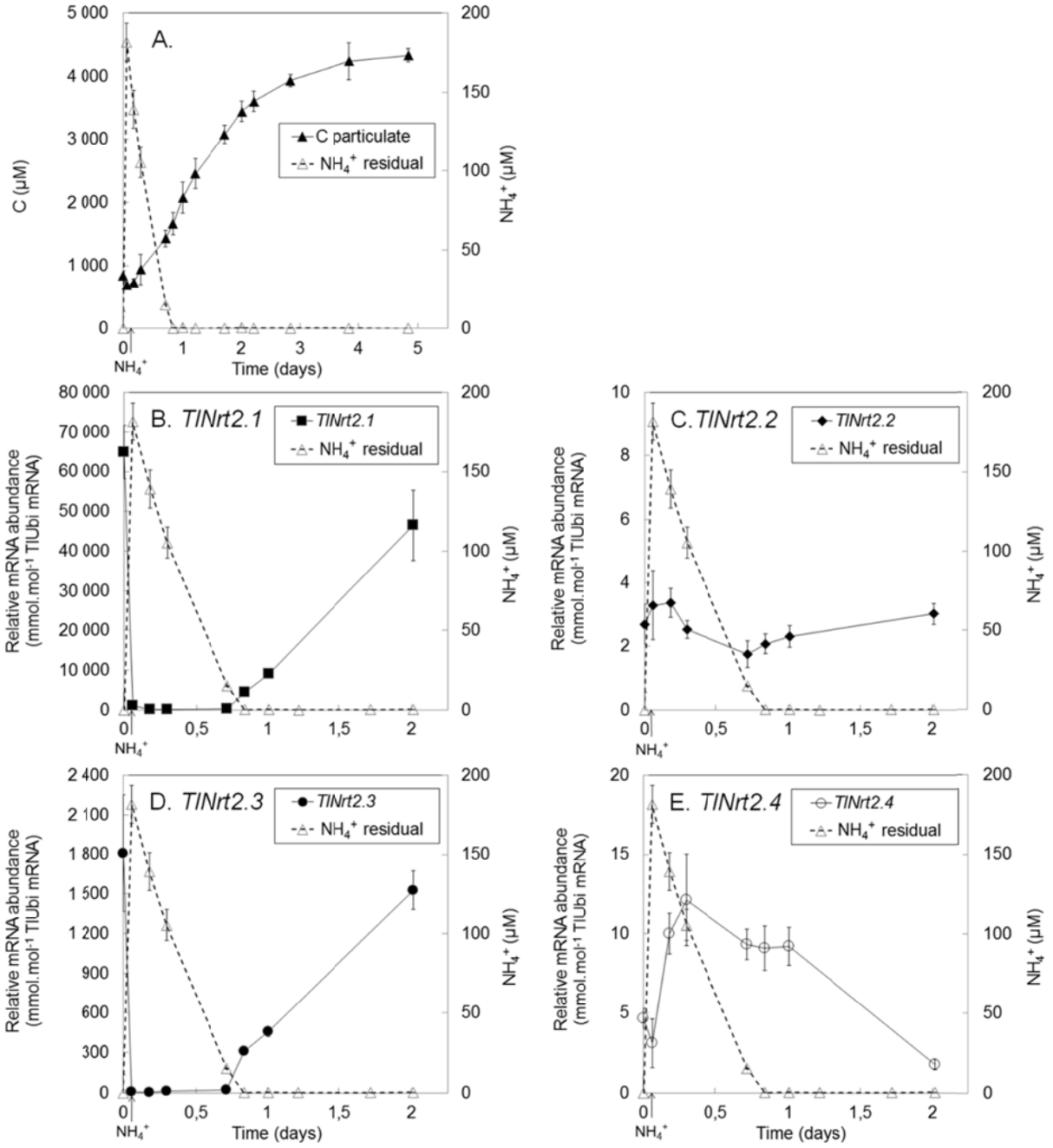
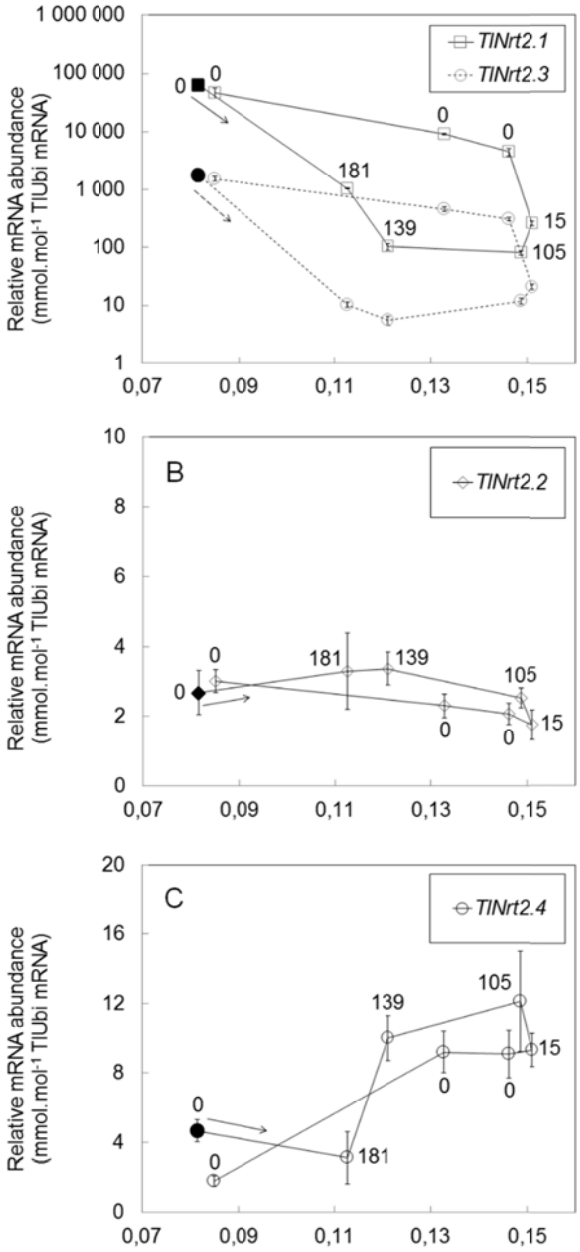
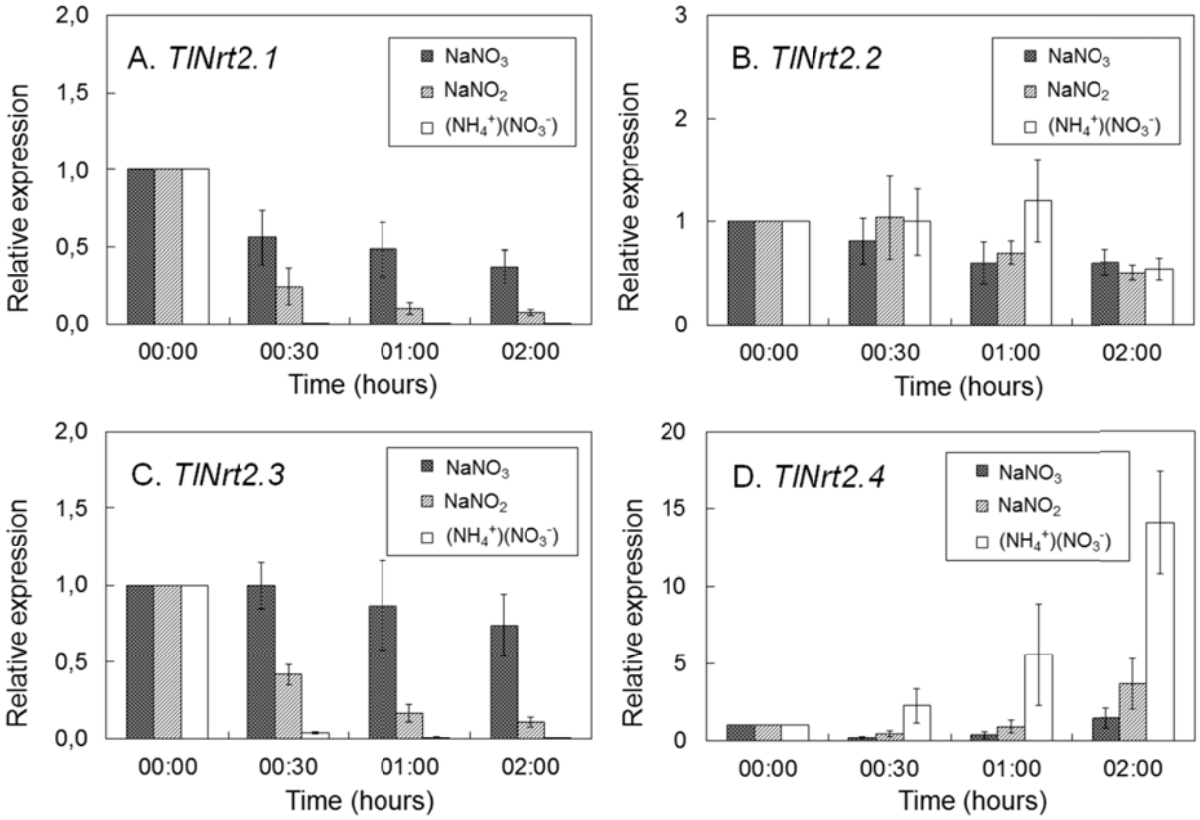
Land plants
Green Algae
Haptophyta
Diatoms
Fungi and Yeast*
Bacteria
Cyanobacteria

2.


A.
C particulate
NO3- residual
C (µM)
NO3- (µM)
B. TlNrt2.1
TlNrt2.1
NO3- residual
Relative mRNA abundance (mmol.mol-1 TlUbi mRNA)
NO3- (µM)
C. TlNrt2.2
TlNrt2.2
NO3- residual
Relative mRNA abundance (mmol.mol-1 TlUbi mRNA)
NO3- (µM)
D. TlNrt2.3
TlNrt2.3
NO3- residual
Relative mRNA abundance (mmol.mol-1 TlUbi mRNA)
NO3- (µM)
Time (days)
E. TlNrt2.4
TlNrt2.4
NO3- residual
Relative mRNA abundance (mmol.mol-1 TlUbi mRNA)
NO3- (µM)
Time (days)


A.
C particulate
NH4+ residual
C (µM)
NH4+ (µM)
Time (days)
B. TlNrt2.1
TlNrt2.1
Relative mRNA abundance (mmol.mol-1 TlUbi mRNA)
C. TlNrt2.2
TlNrt2.2
D. TlNrt2.3
TlNrt2.3
E. TlNrt2.4
TlNrt2.4


1 000 000
100 000
10 000
1 000
100
10
1
Relative mRNA abundance
(mmol.mol-1 TIUbi mRNA)
TlNrt2.1
TlNrt2.3
0
0
0
0
181
139
15
105
0,07
0,09
0,11
0,13
0,15
B
TlNrt2.2
10
8
6
4
2
0
0
0
181
139
105
0
0
15
C
TlNrt2.4
20
16
12
8
4
0
0
0
181
139
105
0
0
15


A. TlNrt2.1
B. TlNrt2.2
C. TlNrt2.3
D. TlNrt2.4
NaNO3
NaNO2
(NH4+)(NO3-)
Relative expression
Time (hours)
00:00
00:30
01:00
02:00
2,0
1,5
1,0
0,5
0,0
3
2
1
0
20
15
10
5

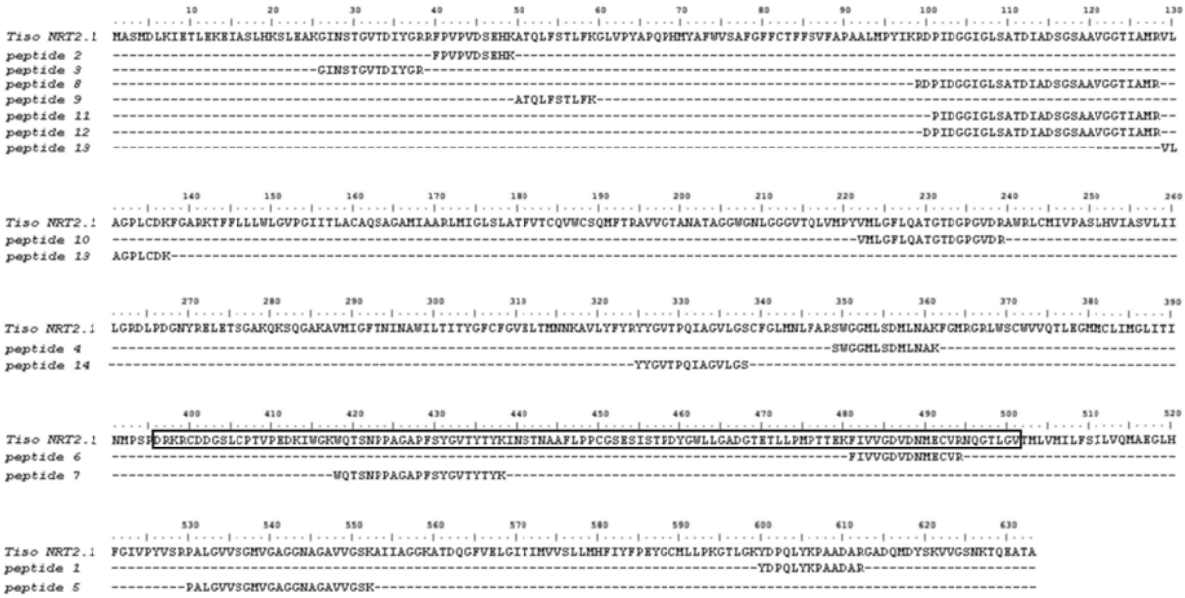
Gene target	Primer	Primer nucleotide sequence (5'- 3')	Amplicon size (bp)
TlNrt2.1	Forward	TCTCCCCAATCGTCTCGC	2115
	Reverse	CAACCCTGCAACGCTCTCAC	
TlNrt2.2	Forward	AACCCTTTGACGGCTCCAG	2463
	Reverse	TGGCGATTGATTCACCTTGC	
TlNrt2.3	Forward	TCCCCTACCACATCCTCG	2278
	Reverse	AATCGTAGCGTGAGACATCG	
TlNrt2.4	Forward	CGCTTCCATCTGTATTGTCC	2333
	Reverse	CGTTGTTAGCTTTGCGTTAGG	

Gene target	Primer	Primer nucleotide sequence (5'- 3')	Amplicon size (bp)
TlNrt2.1	Forward	AAAACCCAAGAGGCTACTGC	100
	Reverse	CGTACGTAGCGGTTAGTCG	
TlNrt2.2	Forward	CAAATTGTGTTTGCGTTGC	193
	Reverse	GGGAAGGCTTCGAATAACC	
TlNrt2.3	Forward	CGACCACCGCTTGAGCC	129
	Reverse	CAATGGGCACCTGAACGG	
TlNrt2.4	Forward	AATGCAGGAAACTGAGTTGG	168
	Reverse	ACTCGAGGCACAAGTTGC	
UBI	Forward	AACTGTGGAGGTGGAGGAGT	141
	Reverse	TTGTAGTCGCCAATCGTCTT	
GAPDH	Forward	CGGTGCTCAATGTAGTGGTT	150
	Reverse	TAGTGATCATGCCCTTCTCG	

A	Gene	*TlNrt2.1*	*TlNrt2.2*	*TlNrt2.3*	*TlNrt2.4*
	TlNrt2.1	100	75.8	82.8	68.4
	TlNrt2.2	75.8	100	79.1	70.1
	TlNrt2.3	82.8	79.1	100	70
	TlNrt2.4	68.4	70.5	70	100

B	Protein	TlNRT2.1	TlNRT2.2	TlNRT2.3	TlNRT2.4
	TlNRT2.1	Id 100 Sim 100	Id 80.0 Sim 89.1	Id 92.9 Sim 95.7	Id 65.1 Sim 76.5
	TlNRT2.2	Id 80.0 Sim 89.1	Id 100 Sim 100	Id 80.6 Sim 88.7	Id 65.1 Sim 74.8
	TlNRT2.3	Id 92.9 Sim 95.7	Id 80.6 Sim 88.7	Id 100 Sim 100	Id 65.2 Sim 76.1
	TlNRT2.4	Id 65.1 Sim 76.5	Id 65.1 Sim 74.8	Id 65.2 Sim 76.1	Id 100 Sim 100

Protein	AnCrnA	HpYNT1	AtNRT2.1	OsNRT2	CrNRT2.3	DsNRT2.1
TlNRT2.1	Id 27.1 Sim 41.8	Id 26.2 Sim 40.2	Id 32.7 Sim 45.6	Id 34.2 Sim 47.2	Id 34.0 Sim 46.5	Id 34 Sim 48.8
TlNRT2.2	Id 28.2 Sim 43.1	Id 26.7 Sim 39.9	Id 34.3 Sim 47.2	Id 34.4 Sim 47.4	Id 34.7 Sim 46.7	Id 33.1 Sim 48.1
TlNRT2.3	Id 26.7 Sim 41.8	Id 26.4 Sim 40.9	Id 32.9 Sim 45.1	Id 34.5 Sim 46.6	Id 32.3 Sim 44.5	Id 34.9 Sim 46.9
TlNRT2.4	Id 26.2 Sim 36.5	Id 23.1 Sim 37.2	Id 28.5 Sim 39.6	Id 32.5 Sim 45.0	Id 31.6 Sim 44.4	Id 32.8 Sim 45.6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peptide 2 -----FPVPVDSEHK-----
peptide 3 -----GINSTGVTDIYGR-----
peptide 8 -----RDPIDGGIGLSATDIADSGSAAVGGTIAMR--
peptide 9 -----ATQLFSTLFK-----
peptide 11 -----PIDGGIGLSATDIADSGSAAVGGTIAMR--
peptide 12 -----DPIDGGIGLSATDIADSGSAAVGGTIAMR--
peptide 13 -----VL
Tiso NRT2.1 AGPLCDKFGARKTFFLLLWLGVPGIITLACAQSAGAMIAARLMIGLSLATFVTCQVWCSQMFTRAVVGTANATAGGWGNLGGGVTQLVMPYVMLGFLQATGTDGPGVDRAWRLCMIVPASLHVIASVLII
peptide 10 -----VMLGFLQATGTDGPGVDR-----
peptide 13 AGPLCDK-----
Tiso NRT2.1 LGRDLPDGNYRELETSGAKQKSQGAKAVMIGFTNINAWILTITYGFCFGVELTMNNKAVLYFYRYYGVTPQIAGVLGSCFGLMNLFARSWGGMLSDMLNAKFGMRGRLWSCWVVQTLEGMMCLIMGLITI
peptide 4 -----SWGGMLSDMLNAK-----
peptide 14 -----YYGVTPQIAGVLGS-----
Tiso NRT2.1 NMPSPDRKRCDDGSLCPTVPEDKIWGKWQTSNPPAGAPFSYGVTYTYKINSTNAAFLPPCGSESISTPDYGWLLGADGTETLLPMPTTEKFIVVGDVDNMECVRNQGTLGVTMLVMILFSILVQMAEGLH
peptide 6 -----FIVVGDVDNMECVR-----
peptide 7 -----WQTSNPPAGAPFSYGVTYTYK-----
Tiso NRT2.1 FGIVPYVSRPALGVVSGMVGAGGNAGAVVGSKAIIAGGKATDQGFVELGITIMVVSLLMHFIYFPEYGCMLLPKGTLGKYDPQLYKPAADARGADQMDYSKVVGSNKTQEATA
peptide 1 -----YDPQLYKPAADAR-----
peptide 5 ----------PALGVVSGMVGAGGNAGAVVGSK-----