Shuttling of (deoxy-) purine nucleotides between compartments of the diatom *Phaeodactylum tricornutum*

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Summary

- Diatom plastids show several peculiarities when compared with primary plastids of higher plants or algae. They are surrounded by four membranes and depend on nucleotide uptake because, unlike in plants, nucleotide *de novo* synthesis exclusively occurs in the cytosol. Previous analyses suggest that two specifically adapted nucleotide transporters (NTTs) facilitate the required passage of nucleotides across the innermost plastid membrane. However, nucleotide transport across the additional plastid membranes remains to be clarified.
- Phylogenetic studies, transport assays with the recombinant protein as well as GFP-based targeting analyses allowed detailed characterization of a novel isoform (*PtNTT5*) of the six NTTs of *Phaeodactylum tricornutum*.
- *PtNTT5* exhibits low amino acid similarities and is only distantly related to all previously characterized NTTs. However, in a heterologous expression system, it acts as a nucleotide antiporter and prefers various (deoxy-) purine nucleotides as substrates. Interestingly, *PtNTT5* is probably located in the endoplasmic reticulum, which in diatoms also represents the outermost plastid membrane.
- *PtNTT5*, with its unusual transport properties, phylogeny and localization, can be taken as further evidence for the establishment of a sophisticated and specifically adapted nucleotide transport system in diatom plastids.

Key words: complex plastid, diatom, endoplasmic reticulum (ER), energy, nucleotide synthesis, nucleotide transport.

Introduction

All three main lineages of photosynthetic organisms, glaucophytes, rhodophytes, and chlorophytes (including land plants), according to the best supported hypothesis, gained their plastids in a process called primary endosymbiosis. During this primary endosymbiosis, a cyanobacterium was engulfed, retained and partially degraded by a heterotrophic host (Keeling, 2013). Massive transfer of cyanobacterial genes to the host nucleus as well as metabolic connection and rearrangement of the consortium resulted in control and functional embedding of the symbiont and finally in the establishment of the photosynthetic organelle (Archibald, 2015). Interestingly, a considerable number of the diverse algal lineages arose from secondary endosymbioses. These processes occurred several times and included the fusion, and thus the genetic and metabolic connection, of two eukaryotes, a host cell and an alga with a primary plastid (Keeling, 2013; Archibald, 2015).

Diatoms are an important group of algae with secondary plastids. They are of ecological relevance and of particular scientific interest because they are highly abundant in the oceans, and they are among the most common species of the phytoplankton and contribute immensely to carbon fixation (Falkowski & Oliver,

2007; Armbrust, 2009). Genome analyses suggest that their rapid diversification, enormous success and predominance in the marine ecosystem might result from an extraordinary genetic composition/flexibility (Bowler *et al.*, 2008; Armbrust, 2009; Archibald, 2015).

The complex plastids of diatoms are of red algal origin and possess four surrounding membranes of which the outermost is continuous with the rough endoplasmic reticulum (ER) (Kroth, 2002; Gould *et al.*, 2008). Compared with plants, diatoms exhibit interesting metabolic peculiarities. For instance, nucleotide *de novo* synthesis is cytosolic in diatoms (Ast *et al.*, 2009), whereas in higher plants, the complete pathway of purine and several steps of pyrimidine nucleotide synthesis take place within the plastid (Zrenner *et al.*, 2006; Witz *et al.*, 2012). Accordingly, in diatom plastids, nucleotides have to be transported across additional membranes and purine nucleotides even in an opposite direction (Ast *et al.*, 2009) compared with higher plant plastids (Zrenner *et al.*, 2006; Witz *et al.*, 2012).

Previous studies indicate that at least two nucleotide transporter (NTT) type carriers mediate nucleotide provision to the diatom plastids (Ast *et al.*, 2009), whereas, in higher plant plastids, transport of purine nucleotides and intermediates of pyrimidine nucleotide synthesis apparently involves different types of

carriers (Leroch *et al.*, 2005; Kirchberger *et al.*, 2008; Witz *et al.*, 2012).

Generally, NTT proteins are restricted to only a few organismic groups; they exist in certain obligate intracellular living bacteria and represent a hallmark of the orders *Chlamydiales* and *Rickettsiales* (Schmitz Esser *et al.*, 2004). *Chlamydiales* and *Rickettsiales* are metabolically impaired (Moulder, 1991; Moran, 2002), lack several anabolic pathways, including *de novo* nucleotide synthesis, and exploit the nucleotide and energy pool of the host via interaction of functionally diverse NTT type proton symporters and exchangers (Krause *et al.*, 1985; Tjaden *et al.*, 1999; Haferkamp *et al.*, 2004, 2006; Audia & Winkler, 2006; Knab *et al.*, 2011).

Among eukaryotes, NTTs have been detected in algae (with primary and secondary plastids) and higher plants (Möhlmann *et al.*, 1998; Tjaden *et al.*, 1998b; Linka *et al.*, 2003; Tyra *et al.*, 2007; Ast *et al.*, 2009) and in protists of the phylum *Microsporidia* (Tsaousis *et al.*, 2008; Heinz *et al.*, 2014), whereas other protists, fungi and animals apparently lack such *ntt* sequences in their genomes. NTTs of primary plastids were shown to operate exclusively as ATP/ADP exchangers (Möhlmann *et al.*, 1998; Tjaden *et al.*, 1998b; Linka *et al.*, 2003) in the inner plastid envelope. Analyses of mutant plants clearly demonstrated their role in energy provision to the stroma, particularly when photosynthesis is insufficient or missing (Tjaden *et al.*, 1998a; Geigenberger *et al.*, 2001; Reiser *et al.*, 2004; Reinhold *et al.*, 2007). *Microsporidial* NTTs reside in the plasma membrane or in the mitosome (a mitochondrial relict unable to produce energy in the form of ATP) and mediate ATP/ADP exchange or purine nucleotide transport (Tsaousis *et al.*, 2008; Heinz *et al.*, 2014).

Although, in recent years, the biochemical characteristics and physiological relevance of NTTs from several organisms have been revealed, our knowledge of NTTs from complex plastids is comparatively limited. This is because (1) metabolite transport into and/or out of complex plastids has not been investigated in detail, (2) diatoms possess an unusually high number of NTTs (six to eight isoforms) compared with other algae or higher plants (one to three isoforms), and finally (3) the two NTTs from diatoms characterized to date (NTT1 and NTT2) exhibit functional properties previously not documented for any plastidial NTT and only rarely shown for NTTs from other organisms.

NTT1, and probably also NTT2, are located in the innermost envelope of the diatom plastid (Ast *et al.*, 2009). The diatom NTT1 isoform acts as a proton symporter and accepts mono-, di-, and triphosphorylated adenine nucleotides as substrates, whereas the NTT2 isoform transports various triphosphorylated nucleotides, including deoxy transports, in a counter exchange mode. Physiological interaction of NTT1 and NTT2 has been suggested to deliver all nucleotides for DNA and RNA synthesis to the stroma. Although NTT1 and NTT2 proteins represent important components of the plastidial nucleotide uptake system in centric (*Thalassiosira pseudonana*) and pennate (*Phaeodactylum tricornutum*) diatoms, until now it has not been clarified how all required (deoxy-) nucleotides pass through the remaining membranes of the complex plastid (Ast *et al.*, 2009).

In this context, it is important to keep in mind that nucleotide transport can connect the sites of nucleotide synthesis and

consumption, and/or play a key role in the adjustment of the subcellular energy states. Just recently, plastids and mitochondria of diatoms were shown to be extraordinarily tightly connected by extensive energetic interaction (Bailleul *et al.*, 2015). This metabolic communication relies on the transport of reducing power from plastids to mitochondria as well as on re-shuttling of energy (from mitochondria to plastids), representing a main factor in the adaptation of cellular ATP/NADPH ratios for optimized photosynthetic performance, carbon fixation and growth (Bailleul *et al.*, 2015). Accordingly, the analysis of putative NTT proteins will help to deduce their possible role in nucleotide and/or energy metabolism.

In this study, we focused on NTT isoform 5 from the diatom *P. tricornutum* (P₅NTT5). P₅NTT5 shows comparatively weak relationships to all previously characterized NTTs and here we report that it is also extraordinary in terms of its biochemical properties and subcellular localization.

Materials and Methods

In silico analyses of NTT proteins

Comparative amino acid sequence analysis of NTT proteins was performed using CLUSTALX (Thompson *et al.*, 1997) and GENEDOC (Nicholas *et al.*, 1997). Presence or absence of putative presequences, signal peptides, cleavage sites, chloroplast transit peptides or transmembrane domains was investigated using the programs SIGNALP 3.0, CHLOROP 1.1, TARGETP 1.1, TMHMM (<http://www.cbs.dtu.dk/services/>) (Emanuelsson *et al.*, 2007) and ASAFIND (<http://rocaplab.ocean.washington.edu/tools/asafind/>) (Gruber *et al.*, 2015) following the methods described by Gruber & Kroth (2014).

NTT amino acid sequences were aligned with MAFFT (Katoh & Standley, 2013), and phylogenetic trees were reconstructed with MEGA6 (Tamura *et al.*, 2013) using the neighbor joining method and Poisson correction, the parsimony bootstrap method, and the maximum likelihood method (using the Jones–Taylor–Thornton (JTT) amino acid substitution model); all trees were calculated with 1000× bootstrapping. All positions containing gaps and missing data were eliminated. There were a total of 363 positions in the final data set.

Cultivation of *Phaeodactylum tricornutum*

Phaeodactylum tricornutum Bohlin 1897, strain 646 (available at UTEX Culture Collection of Algae, University of Texas, Austin, TX, USA; <https://utex.org>) was cultivated in f/2 medium at a concentration of 50% sea water (16.6 g of sea salt; Tropic Marin Dr. Biener, GmbH, Wartenberg, Germany) in 1 l of distilled water, adjusted to pH 7.0. Supplements were added as described in Kroth (2007). For agar plate cultivation, corresponding solid media were complemented with 1.2% Bacto Agar (Becton, Dickinson & Co., Le Pont de Claix, France). Cells were grown under continuous illumination at 35 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ or with a day–night cycle (16 h : 8 h, light : dark; for protein preparation and western blotting).

Preparation of cDNA and generation of expression constructs

RNA isolation was performed with the RNeasy plant mini kit (Qiagen, Hilden, Germany) and poly(A⁺) RNA was enriched from this preparation with the Oligotex kit (Qiagen). Production of cDNA from mRNA templates was conducted with the reverse transcriptase SuperscriptII (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The coding sequence of *PtNTT5* was amplified via PCR and oligonucleotides (MWG, Ebersberg, Germany or Sigma Aldrich, Munich, Germany) that allow compatible insertion into the expression vectors. The amplification products were restricted with specific endonucleases, purified from the agarose gel with the NucleoSpin Extract II Kit (Macherey & Nagel, Düren, Germany).

For generation of the *Escherichia coli* expression construct, the coding sequence of full length *PtNTT5* was inserted via the restriction sites *NdeI* and *XhoI* in frame with the histidine tag into the Isopropyl β -D-thiogalactoside inducible expression vector pET16b (Novagen, Heidelberg, Germany).

For the localization studies, the start codon of the eGFP gene of the pPha T1 GFP plasmid (Gruber *et al.*, 2007) (a modified version of the shuttle vector pPha T1; GenBank AF219942; Zaslavskaja *et al.*, 2000) was replaced by the codon GGA (coding for glycine) via site directed mutagenesis to inactivate the potential translation start. The *PtNTT5* amplicon was ligated into the *StuI* site of this modified pPha T1 GFP plasmid. Truncated versions of *PtNTT5* were produced via PCR or deletion PCR with specific oligonucleotides (Supporting Information Table S1) and using the vector containing full length *PtNTT5* with either N or C terminal GFP fusion as a template.

Amplification products were purified and either inserted into the pPha T1 shuttle vectors or re ligated (deletion PCR products). Correctness of the respective constructs was verified by restriction analyses (Fermentas, St Leon Rot, Germany) and sequencing (SEQ IT, Kaiserslautern, Germany or GATC Biotech AG, Konstanz, Germany).

Heterologous expression in *Escherichia coli* and import measurements

The *E. coli* strain Rosetta 2(DE3)pLysS (Novagen) was used for heterologous synthesis of *PtNTT5*. To analyze transport properties of the recombinant *PtNTT5*, either induced or noninduced *E. coli* cells harboring the expression plasmid pET16b *PtNTT5* were incubated in phosphate buffer (KP_i) supplemented with the respective [α ³²P] labeled substrates. Import was conducted at 30°C and terminated by removal of external substrate using vacuum filtration and washing. Radioactivity was quantified by scintillation counting (Tricarb 2500; Canberra Packard, Heidelberg, Germany).

Nuclear transformation and microscopy

Nuclear transformation of *P. tricornutum* was performed as described previously (Kroth, 2007). Cellular localization of GFP

fusion proteins was analyzed with confocal laser scanning microscope LSM 780 or LSM 510 META (Carl Zeiss, Oberkochen, Germany) using a Plan Apochromat 63 $\times$ 1.4 oil immersion Nomarski differential interference contrast (DIC) objective (Carl Zeiss) or an epifluorescence microscope Olympus BX51 (Olympus Europe, Hamburg, Germany) equipped with a Zeiss AxioCam MRm digital camera (Carl Zeiss) and an Olympus PLN $\times$ 40 objective (Olympus). Nucleic acids were stained with the dye Hoechst 33342 (Calbiochem[®]; Behring Diagnostics, La Jolla, CA, USA) to indicate the location of the nucleus, while MitoTracker[®] Orange CM H₂TMRos (Molecular Probes, Inc., Eugene, OR, USA) showed the location of the mitochondria. Image processing was conducted using ZEN LITE and AXIOVISION REL. 4.7 (Carl Zeiss).

Western blot analyses and SDS-PAGE

Cells were harvested during the exponential growth phase by centrifugation (3000 g for 10 min at 4 °C) and the pellet was resuspended in 1 ml of lysis buffer containing protease inhibitor 'complete EDTA free' (Roche, Mannheim, Germany), 50 mM Tris HCl, pH 8, 1 mM EDTA, 8 M urea and 1% SDS. A mixture of glass beads (0.1–1 mm diameter) was added and cells were homogenized in Fast Prep FP 120 Bio 101 Savant (Qbiogene Inc., Carlsbad, CA, USA) at maximum speed six times for 20 s each, being kept on ice for 1 min between each homogenization. Samples were again centrifuged to remove nondisrupted cells. The total protein concentration in the supernatant was determined using the 660 nm Pierce Protein Assay (Thermo Scientific, Schwerte, Germany). Five micrograms of this protein extract was analyzed in a discontinuous, denaturing system on 10% or 12% separating polyacrylamide gels (Laemmli, 1970) and further transferred onto a nitrocellulose membrane (Whatman Protran BA 79; Whatman Inc., Maidstone, UK) electrophoretically using a Trans Blot Turbo (Bio Rad, Hercules, CA, USA). The Page Ruler Prestained Protein Ladder (Thermo Scientific) was used as a marker.

Immunodetection was performed using an anti GFP antibody (Invitrogen, Molecular Probes, Eugene, OR, USA) combined with anti Rabbit IgG peroxidase antibody (Sigma Aldrich). The western blots were treated with Roti[®] Block, Roti[®] Blue and Roti[®] Lumin plus, as described in the manuals (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Chemoluminescence was documented with Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, UK) and Konica SRX 201 (Konica Minolta, Chiyoda, Japan).

Results

The evolutionary origin of *PtNTT5*

To elucidate the possible evolutionary origin of diatom NTTs, we performed a phylogenetic analysis with published and biochemically characterized NTTs from various organisms as well as NTT isoforms from *Thalassiosira pseudonana* (*TpNTT1* 8) and *P. tricornutum* (*PtNTT1* 6).

The overall topology of the obtained phylogenetic tree (Fig. 1) is in agreement with previous studies performed with larger data sets including also various NTTs of unknown function (Tyra *et al.*, 2007; Tsaousis *et al.*, 2008; Ast *et al.*, 2009; Heinz *et al.*, 2014).

Microsporidial isoforms generally constitute a separate branch among the NTTs (Fig. 1; Tsaousis *et al.*, 2008; Heinz *et al.*, 2014). The isoforms of *Trachipleistophora hominis* and *Encephalitozoon cuniculi* form two distinct clusters. This is in line with recent analyses suggesting that the different NTT isoforms of contemporary microsporidians arose from repeated, lineage specific gene duplications whereas the ancestor of

microsporidians probably possessed only a single NTT gene (Heinz *et al.*, 2014).

It is widely accepted that early primary photosynthetic eukaryotes probably acquired the nucleotide transporter gene from a donor closely related to extant chlamydiae (Greub & Raoult, 2003; Schmitz Esser *et al.*, 2004; Tyra *et al.*, 2007), and also in our analysis a monophyletic grouping of plastidial and chlamydial ATP/ADP transporters (bacterial NTT1 isoforms) was evident (Fig. 1). Moreover, plastidial NTTs from higher plants, red algae and diatom NTT1 isoforms form a monophyletic cluster and their close relationship as sister groups suggests that diatom NTT1 proteins originated from the red algal

Fig. 1 Phylogenetic relationships of nucleotide transporters (NTTs) from various organisms. An amino acid based phylogenetic tree calculated with MEGA6 using the maximum likelihood algorithm with the Jones–Taylor–Thornton (JTT) model is shown. Black dots indicate nodes supported by maximum likelihood, maximum parsimony, and neighbor joining bootstrap values (1000× resampling) > 90%. GenBank accession numbers are given in brackets. The bar represents 20% estimated evolutionary distance. Organismic groups are marked by shading: diatoms, orange; red alga, red; higher plants, green; *Chlamydiales*, *Rickettsiales* and *Lawsonia intracellularis*, grayscale; microsporidia, blue. Basic transport features were taken from Schmitz Esser *et al.* (2004), Audia & Winkler (2006), Schmitz Esser *et al.* (2008), Ast *et al.* (2009), Knab *et al.* (2011), Heinz *et al.* (2014) and literature therein.

progenitor engulfed during secondary endosymbiosis (Fig. 1; Ast *et al.*, 2009).

Diatom NTT2 proteins and *Tp*NTT3 form a deep branch together with functionally diverse NTTs from rickettsiae (Ast *et al.*, 2009) or even constitute a separate group among various bacterial and plastidial NTTs (Fig. 1). The remaining diatom NTT isoforms also form a distinct subgroup of NTTs with two well supported subclusters (Fig. 1). One subcluster is formed by *Pt*NTT3, *Pt*NTT4 and the respective homologs from *T. pseudonana*, *Tp*NTT4 and *Tp*NTT5. *Pt*NTT5 and its possible homolog *Tp*NTT6 constitute the second subcluster together with *Pt*NTT6, *Tp*NTT7 and *Tp*NTT8. Therefore, these diatom NTTs seem to be only weakly related to other bacterial and plastidial NTTs and only the cluster formed by the microsporidial NTTs exhibits a higher phylogenetic distance.

The branching pattern indicates that the diatom NTT1 isoform originated from endosymbiotic gene transfer, whereas the ancestor of the remaining diatom NTTs cannot be resolved. It is possible that those proteins arose from the red algal derived NTT1 isoform via several gene duplication events. If this is the case, the origin of these isoforms is apparently masked by considerable alterations in the amino acid sequences, maybe as a consequence of functional constraints. Alternatively, the remaining diatom NTTs may have resulted from horizontal gene transfer from bacterial (or maybe also microsporidial) donors, and subsequent gene duplications led to the establishment of the respective subgroups of NTTs in diatoms. Their distant relationship to the plant NTTs, including the diatom NTT1 isoforms, and the fact that horizontal gene transfer is an important and common phenomenon in diatom evolution, might be taken to support the second scenario (Bowler *et al.*, 2008).

In fact, the nuclear genome of diatoms appears to be a patchwork and comprises a comparatively high number of genes from different bacterial sources (Bowler *et al.*, 2008). The higher GC content of *Pt*NTT5 and *Pt*NTT6 (Table S2), but most importantly the different numbers of introns (Fig. S1) suggest that the remaining NTT isoforms (with no or, in a few cases, one or two introns) do not represent modified duplications of NTT1 (with three introns), but instead were gained by at least one or by several independent horizontal gene transfers. The intron number of putative homologs also differs between the two diatom species and suggests a slightly higher tendency for intron gain in *T. pseudonana*. This is in line with comparative genome analyses documenting a higher number of introns in *T. pseudonana* compared with *P. tricornutum* (Bowler *et al.*, 2008). The diatom isoforms *Pt*NTT3 6 and *Tp*NTT4 8 are phylogenetically clearly separated from the previously characterized NTTs. This is why we were interested in a detailed analysis of *Pt*NTT5 as a first representative member of this particular group of diatom NTTs.

Peculiarities in the amino acid sequence of *Pt*NTT5

From our sequence alignment of *Pt*NTT5 and representative, biochemically characterized NTTs from diverse organisms, it became evident that the amino acid sequences of NTTs from plants and algae possess an N terminal extension compared with

the remaining NTTs (Fig. S2). These extensions generally contain information supporting plastid targeting. In diatoms, nuclear encoded proteins with a stromal destination exhibit a characteristic bipartite arrangement of the N terminal extension (pre sequence) starting with a signal peptide followed by a transit peptide. Moreover, a conserved motif, the so called 'ASAFAP' motif, mostly flanks/overlaps with the predicted signal peptide cleavage site (Kilian & Kroth, 2005; Gruber *et al.*, 2007). NTT1 and NTT2 from diatoms do show such a typically structured N terminal targeting sequence and NTT1 isoforms even contain an authentic 'ASAFAP' motif at the signal peptide (Fig. S2; Ast *et al.*, 2009).

*Pt*NTT5 lacks a comparable N terminal extension and in this context rather resembles microsporidial NTTs which are mainly located in the plasma membrane. *In silico* analyses also failed to detect a possible N terminal signal peptide with a corresponding peptide cleavage site (Ast *et al.*, 2009). Moreover, prediction of the subcellular localization of *Pt*NTT5 was unclear (Ast *et al.*, 2009). However, the absence of characteristic plastidial targeting motifs (Fig. S2; Ast *et al.*, 2009) implies that *Pt*NTT5 resides in a different compartment/membrane from isoforms 1 and 2. A detailed inspection of the *Pt*NTT5 sequence revealed a comparatively high accumulation of positively charged amino acid residues at its far C terminus; however, so far it is unclear whether this finding is of any relevance for correct protein targeting or membrane insertion.

*Pt*NTT5 generally exhibits only moderate amino acid sequence similarities (24–31%) to previously described NTTs, including diatom NTT1 and NTT2 isoforms (Table S3). Several regions that are conserved in plant and bacterial NTTs show higher variations in *Pt*NTT5 as well as in microsporidial NTTs (Fig. S2). Moreover, not all predicted transmembrane domains of *Pt*NTT5 did match with the hydrophobic regions of representative characterized NTTs. Based on sequence similarities, it is hard to judge whether *Pt*NTT5 is involved in plastidial nucleotide transport or whether it even fulfills a different physiological function.

*Pt*NTT5 accepts various purine nucleotides as substrates

We determined the functionality and biochemical properties of *Pt*NTT5 using transport measurements with radioactively labeled substrates and *E. coli* cells expressing the recombinant carrier. Initial studies with [α^{32} P] ATP and [α^{32} P] ADP revealed that *Pt*NTT5 in fact is able to transport nucleotides (Fig. 2). ATP and ADP were efficiently imported into *E. coli* via *Pt*NTT5, whereas noninduced cells (control) showed no or only marginal accumulation of radioactively labeled nucleotides.

To determine the general substrate specificity of *Pt*NTT5, we conducted competition experiments with different nonlabeled nucleotides or nucleotide derivatives. In addition to nonlabeled ATP and ADP, dATP and AMP, as well as the corresponding guanidine nucleotides, markedly reduced [α^{32} P] ATP uptake (Table 1). Among the tested purine nucleotides, GDP addition resulted in the lowest decrease of [α^{32} P] ATP uptake, and the corresponding reduction was similar to that caused by TTP, but

Fig. 2 Time dependent uptake of ATP and ADP via nucleotide transporter isoform 5 from *Phaeodactylum tricornutum* (*PtNTT5*). Transport of [α^{32} P] ATP (a) and [α^{32} P] ADP (b) into Isopropyl β -D-thiogalactoside induced *Escherichia coli* cells synthesizing *PtNTT5* (black symbols) in comparison to nucleotide uptake into corresponding noninduced *E. coli* cells (gray symbols) is shown. Data are the mean of at least three independent experiments, $\pm$ SE.

Table 1 Effects of various nucleotides and nucleotide derivatives on [α^{32} P] ATP import via nucleotide transporter 5 from *Phaeodactylum tricornutum* (*PtNTT5*)

Effector	Import (%)	SE (%)	Effector	Import (%)	SE (%)
(-)	100	0	CDP	71.5	2.9
ATP	26.0	1.0	TMP	99.7	4.5
ADP	28.3	2.7	dATP	33.9	2.2
AMP	21.7	1.7	dGTP	30.1	1.8
GTP	43.0	1.5	dCTP	70.0	1.8
GDP	53.6	1.8	TTP	50.5	6.2
GMP	37.5	2.9	cAMP	104.5	5.3
ITP	67.1	3.2	cGMP	91.2	4.2
IDP	81.9	3.4	NAD	88.2	5.5
IMP	82.5	5.0	PAP	93.6	4.3
UTP	62.8	2.3	PAPS	97.8	11.5
UDP	81.6	3.4	NADP	95.2	6.2
UMP	96.7	3.4	CoA	89.3	4.7
CTP	78.0	3.6	FAD	88.5	3.0

Uptake of [α^{32} P] ATP by recombinant *PtNTT5* at a substrate concentration of 50 μ M was set to 100% (control). Corresponding transport in the presence of nonlabeled effectors (10 fold excess) was calculated according to the control. Import was stopped after 3 min. Nucleotide uptake rates are net values (minus control: ATP uptake into noninduced *Escherichia coli* cells) and the mean of three independent experiments. Bold type is used to highlight significantly reduced import rates (< 50% residual activity).

higher than that produced by UTP addition. To demonstrate that the given molecules do represent substrates competing with ATP for import, but are not inhibitors of the transport protein, we performed uptake studies with the corresponding radiolabeled isotopes. All tested nucleotides were imported and most of them exhibited rates comparable to the transport rates of ATP and ADP; however, GMP caused slightly lower, whereas deoxy purine nucleotides caused higher transport rates (Fig. S3). Therefore, a broad set of purine nucleotides, including dATP and dGTP, were identified as substrates of *PtNTT5*.

To examine the biochemical properties of *PtNTT5* in more detail, we determined the substrate affinities (Michaelis constant, K_M) as well as the corresponding maximal transport velocities (V_{max}). For this purpose, transport measurements were conducted in the presence of rising concentrations of the respective labeled nucleotides (Table 2). *PtNTT5* exhibited the highest affinities for adenine nucleotides (c. 120–150 μ M) followed by

dATP and dGTP (c. 210 μ M), whereas guanine nucleotides were generally imported with lower affinities (c. 310–350 μ M). GDP and ATP were imported with the lowest maximal velocity (c. 30 and 37 nmol mg⁻¹ protein h⁻¹, respectively) compared with the remaining (deoxy) nucleotides (c. 60–84 nmol mg⁻¹ protein h⁻¹). These results demonstrate that not only tri-, di-, and monophosphorylated adenine and guanine nucleotides, but also dATP and dGTP represent relevant substrates of *PtNTT5*.

PtNTT5 is an antiporter

To reveal the NTT function in the cellular context, it is important to determine its mode of transport. In principle, NTT type proton symporters enable net gain of nucleotides, whereas NTT type antiporters either allow energy uptake by the exchange of ATP and ADP or the adaptation of the composition of the respective nucleotide pools by exchanging internal with external (deoxy) nucleotides.

To analyze whether recombinant *PtNTT5* is driven by the proton gradient across the *E. coli* membrane, we investigated the influence of the protonophore m-chlorophenylhydrazine (CCCP) on ATP import (Fig. 3a). Interestingly, nucleotide import was not inhibited, but rather slightly stimulated by moderate CCCP concentrations (at 50–100 μ M CCCP: transport rate > 100%), while rather high concentrations (> 250 μ M CCCP) were required to cause a visible reduction of transport compared with unaffected transport (set to 100%). Even in the presence of 750 μ M CCCP, *PtNTT5* showed quite high transport activity of 68% (inhibition by 32%). Proton driven NTTs are generally known to be highly inhibited (by 65–80%) already at moderate CCCP concentrations (10–100 μ M CCCP: transport rates 20–35%) (Haferkamp *et al.*, 2006; Ast *et al.*, 2009; Knab *et al.*, 2011), and thus we can conclude that *PtNTT5* does not act as a proton symporter.

In a next step, we investigated the capacity of nonlabeled import substrates to induce export of radioactively labeled nucleotides (previously loaded into *E. coli*) in a chase experiment. *Escherichia coli* expressing *PtNTT5* was incubated in 50 μ M [α^{32} P] ATP and the radioactivity in the cells was quantified over time. After 5 min of import, significant amounts of [α^{32} P] ATP accumulated in the cells and possible efflux was started by

Table 2 Determination of apparent Michaelis constant (K_M) and maximal velocity (V_{max}) values of purine nucleotide transport mediated by nucleotide transporter 5 *PtNTT5* from *Phaeodactylum tricornutum* (*PtNTT*)

Substrate	Adenine nucleotides		Substrate	Guanidine nucleotides	
	K_M	V_{max}		K_M	V_{max}
ATP	120.7 (13.4)	36.8 (3.7)	GTP	311.5 (19.6)	73.6 (9.6)
ADP	137.0 (1.8)	59.9 (4.8)	GDP	304.0 (23.1)	29.2 (2.4)
AMP	151.0 (8.9)	71.9 (8.3)	GMP	348.0 (31.0)	83.8 (9.1)
dATP	207.2 (17.6)	84.1 (6.4)	dGTP	211.3 (26.7)	82.1 (6.3)

Nucleotide uptake in the presence of rising substrate concentrations (5–1000 μM) was allowed for time spans in the linear phase of the corresponding transport at 50 μM . Apparent K_M values are given in μM ; V_{max} values are given in $\text{nmol mg}^{-1} \text{protein h}^{-1}$. Data are the mean of at least three independent experiments. Corresponding standard errors are given in brackets.

Fig. 3 Analysis of the transport mode of nucleotide transporter 5 (NTT5) from *Phaeodactylum tricornutum*. (a) Influence of membrane potential depletion by protonophore addition on the transport activity of *PtNTT5*. Unaffected net $[\alpha^{32}\text{P}]$ ATP import (import into induced cells minus rates of noninduced cells) was set to 100%. $[\alpha^{32}\text{P}]$ ATP import in the presence of rising m chlorophenylhydrazone (CCCP) concentrations was calculated as a percentage of the unaffected transport. (b) Investigation of a possible counter exchange transport mode. Time dependent accumulation of 50 μM ATP in *Escherichia coli* cells expressing *PtNTT5* is shown (black circles). After 5 min of ATP import, possible efflux of internal label was induced by the addition of a 20 fold excess of nonlabeled substrates (red arrow). Efflux induction was tested with GTP (white circles) and dGTP (gray squares) as representative counter exchange substrates. Data are the mean of at least three independent experiments, $\pm$ SE.

addition of nonlabeled GTP or dGTP. Internal radioactivity subsequently markedly decreased, indicating that uptake of non labeled nucleotides is accompanied by the export of interior label. This observation indicates that *PtNTT5* operates in an antiport mode (Fig. 3b).

GFP fusions suggest targeting of *PtNTT5* to the ER membrane

The amino acid sequence of *PtNTT5* lacks the N terminal signal peptide typical of proteins with plastidial destination (stroma/innermost membrane) and also *in silico* analyses failed to clearly predict a specific subcellular targeting. We therefore expressed different GFP fusion proteins of *PtNTT5* in *P. tricornutum* to deduce the possible *in vivo* localization of the transporter. We fused GFP to either the N or the C terminus of *PtNTT5*. Fluorescence of the N terminal GFP fusion labeled a network like structure distributed throughout the cell, surrounding the plastid and the nucleus (Fig. 4a,b). This fluorescence pattern is very characteristic for proteins located in the continuous ER/chloroplast ER (cER) (Apt *et al.*, 2002). As a reference protein for the ER, we re investigated a *P. tricornutum* cell line (generated by Apt *et al.*,

2002), which expresses GFP fused to the pre sequence of the ER luminal binding protein precursor (BIP) (Fig. S4). By staining intracellular DNA with the dye Hoechst 33342, we confirmed that the GFP signal (of the ER) surrounded the nucleus (Fig. 4a), as expected because of the continuity of the ER, nuclear envelope and cER lumen.

The C terminal GFP fusion protein did not label a cellular network, but instead accumulated in rather diffuse patches close to the plastid/nucleus (Fig. 4c). This pattern differed from that of ER targeted proteins (Fig. S4) and did not totally cover or surround the chlorophyll autofluorescence area of the plastid. Moreover, it did not overlap with the MitoTracker signal, which argues against a potential mitochondrial localization.

To investigate the subcellular localization of *PtNTT5* in independent systems, we transiently expressed *PtNTT5* as C terminal and N terminal GFP fusions (Grefen *et al.*, 2010) in *Nicotiana benthamiana* and in *Arabidopsis thaliana*. Also in higher plant cells, *PtNTT5* with N terminal GFP was targeted to a network like structure (Fig. S5) and the GFP signal overlapped with that of the co expressed ER marker protein (ER rk; Nelson *et al.*, 2007). By contrast, *PtNTT5* with C terminal GFP showed quite heterogeneous and ambiguous fluorescence patterns in both

Fig. 4 Cellular localization of nucleotide transporter 5 (NTT5) in *Phaeodactylum tricornutum* cells. (a) GFP fused N terminally to PtNTT5. (b) Schematic illustration of a *P. tricornutum* cell depicting the stained organelles. (c) GFP fused C terminally to PtNTT5. GFP fluorescence, green; autofluorescence of the chlorophyll, red; Hoechst 33342 fluorescence, blue; MitoTracker fluorescence, yellow; Nomarski differential interference contrast (DIC), grayscale. The position of the Hoechst stain is marked with an arrow. Bars, 5 μm. N, nucleus; M, mitochondrion; ER, endoplasmic reticulum; P, plastid.

tobacco and *A. thaliana* (Fig. S6). Diffuse GFP signals and punctuated structures appeared in the cytosol and at the nucleus. The fluorescence also surrounded the plastids, but in quite an irregular manner with alternating regions of higher and lower intensity. In contrast to PtNTT5 with N terminal GFP, which labeled the entire ER network, the apparent ER localization of the C terminal GFP fusion protein was mainly restricted to the nuclear region and was not as constant and precise as observed with the N terminal GFP construct.

The finding that N and C terminal GFP constructs caused different fluorescence patterns led us to the assumption that the position of the marker protein affects subcellular targeting or membrane insertion. In order to identify possible targeting domains and to determine whether the C or the N terminal GFP fusion reflects the localization of the native transporter, we performed targeting experiments with truncated versions of PtNTT5 carrying GFP either at the C terminus or at the N terminus (Figs S7–S11).

Microscopy analyses of the respective transformed cell lines revealed that almost all fusion proteins caused GFP signals. Expression of selected GFP fusions was additionally monitored by western blotting and immuno staining using an GFP antibody. This method not only verified the successful expression of

the various fusion proteins (including one construct that did not cause visible GFP fluorescence) but also documented their different molecular weights (Fig. S12).

The fluorescence pattern resulting from the truncated versions of PtNTT5 with C terminal GFP resembled that of the corresponding full length protein, no matter which part of the protein was deleted (Figs S8, S9). Therefore, no specific region(s) in the sequence of PtNTT5 could be detected that may have caused the accumulation of GFP in proximity to the plastid, suggesting that this localization might be attributable to the C terminal position of GFP. By contrast, C terminally truncated PtNTT5 proteins with N terminal GFP caused different labeling patterns. Accordingly, the subcellular targeting of these fusion proteins depends on the length of the amino acid sequence of PtNTT5. The GFP signal caused by the shorter versions of PtNTT5 (up to 361 amino acids in length) appeared somewhere in the cytosol, whereas longer versions (at least 390 amino acids in length) were targeted to the (chloroplast) ER (Figs S10, S11). Thus, the longer the fragment, the more the labeling pattern resembled that of the corresponding full length protein.

These results suggest that adding GFP to the C terminus of PtNTT5 may prevent targeting to the ER by masking an as yet unknown motif or a domain required for (chloroplast) ER

targeting. Furthermore, both a (nearly) full length protein sequence and the unblocked C terminal part of *PtNTT5* may be essential for correct targeting. In this context, it is important to mention that potential targeting information within the protein sequence (particularly the remarkable accumulation of positive charges) at the far C terminus could not be clearly associated with ER targeting, because the correspondingly truncated *PtNTT5* versions with N terminal GFP were also detectable in the ER.

Discussion

Diatoms possess the highest number of putative NTTs (up to eight) among all known organisms (Ast *et al.*, 2009) which immediately raises questions regarding the phylogeny and physiological benefits of the different isoforms.

In phylogenetic studies, diatom NTT1 isoforms have been found to be most closely related to ATP/ADP transporters from red algae (Fig. 1; Ast *et al.*, 2009). Therefore, NTT1 was probably introduced into the diatom genome via gene transfer from the red algal endosymbiont. The remaining diatom NTTs form separate clusters, more distantly related to the plastidial ATP/ADP transporters from plants and algae (Fig. 1). Although the direct ancestor(s) of these NTTs cannot be identified, their low number of introns and the high GC content of at least *PtNTT5* and *PtNTT6* argue against a possible origin from the red algal derived NTT1 isoform by gene duplication and subsequent modification (Table S2; Fig. S1). The origin of almost all diatom NTTs (apart from NTT1 isoforms) by horizontal gene transfer from bacterial sources thus appears more likely. The branching pattern might indicate that the group comprising diatom NTT2 and *TpNTT3* and the other group comprising *PtNTT3* 6 and *TpNTT4* 8 originated from two different events of horizontal gene transfer followed by gene duplications.

Although showing significant similarities to plastidial ATP/ADP transporters, diatom NTT1 isoforms act as proton symporters and mediate net uptake of diverse adenine nucleotides across the innermost plastid envelope (Ast *et al.*, 2009). Accordingly, the substrate spectrum and the transport mode of the ancestral red algal carrier were modified to fulfill a new function in diatom plastids. Diatom NTT2 isoforms catalyze the antiport of diverse (deoxy) nucleotides (Ast *et al.*, 2009). Interestingly, the functional properties of diatom NTT1 and NTT2 resemble that of NTTs in intracellular bacteria. These bacteria parasitize nucleotides from the host (via interaction of functionally different NTTs) and compensate their missing capacity for nucleotide synthesis (Haferkamp *et al.*, 2004, 2006; Audia & Winkler, 2006; Knab *et al.*, 2011). Similarly, interaction of diatom NTT1 and NTT2 in the innermost plastid envelope was suggested to allow net gain of all substrates for DNA and RNA synthesis in the stroma (Fig. 5; Ast *et al.*, 2009). Adenine nucleotides imported via NTT1 can in turn (in the form of ATP) drive the uptake of diverse (deoxy) nucleotides via NTT2.

This finding was in some ways unexpected because it contrasts with the strong expectation that all NTTs from plants and algae exclusively act as ATP/ADP exchangers, with a specific relevance

Fig. 5 Schematic illustration of the proposed interaction of the biochemically characterized nucleotide transporters (NTTs) from *Phaeodactylum tricornutum*. Interaction of *PtNTT1* (gray circle, 1) and *PtNTT2* (gray circle, 2) in the innermost plastid membrane provides substrates for DNA and RNA synthesis in the stroma. Pyrimidine and purine nucleotide synthesis takes place in the cytosol. The existence of ATP dependent reactions, such as activity of the luminal binding protein precursor (BIP) or protein folding, indicates that ATP is present in the ER and chloroplast ER (CER) lumen. However, net uptake of adenine nucleotides into the ER has generally not yet been clarified (gray circle, ?). *PtNTT5* (gray circle, 5) can use luminal adenine nucleotides (AXP) to drive the uptake of guanine nucleotides (GXP), dATP and dGTP from the cytosol. Moreover, the antiport of substrates with different phosphorylation states has an impact on the organellar energy state. Passage of nucleotides (NTPs) and deoxynucleotides (dNTPs) across the remaining plastid membranes and their entry into the periplastid compartment (PPC) and the inter envelope space (IES) as well as the entry of pyrimidine nucleotides (PyrNuc) into the ER are unclear (gray circles, ?).

for energy supply to the plastid. However, it is completely in line with the requirement for net nucleotide uptake into the complex diatom plastid. In diatoms, purine and pyrimidine nucleotides are produced exclusively in the cytosol and not, like in higher plants, mainly in the plastid stroma, which necessitates a specifically adapted nucleotide transport system in diatom plastids (Ast *et al.*, 2009).

The intriguing question that arose from the previous study (Ast *et al.*, 2009) is how nucleotides pass through the remaining plastidial membranes. The recruitment of bacterial NTTs with appropriate properties might be a valuable process to establish a transport system in the different plastid membranes that connects nucleotide synthesis in the cytosol with nucleotide consumption in the plastid stroma. A further hypothesis is that all plastid membranes except the innermost one are selectively permeable for

various molecules and allow more or less uncontrolled passage of nucleotides. This assumption would imply that not all diatom NTTs are involved in plastidial nucleotide uptake but fulfill diverse physiological functions.

In this study, we performed a detailed analysis of *PnNTT5*. In view of the low similarity of *PnNTT5* to previously described NTTs (Fig. S2), it was unclear whether this protein may act as a nucleotide transporter at all. Import experiments, however, clearly demonstrated its capacity for nucleotide transport. It is specific for tri-, di- and monophosphorylated purine nucleotides, and dATP and dGTP are among the most favored substrates (Figs 2, S3; Tables 1, 2). Moreover, we discovered that transport activity of *PnNTT5* is not dependent upon a proton gradient but on the presence of suitable exchange substrates (Fig. 3). Accordingly, *PnNTT5* represents a purine nucleotide antiporter.

The determined biochemical features of *PnNTT5* are not identical, but closely related to those of certain bacterial and microsporidial NTTs as well as to the NTT1 isoforms of diatoms (Haferkamp *et al.*, 2006; Audia & Winkler, 2006; Knab *et al.*, 2011; Ast *et al.*, 2009; Heinz *et al.*, 2014). This implies that not, or not only, slight modification of the transport properties, but rather other factors put specific evolutionary pressure on *PnNTT5* which is reflected in the distant relationship to bacterial, plastidial and microsporidial NTTs. In this context, it is important to consider that *PnNTT5* is apparently located in the ER membrane (Fig. 4), and therefore in an environment not typical for NTTs. It is likely that considerable structural adaptations and hence alterations in the amino acid sequence were necessary to allow appropriate targeting and embedding of an NTT in the ER membrane. Similarly, the microsporidial NTTs also exhibit biochemical properties that are identical to or at least highly resemble those of NTTs from bacteria or primary plastids while residing in an NTT atypical membrane. The amino acid sequences of microsporidial NTTs and *PnNTT5* apparently harbor interior targeting information. Although specific motifs or domains cannot be assigned at first glance, the corresponding sequence modifications might hamper the determination of the direct ancestor of these NTTs (Fig. 1).

Phylogenetic analyses suggest that plastidial NTTs arose from an ancient chlamydial ATP/ADP transporter by horizontal gene transfer (Fig. 1; Greub & Raoult, 2003; Schmitz Esser *et al.*, 2004; Tyra *et al.*, 2007). The subsequent adaptation to a new targeting mode of the bacterial type NTT towards the plastid envelope membrane, including membrane insertion, apparently did not require substantial modifications within the amino acid sequence (Fig. S2). The most striking difference between plastidial and chlamydial ATP/ADP transporters is the presence (addition) of an N terminal sequence extension that directs the protein to the plastid and that is cleaved off after translocation (Neuhaus *et al.*, 1997).

To investigate the subcellular localization of *PnNTT5* and, in particular, to identify possible internal targeting information, we expressed full length as well as truncated versions of this transporter with GFP fused either N- or C terminally.

Shorter versions of the N terminal GFP fusions ended up in the cytosol, whereas longer versions were targeted to the ER

membrane (Figs 4a, S5, S10, S11). Therefore, a minimal protein length or even specific information in the C terminal part of *PnNTT5* is required for its targeting to the ER. We therefore conclude that ER targeting of *PnNTT5* takes place post translationally. We suggest that a translocation pathway similar to that of C terminally anchored proteins might be used for post translational insertion into the ER membrane. C terminal or tail anchored (TA) proteins are transmembrane polypeptides that are held in the phospholipid bilayer by hydrophobic amino acids at the C terminus (Borgese *et al.*, 2003). Direct insertion of newly synthesized TA proteins may occur in mitochondrial membranes, but also in the membranes of ER, peroxisomes or the chloroplast (Borgese *et al.*, 2007; Borgese & Fasana, 2011). *In vitro* analyses revealed the existence of different insertion pathways for TA proteins: the unassisted spontaneous integration into lipid bilayers or the energy requiring chaperone mediated pathway (Borgese & Fasana, 2011). Import competition assays suggest that the insertion pathways of TA and multi span proteins may overlap (Otera *et al.*, 2007; Dukanovic & Rapaport, 2011).

In the case of C terminal attachment of GFP to full length or truncated versions of *PnNTT5*, the GFP signals always appeared in diffuse patches close to the plastid/nucleus (Figs 4c, S8, S9). Therefore, this localization is independent of the used part or length of the *PnNTT5* sequence. Accordingly, no specific regions in the fusion protein, apart from the C terminal GFP, could be identified that might be associated with the corresponding localization. The fluorescence pattern of the C terminal GFP fusions is indicative of accumulation rather than insertion in a specific membrane. It is conceivable that the diffuse GFP signals are caused by degradation of the hydrophobic *PnNTT5* moiety and delivery of GFP. However, western blot analyses indicate that this is not the case (Fig. S12). Alternatively, one might envision that mal targeted fusion proteins accumulate in membranous structures such as the Golgi apparatus or form aggregates by hydrophobic interaction in an as yet unknown compartment.

Although we cannot rule out completely the possibility that the diffuse patches reflect the correct localization, the GFP analyses in sum suggest that *PnNTT5* represents a membrane protein that is post translationally inserted into the ER.

Activity of the (deoxy-) purine nucleotide shuttle *PnNTT5* in the ER membrane (which is continuous with the outermost plastid membrane) would presuppose the presence of suitable exchange substrates in the lumen. Adenine nucleotides are definitely present in the lumen because multiple ATP dependent reactions (e.g. protein import and folding, and ATP hydrolysis via BIP) take place in the ER (Hirschberg *et al.*, 1998; Apt *et al.*, 2002; Csala *et al.*, 2007). It is not clear which proteins catalyze net adenine nucleotide loading of the ER in all eukaryotes, but members of the mitochondrial carrier family are promising candidates (Hirschberg *et al.*, 1998; Csala *et al.*, 2007; Leroch *et al.*, 2008). Independent of the nature of the carriers involved, the adenine nucleotides imported into the ER can drive the antiporter *PnNTT5* (Fig. 5). When substrates of different phosphorylation states are exchanged (e.g. ATP for ADP), *PnNTT5* alters the organellar energy state. Interestingly, in contrast to transporters commonly known to be involved in energy

metabolism, the substrate spectrum of *P*NTT5 comprises deoxy nucleotides as additional important substrates. This feature can be considered an important indicator of an additional and possibly major role of *P*NTT5 as a component of the nucleotide uptake system of the complex plastid: import of GTP, dATP and dGTP in exchange for the luminal adenine nucleotides not only alters the nucleotide composition of the ER, but also allows the passage of building blocks for DNA and RNA synthesis across the outermost plastid envelope (Fig. 5).

These findings indicate that further components of the nucleotide uptake system of diatom plastids await identification. It is unclear which factors mediate (deoxy) pyrimidine nucleotide entry into the ER/translocation across the outermost plastid envelope and how various (deoxy) nucleotides pass through membranes two and three (Fig. 5). NTT proteins can be considered as important candidates for these tasks and three of the NTT isoforms found in *P. tricornutum* have not been characterized yet. The analysis of NTT isoforms from centric diatoms might additionally help to identify common principles or discrepancies in nucleotide transport of diatoms. In fact, at least slight differences in nucleotide transport can be expected because the centric *T. pseudonana* possesses a different number of putative NTT isoforms from the pennate *P. tricornutum* (Ast *et al.*, 2009). Interestingly, the identification of nucleotide transporters with extraordinary properties was recently shown to pave the way for sophisticated biotechnical approaches. Heterologous expression of *P*NTT2 allowed the feeding of artificial deoxy nucleotides into *E. coli* and finally resulted in a semi synthetic organism with an extended genetic alphabet (Malyshev *et al.*, 2014).

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Author contributions

L.C. designed and carried out the localization studies in diatoms and participated in the biochemical characterization and in drafting and finalizing the manuscript. A.G. carried out microscopic analyses and participated in designing the localization studies and in drafting and finalizing the manuscript. M.A. participated in the biochemical characterization. S.S.E. designed and carried out the phylogenetic analyses. J.A. performed localization studies in higher plants. H.E.N. participated in designing the biochemical studies. P.G.K. conceived of the study and participated in designing the localization studies and in finalizing the manuscript. I.H. conceived of the study, drafted the manuscript and participated in the biochemical characterization

and in finalizing the manuscript. All authors read and approved the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Exon–intron structure of diatom NTTs.

Fig. S2 Amino acid sequence alignment of biochemically characterized NTTs.

Fig. S3 Time dependent uptake of purine nucleotides via *PtNTT5*.

Fig. S4 Cellular localization of *PtBIP::GFP* in *Phaeodactylum tricornutum* cells.

Fig. S5 Cellular localization of *PtNTT5* with N terminal GFP in plant cells.

Fig. S6 Cellular localization of *PtNTT5* with C terminal GFP in plant cells.

Fig. S7 Schematic illustration of GFP fusion proteins of full length and truncated *PtNTT5*.

Fig. S8 Localization of *PtNTT5* in *Phaeodactylum tricornutum* (C terminal deletion and C terminal GFP fusion).

Fig. S9 Localization of *PtNTT5* in *Phaeodactylum tricornutum* (N terminal deletion and C terminal GFP fusion).

Fig. S10 Localization of *PtNTT5* in *Phaeodactylum tricornutum* (C terminal deletion and N terminal GFP fusion).

Fig. S11 Localization of *PtNTT5* in *Phaeodactylum tricornutum* (C terminal deletion and N terminal GFP fusion).

Fig. S12 Expression analysis of selected C terminal and N terminal GFP fusions of *PtNTT5*.

Table S1 Oligonucleotides used to generate *PtNTT5* GFP fusion constructs

Table S2 GC content of diatom NTTs

Table S3 Amino acid similarities of diverse functionally characterized NTTs (in %)

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