Evolutionary Maintenance of the PTS2 Protein Import Pathway in the Stramenopile Alga *Nannochloropsis*

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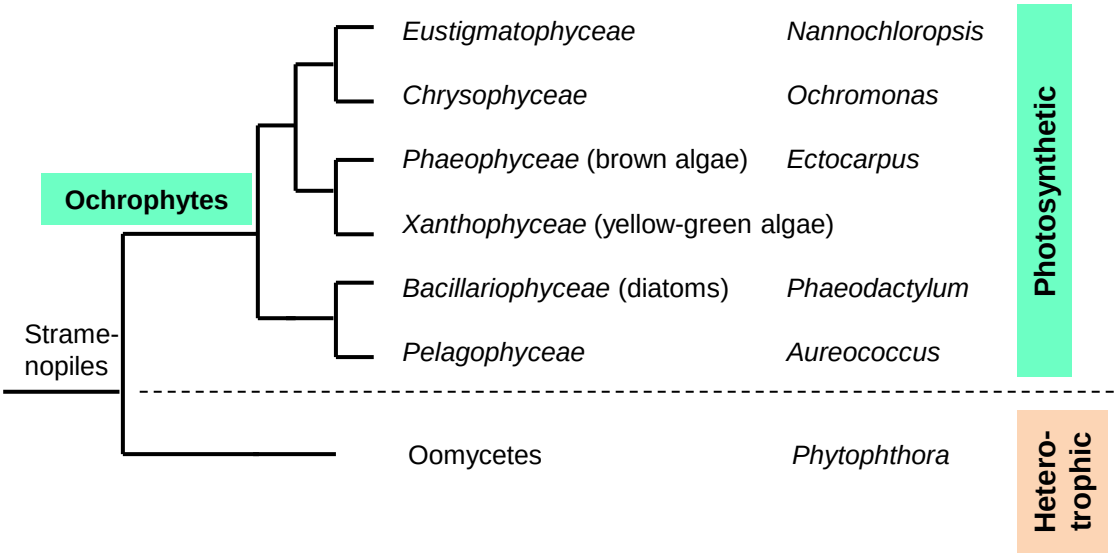
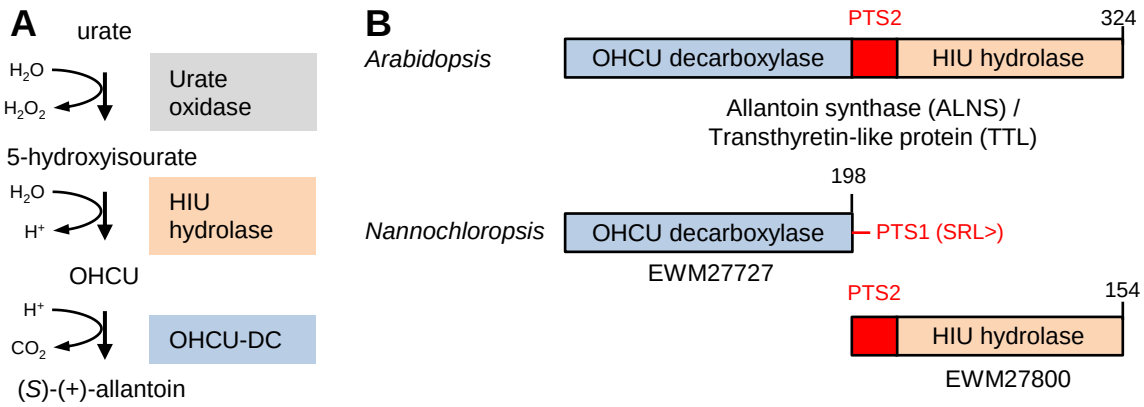
Supplementary Figures

Suppl. Figure S1



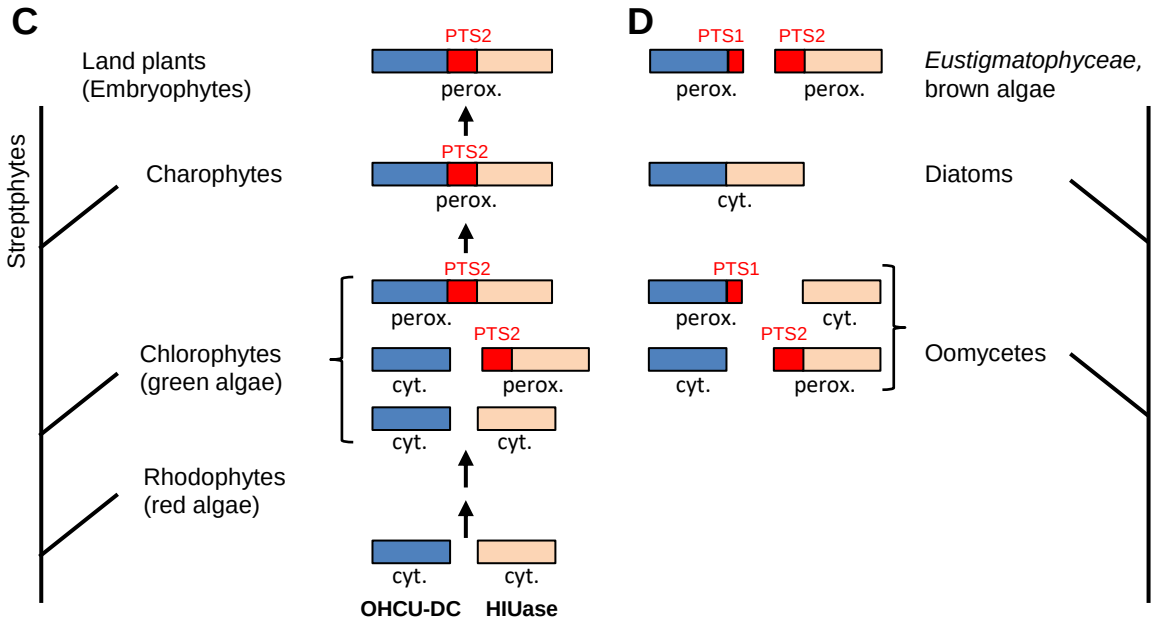
Suppl. Figure S1: Schematic phylogenetic tree of stramenopiles according to multiple gene analyses by Yoon et al. (2012). Stramenopiles contain both photosynthetic members (the ochrophytes), which possess complex plastids of red algal origin, and aplastidic and non-photosynthetic members (e.g. oomycetes). Ochrophytes include many ecologically important lineages (diatoms, kelps, pelagophytes) and *Nannochloropsis* as a potential model lineages for biofuels research. Ochrophytes form the most significant component of eukaryotic marine phytoplankton (Dorrel et al., 2017).

Suppl. Figure S2 A, B



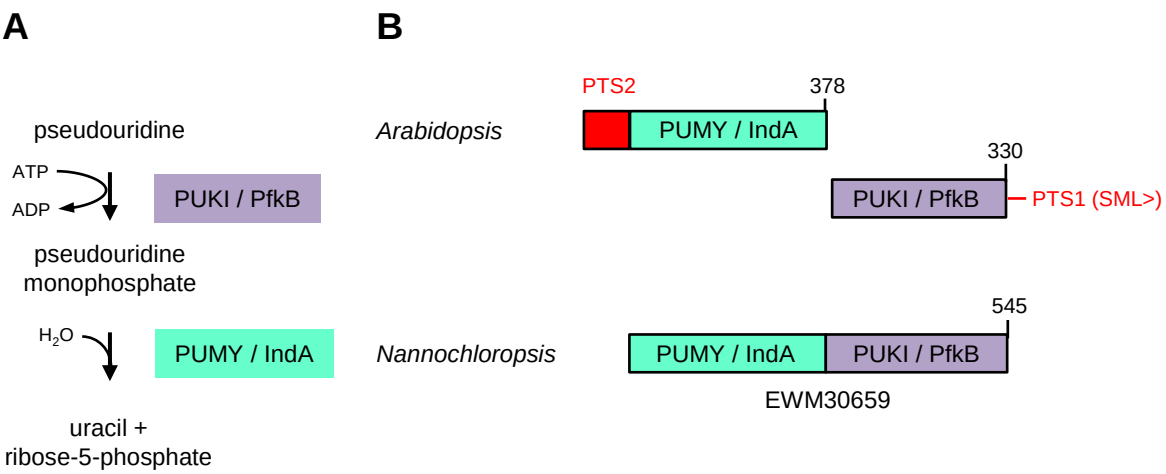
Suppl. Figure S2: Activity, domain architecture and evolution of peroxisomal enzymes of urate catabolism in *A. thaliana* and *N. gaditana*. (A) Urate degradation to (*S*)-(+)-allantoin is catalyzed by urate oxidase, 5-hydroxyisourate (HIU) hydrolase and (*S*)-2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) decarboxylase (DC). **(B)** The domain architecture of OHCU-DC and HIU hydrolase shows for *A. thaliana* a bifunctional fusion protein with an unusual internal PTS2 and for *N. gaditana* two distinct PTS1- or PTS2-carrying enzymes. **(C)** and **(D)** are provided on the next page.

Suppl. Figure S2 C, D



Suppl. Figure S2: Activity, domain architecture and evolution of peroxisomal enzymes of urate catabolism in *A. thaliana* and *N. gaditana*. (A) and **(B)** are shown on the previous page. **(C)** Schematic presentation of enzyme evolution in *Viridiplantae*. Initially both enzymes (OHCU-DC and HIUase) were separated and cytosolic, as still nowadays in Rhodophytes and few species of chlorophytes (*Trebouxiophyceae*). For instance, *Chlorella sorokiniana* still uses the ancient forms of single cytosolic enzymes. In the last common ancestor of Chlorophyceae and Streptophytes, HIUase first evolved a PTS2 (as found today in some *Trebouxiophyceae*). Examples include HIUase of *Micractinium conductrix* (RAx_5QL) and *Chlorella variabilis (*RAx_5QL). Subsequently, HIUase merged N-terminally with OHCU-DC, thereby leading to the unusual internal PTS2. Gene fusions (OHCU-DC-HIUase), all with the internal PTS2, are indeed detectable in two *Trebouxiophycean* species (*Trebouxia* sp. A1-2, *Coccomyxa subellipsoidea*, both RAx_5HL). **(D)** Schematic presentation of enzyme evolution in Stramenopiles with different variants in *Nannochloropsis* (*Eustigmatophyceae*, OHCU-DC with PTS1, HIUase with PTS2), diatoms (cytosolic protein fusion) and Oomycetes (separate enzymes with generally only one PTS-carrying enzyme). The constellation in Oomycetes implies peroxisome import of the other enzyme by oligomerization and piggyback mechanism.

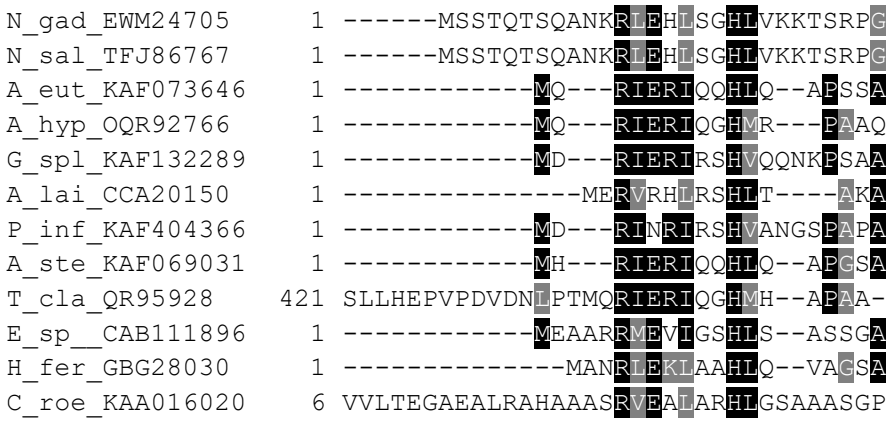
Suppl. Figure S3



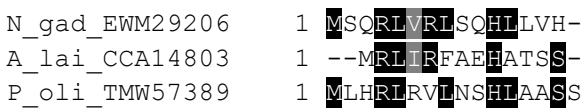
Suppl. Figure S3: Comparision of pseudouridine degradation catalyzed by single peroxisomal (*A. thaliana*) or a bifunctional cytosolic fusion enzyme (*N. gaditana*). (A) In *Arabidopsis*, pseudouridine degradation to uracil was recently demonstrated to be catalyzed by two peroxisomal enzymes, pseudouridine kinase (PUKI/PfkB) and pseudouridine monophosphate glycosylase (PUMY/IndA, Chen and Witte, 2020). **(B)** Comparative schematic diagram of the protein structures of PUKI/PfkB and PUMY/IndA in *A. thaliana* (two distinct PTS1- or PTS2-carrying enzymes) and *N. gaditana* (bifunctional fusion protein) without predicted PTS1/2 (but the decapeptide RLx_6HV).

Suppl. Figure S4

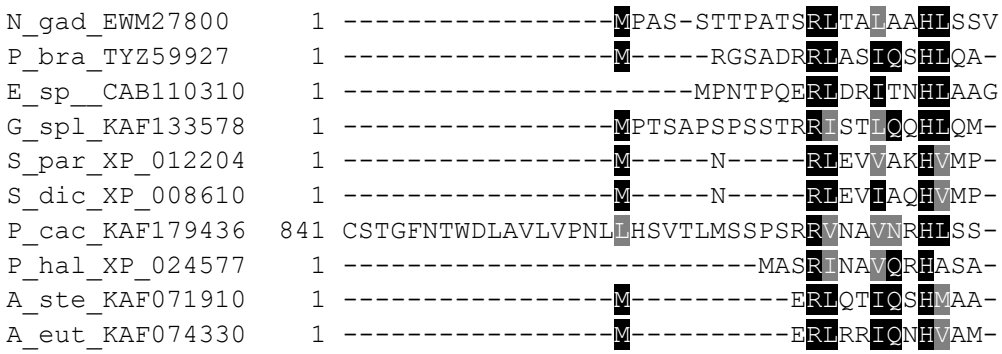
A: Thiolase (NgPKT)



B: HIT1 (NgHIT1)



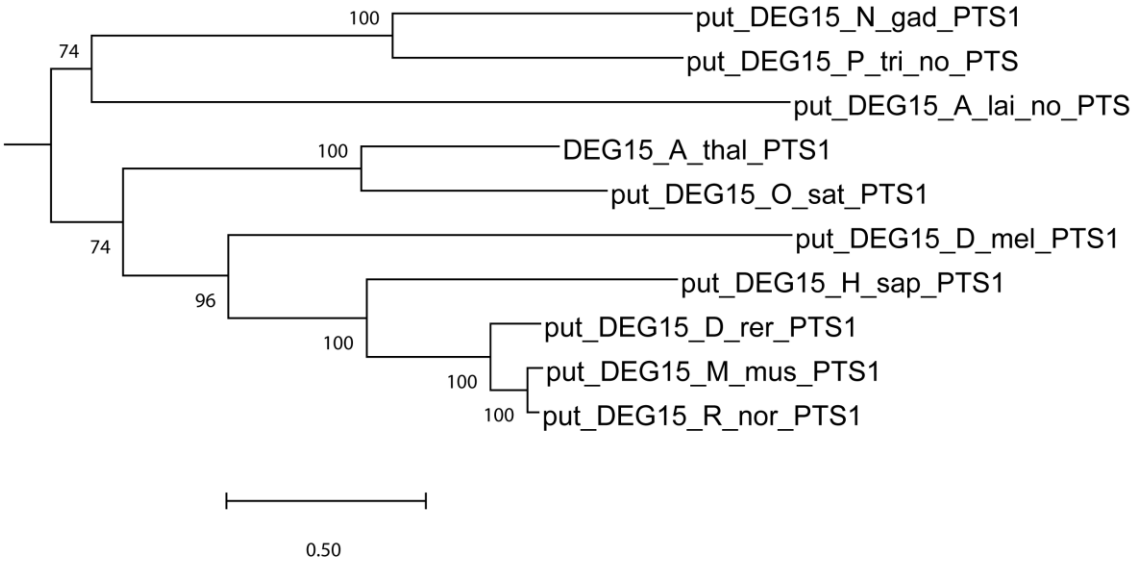
C: HIUase (NgHIUase)



Suppl. Figure S4: Analysis of PTS2 conservation of predicted *N. gaditana* PTS2 proteins in Stramenopiles. Putative orthologs of the PTS2-carrying thiolase (NgPKT, A), NgHIT1 (B) and HIUase (C) were identified by protein BLAST searches at NCBI using Genbank focusing on Stramenopiles. Homologs were aligned by COBALT (Papadopoulos and Agarwala, 2007) and sequence conservation labeled by Boxshade. Acronyms: A_eut, *Aphanomyces euteiches*; A_hyp, *Achlya hypogyna*; A_lai, *Albugo laibachii* Nc14; A_ste, *Aphanomyces stellatus*; C_roe, *Cafeteria roenbergensis*; E_sp, *Ectocarpus* sp. CCAP 1310/34; G_spl, *Globisporangium splendens*; H_fer, *Hondaea fermentalgiana*; N_gad, *Nannochloropsis gaditana* B-31; N_sal, *Nannochloropsis salina* CCMP1776; P_bra, *Pythium brassicum*; P_cac, *Phytophthora cactorum*; P_hal, *Plasmopara halstedii*; P_inf, *Phytophthora infestans*; P_oli, *Pythium oligandrum*; S_dic, *Saprolegnia diclina* VS20; S_par, *Saprolegnia parasitica* CBS 223.65; T_cla, *Thraustotheca clavata.*

Suppl. Figure S5 A

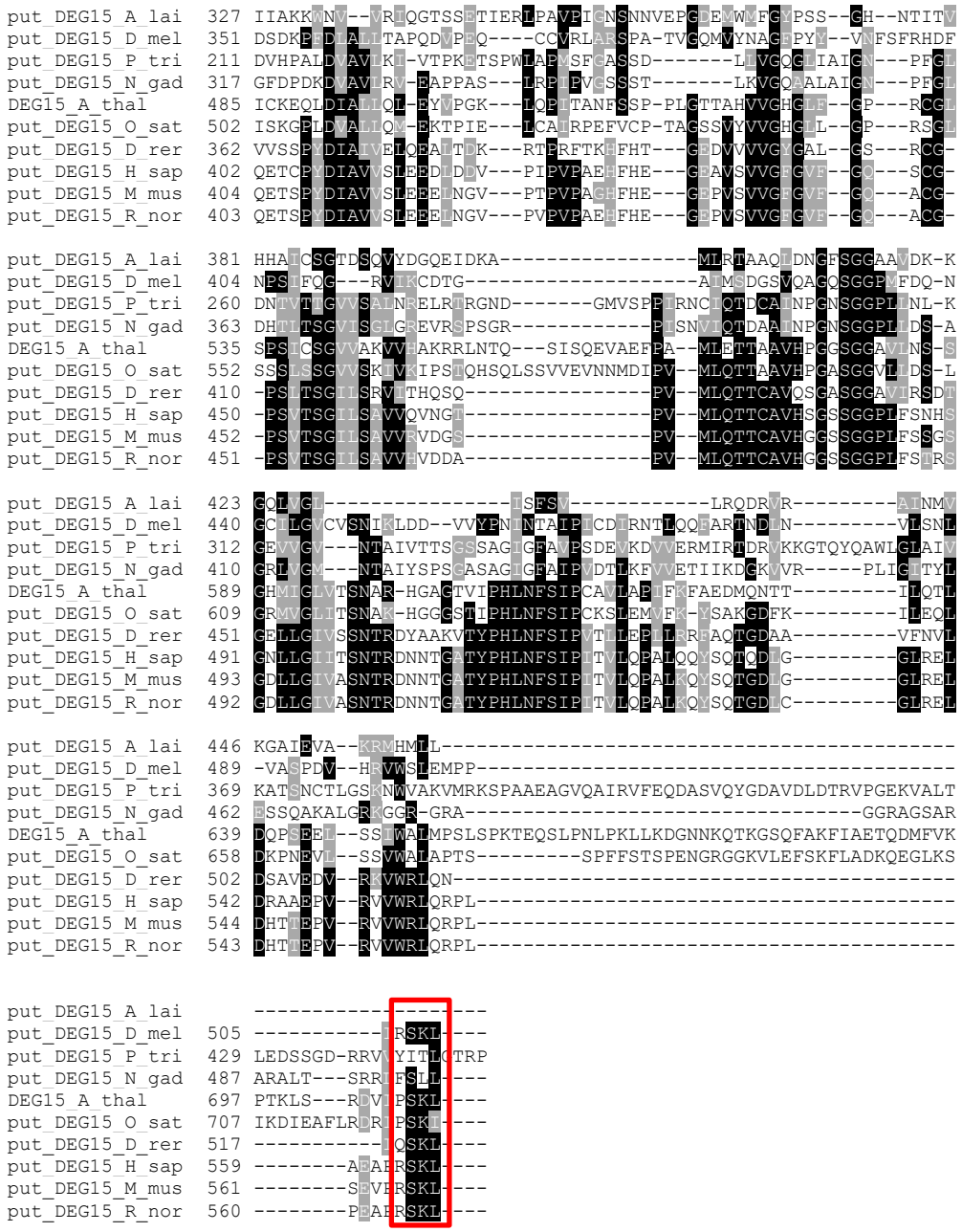
A



Suppl. Figure S5: Phylogenetic and homology analysis of DEG15 in *A. thaliana*, Stramenopiles and other eukaryotes. (A) Homologs of the PTS1-carrying DEG15 homolog of *N. gaditana* (putNgDEG15, EWM21659, SLL>) were identified by protein BLAST searches at NCBI using Genbank and focusing on Stramenopiles, green and red algae, land plants and animals. Sequences were aligned by MUSCLE (Edgar 2004) and the phylogenetic tree was constructed using the Bayesian inference method (Ronquist 2012). The branch support values were calculated as Bayesian posterior probabilities and are shown next to the branches. The platform Phylogeny.fr (Dereeper et al., 2008) was used for phylogenetic analysis, and MEGA X was used for tree visualization (Kumar, 2018). **(B)** is shown on the next page.

Suppl. Figure S5 B

B



Suppl. Figure S5: Phylogenetic and homology analysis of DEG15 in *A. thaliana*, stramenopiles and other eukaryotes. (A) is shown on the previous page. **(B)** The alignment was performed with MUSCLE (v3.8.31, Edgar 2004). Only the conserved C-terminal domain is shown (amino acid residues 485 to 720 of AtDEG15). Acronyms: A_lai, *Albugo laibachii* Nc14 (CCA14173.1); A_thal, *Arabidopsis thaliana (*NP_174153.2); D_mel, *Drosophila melanogaster (*NP_611968.1); D_rer, *Danio rerio (*NP_001122182.1); H_sap, *Homo sapiens (*NP_775826.2); P_tri, *Phaeodactylum tricornutum* CCAP 1055/1 (XP_002183960.1); M_mus, *Mus musculus (*NP_082188.1); N_gad, *Nannochloropsis gaditana* B-31 (EWM21659.1); O_sat, *Oryza sativa* Japonica (XP_015640602.1); R_nor, *Rattus norvegicus (*NP_001102402.1).

Suppl. Figure S6

A. PEX7(NgPEX7, EWM28214.1):

MKRESFHAANTGGGGLGGPIPDAVFQTQFQCCS (intron 1) VEFSPFQEARLAVATAQYFGIIG
NGRQHILE (intron 2) IGPDGNLREIRSFLTQEGLYDCCWSEANQNQLVSASADGSLKLWDVMT
SDGYPVAH (intron 3) WQEHSAEVSSVHWNQVVKTNFLSASWDGSIKLWDPHHPTSLSTYCGHT
GCVYAGIHSPRHPHRFLSCGTDGSLRIWDTKLPPSHATSLGLGRAEGGAVQVVRAHEGEVLSADWDKY
QDFLVYTGGVDRSIKIWDLRRPSLPLGFLHGHGYAVRRLKTSPHQEGVLGSVSYDMSCRVWGPTRAGG
RAGELWRCEEHTEFVQGLDFHLFWPGRIATCGWDRRVCVWTLPLP

B. Malate synthase 2 (NgMLS2, EWM30341.1):

MFLSGSMAQ**RL**QVISR**HL**QQKKAFEEKNGATRRLTSFEAGPHI (intron 1) IEYDADVDGLHSS
IVDEVLTEGALSFLAELVQHFNKDVLE (intron 2)LYRRRAEVQTKIETGTYEFGFSPETAQIRK
(intron 3) GIWQVEPVPSVLLDRRVDVGDVAPDDARALVSALNSGAQGVQCDFDDGFCPTWRNV
LLGIRNVMDASQGVLCYPDSVRAHSVAIVSNAAVMMLRPRAWCMTEMHFYVNGRAIPGPLLDY(int-
ron 4) GLLIYHCGAELEMRGKGPFFYCSKVENYLEARLWNSIFTWSEVKLGLKK (intron 5)
NSVKACVLIENITASFQMDEILYELRNHSAGLNCGIWDYSASFIARFAQRSDMIFPDRTKYVTMECGF
MRNYMRLLVDTCHKRGAIATTGMAGLVLDPKWDKATRQAKLQEVRLAKHFEAEKGGSDGALIYDLNLK
GLVAEVFGGGLGRLNQLNRPLSSASIGPADLLEVPPGGITLEGVRFNTEIVVRFIDSWLRGRGSFVYR
NSAEDSATAEISRSQIWQWVRHGLYTEGKDAITLSLVMEFACETADELAQEGREAQIPPALVDDRVAS
ALSLYRMLVSVPVFPRFITTFLYEQALFHEFARRRSPIM

C. 3-Ketoacyl thiolase (NgPKT, EWM24705.1):

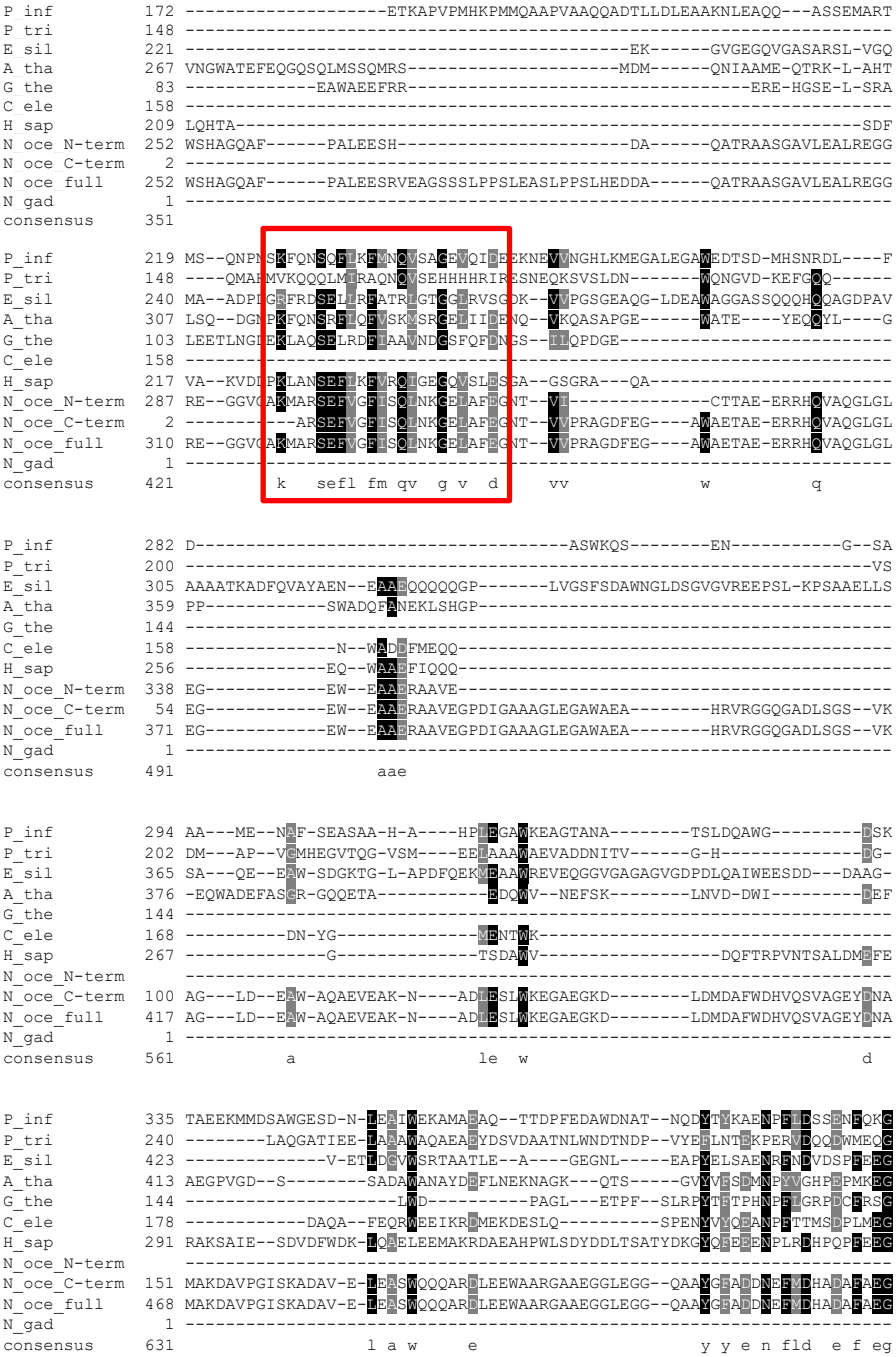
MSSTQTSQANK**RL**EHLSG**HL**VKK (intron 1) TSRPGFLGGDVVVVSALRTPL**C**KAKRGAFKSTT
TDDLLAPVLEAVVKQSGVDAATLGDIVVGNVLQPGSGAVGARMAQFYAGIPYQ (intron 2) VPL
CTLNRQCSSGLQAFLQVAASIQSGLYEAGIAGGVESMSLTDMSTSVPDVNFERVSENALSKDCTIPMG
QTSDEVATRFQVSREDQDRFAAASHAKAEAAVKAGKFAEEIVPVRVSSGGEDGGEDEVTVVREDEGIR
PGTTPEKLGGLRSSFSEGGSTTAGNSSQMTDGAAAVLVMSRGAATAQGMPVMGVLRGAAVVGVPPDIM
GIGPAVAIPAALAQARLRVEDVDVFEINEAFASQCLYCVRELKIPMEKVNPNGGAIALGHPLGATGAR
QIATLLHEMRRTRKRWGVVSMCIGTGMGAAAVFENEAAAN

D. Histidine triad family protein 1 (NgHIT1, EWM29206.1):

MSQ**RL**VRLSQ**HL**LVHRTSLHATSIAVRLSRTTRR**C**LVTGNQANTMADEVAESRKAAAKKMEEAADAGE
(intron 1) PTVFDKIVKKEIPSNFIYEDDDCVAFHDLSPQGPVHFLVIPKDRAGLSRLSKAEESH
KALLGHLLYVAQQVAKQEGLVPGGFRVVINDGPDGSQSVYHLHIHVIGGRQ (intron 2)MGWPPG

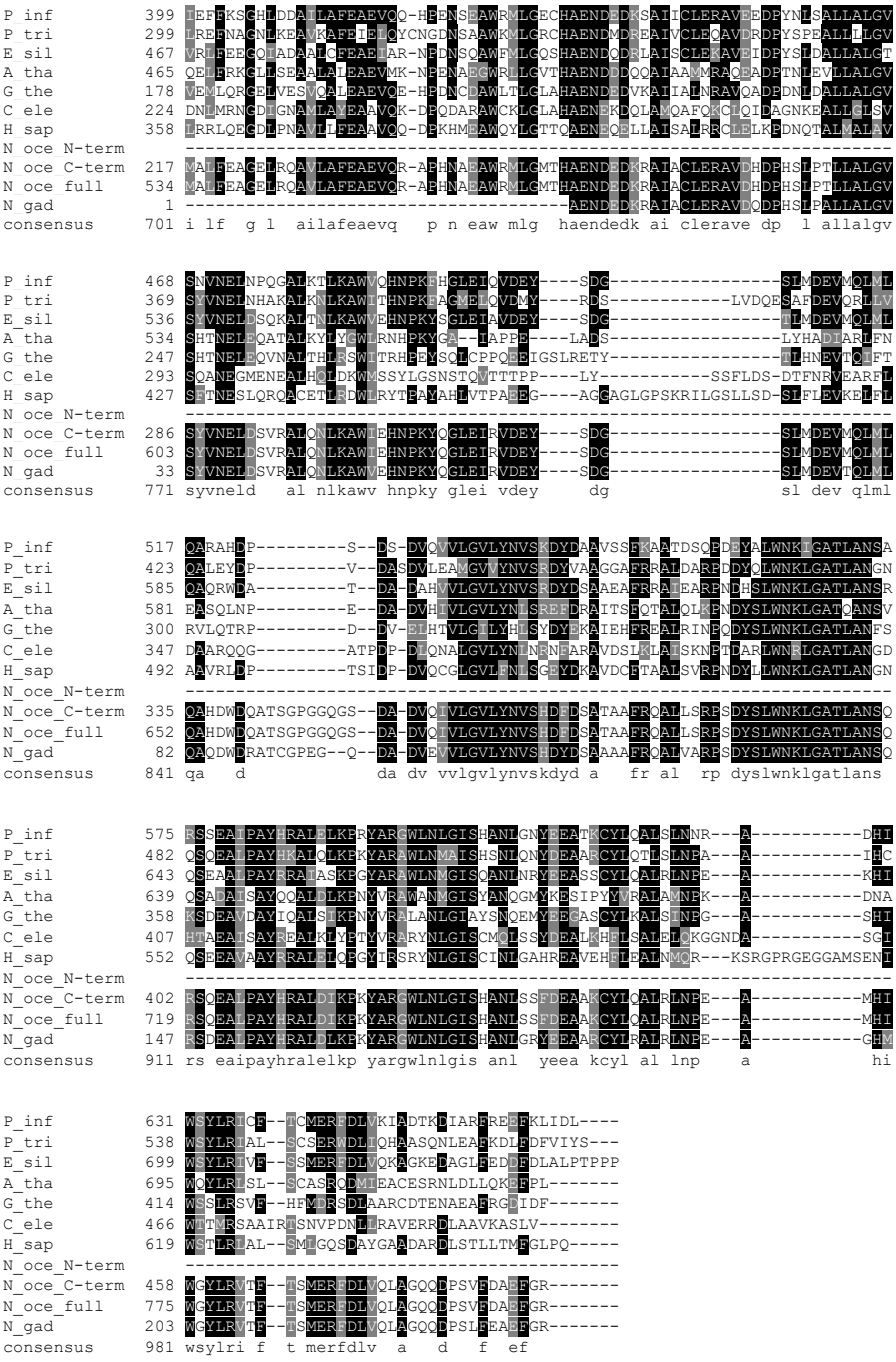
Suppl. Figure S6: Exon-intron structure of the PEX7 ortholog and three predicted PTS2 proteins from *N. gaditana* investigated experimentally in this study. For each *N. gaditana* protein, the Genbank accession number is provided for strain B-31 and intron positions are indicated. For the three PTS2 proteins investigated experimentally, the CDS of the first exon (underlined) was cloned from strain CCMP526 and placed upstream of the fluorescent reporter gene. The predicted PTS2 are highlighted in red and the most conserved four amino acid residues (e.g. RLx_5HL) are marked in bold. Cys residues of putative PTS2 cleavage sites are indicated (blue, C, D).

Suppl. Figure S7



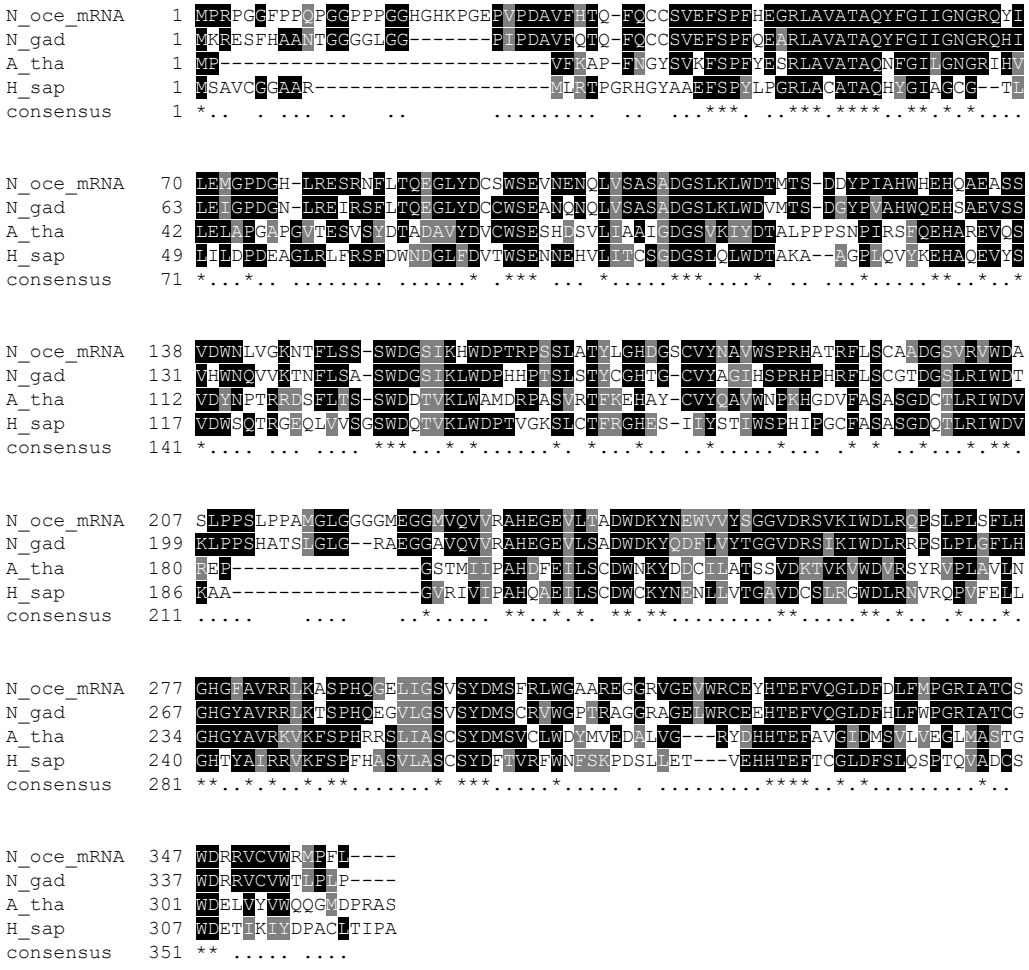
Suppl. Figure S7: Analysis of *Nannochloropsis* PEX5 orthologs for the presence of the PEX7 binding domain in Stramenopiles. (Legend on next page).

Suppl. Figure S7 (cont.)



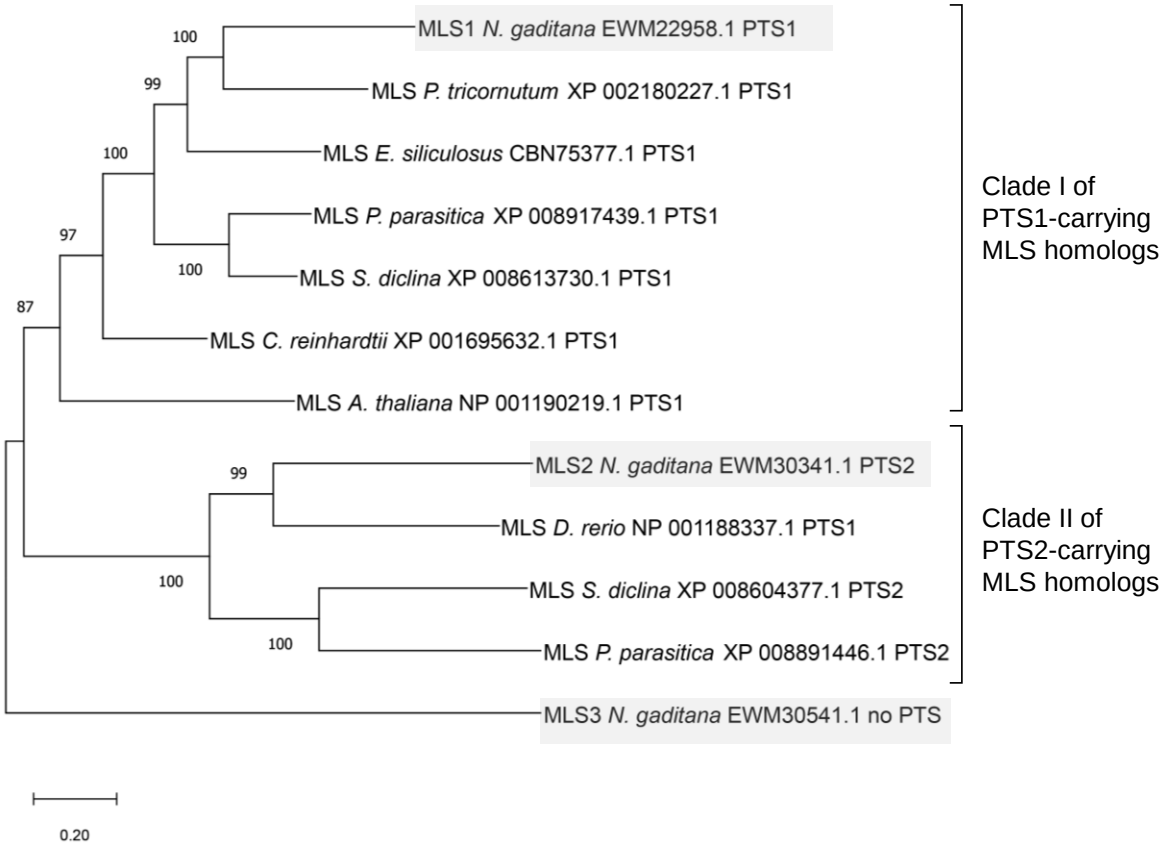
Suppl. Figure S7: Analysis of *Nannochloropsis* PEX5 orthologs for the presence of the PEX7 binding domain in Stramenopiles. The predicted genomic PEX5 CDS from *N. gaditana* (N_gad, EWM20982.1) and *N. oceanica* (626268) were aligned with PEX5 homologs from *A. thaliana* (A_tha, NP_200440), *H. sapiens* (H_sap, NP_001124495) and others by ClustalW (v2.1, at Phylogeny.fr). Sequence conservation was labelled by Boxshade. The alignment confirms that the four *Nannochloropsis* proteins are indeed PEX5 homologs and possess the conserved middle domain (boxed in red) that binds PEX7 in *Arabidopsis* and humans. The alignment of the weakly conserved N-terminal domain (approx. 260 aa of *A. thaliana* PEX5) is not shown.

Suppl. Figure S8



Suppl. Figure S8: Homology analysis of two putative PEX7 homologs from *Nannochloropsis* by multiple sequence alignment with *Arabidopsis* and human PEX7. The translated CDS of a full-length mRNA of a putative PEX7 homolog from *N. oceanica* CCMP1779 (N_oce_mRNA, 583661) was aligned with the predicted PEX7 from *N. gaditana* (N_gad, EWM28214.1) and with PEX7 from *A. thaliana* (A_tha, NP_174220) and *H. sapiens* (H_sap, NP_000279) by ClustalW (v2.1, at Phylogeny.fr). Sequence conservation was labelled by Boxshade. The alignment confirms that both *Nannochloropsis* proteins are indeed PEX7 homologs of *Arabidopsis* and human PEX7 and that *N. oceanica* expresses a full-length PEX7 mRNA.

Suppl. Figure S9



Suppl. Figure S9: Phylogenetic analysis of peroxisomal MLS isoforms from *N. gaditana*. Homologs of the PTS1- and PTS2-carrying MLS isoforms of *N. gaditana* (NgMLS1, EWM22958.1, SRL>; NgMLS2, EWM30341.1, RLx_5HL) were identified by protein BLAST searches at NCBI using Genbank and focusing on Stramenopiles, green and red algae, land plants and animals. Multiple sequence alignment of homologous proteins was performed in MUSCLE (Edgar 2004) and the proteins were analyzed for predicted PTS1 using the PredPlantPTS1 prediction server (http://ppp.gobics.de) and for predicted PTS2 using a manual motif search algorithm. The phylogenetic tree was constructed using the Bayesian inference method (Ronquist 2012). The branch support values were calculated as Bayesian posterior probabilities and are shown next to the branches. The platform Phylogeny.fr (Dereeper et al., 2008) was used for phylogenetic analysis, and the tree was visualized in MEGA X (Kumar, 2018). Among selected organisms, only Nannochloropsis and two oomycete species (*Saprolegnia diclina* VS20 and *Phytophthora parasitica*) had both, a predicted PTS1- and a PTS2-carrying MLS isoforms. The phylogenetic analysis revealed two distinct clades of PTS1-carrying and PTS2-carrying MLS isoforms (except for *D. rerio*). The non-peroxisomal MLS isoform of *N. gaditana* was used as an out-group.