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(71) Applicant *(for all designated States except US)*: **J. CRAIG VENTER INSTITUTE** [US/US]; 10355 Science Center Drive, San Diego, CA 92121 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ALLEN, Andrew** [US/US]; 2927 Copley Avenue, San Diego, CA 92116 (US). **DUPONT, Christopher, L.** [US/US]; 904 Missouri Street, San Diego, CA 92109 (US).

(74) Agents: **ARNOLD, Beth, E.** et al.; Foley Hoag LLP, Seaport West, 155 Seaport Boulevard, Boston, MA 02210-2600 (US).

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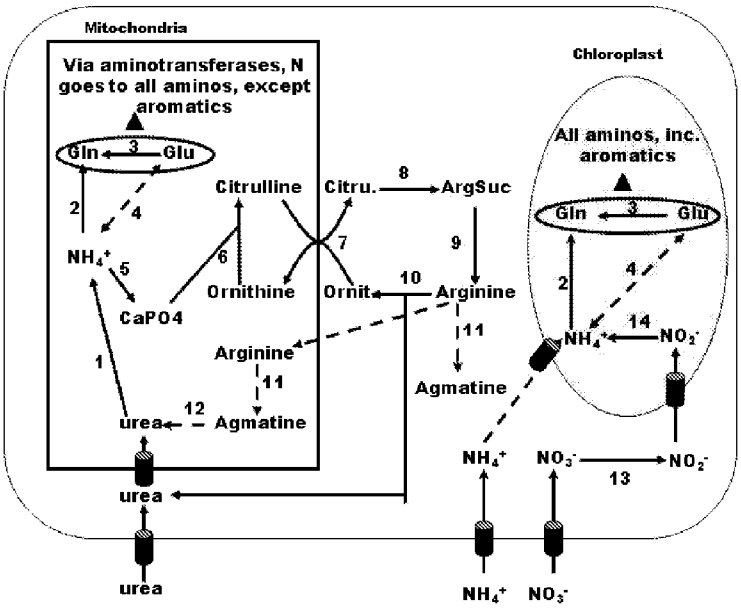
[Continued on next page]

(54) Title: ENGINEERED MICROALGAE WITH ENHANCED LIPID PRODUCTION

Figure 1



(57) Abstract: Described herein are engineered microalgae that exhibit enhanced lipid production during exponential growth. Such engineered microalgae are useful, for example, for the production of biofuels.

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ENGINEERED MICROALGAE WITH ENHANCED LIPID PRODUCTION

RELATED APPLICATION

This application claims benefit under 35 U.S.C. § 119(e) of United States Provisional Patent Application No. 61/422,462, filed on December 13, 2010.

BACKGROUND

Because of their high lipid and fatty acid content, microalgae, including diatoms, are regarded as a potentially useful source of neutral lipids for use in biodiesel fuel production. Theoretical calculations suggest that an annual oil production of greater than 200 barrels of algal oil per hectare of land may be achievable through mass culture of microalgae such as diatoms, which is 100-fold greater than that of soybeans, a major feedstock currently being used for biodiesel in the USA (Hu *et al. The Plant Journal*, 54:621-639 (2008)).

Under optimal growth conditions, diatoms and other microalgae synthesize fatty acids primarily for esterification into glycerol-based membrane lipids, which constitute about 5-20% of their dry cell weight. However, under unfavorable environmental conditions, such as during nitrogen deprivation, many algae shift their lipid profile towards the formation and accumulation of neutral lipids, principally in the form of triacylglycerol. Under such unfavorable growth conditions, the total lipid composition of certain microalgae can increase to above 50% of the algae's dry cell weight.

However, in addition to increasing lipid production, culture of microalgae under nutrient deprivation conditions also results in the halt of algal cell division. As a result, the increased lipid content of nutrient starved algae does not lead to an overall increase in lipid productivity. In fact, total rates of lipid production are typically lower under periods of nutrient starvation because higher cellular levels of lipid are offset by crashes in cell division. Thus, using existing technologies, it is possible either to culture microalgae under conditions that promote a high growth rate, or to culture microalgae under conditions that

promote an elevated cellular TAG content, but it is not possible to do both simultaneously (Sheehan *et al.,* 1998. A look back at the US Department of Energy's Aquatic Species Program-biodiesal from algae. National Renewable Energy Laboratory, Golden, Colorado; and Yu *et al., Journal of Applied Phycology*, 21:669-681 (2009)).

The development of microalgae cable of maintaining a lipid-rich phenotype under culture conditions that permit cell division would greatly enhance the economic viability of microalgae, including diatoms, as a source of biofuel precursors. Thus, there is a great need for engineered microalgae with enhanced lipid production during exponential growth.

SUMMARY

In one aspect, the invention features microalgae that have been engineered to assimilate carbon at a higher rate than nitrogen, thereby producing more lipid than wild-type microalgae during the exponential phase of cellular growth. In certain embodiments, the engineered microalgae is a diatom, such as *Phaeodactylum triconutum* or *Thalassiosira pseudonana.*

In certain embodiments, the microalgae has been engineered to produces more of a protein that facilitates carbon assimilation or a more active version of a protein that facilitates carbon assimilation. In certain embodiments the protein is selected from the group consisting of a pyr-decarboxylase, rubsico, beta CA or the chloroplast HCO_3 transporter.

In another embodiment, the microalgae has been engineered to produce less of a protein that facilitates nitrogen assimilation or a less active version of a protein that facilitates nitrogen assimilation. In certain embodiments, the protein that facilitates nitrogen assimilation is selected from the group consisting of nitrate reductase, nitrate transporter, NADPH nitrate reductase, ferredoxin nitrate reductase or glutamine synthetase

II In certain embodiments the engineered microalgae include a nucleic acid encoding an RNAi molecule that is an inhibitor of the expression of the protein that facilitates nitrogen assimilation. In another embodiment, the engineered microalgae includes a gene encoding a nitrogen assimilation protein that contains a mutation that reduces the expression or activity of the protein.

In another aspect, the invention features methods for producing lipids from microalgae. In one embodiment, the method comprises the steps of: a) growing a culture of the engineered microalgae for a sufficient period of time and under appropriate conditions to produce lipid during exponential growth of the microalgae culture; and b) harvesting lipids from the microalgae culture. In certain embodiments, the microalgae culture may be a biofilm.

In yet another aspect, the invention features expression vectors, which are comprised of a nucleic acid sequence encoding a protein that facilitates carbon assimilation operably linked to a transcription control element.

The engineered microalgae described herein are useful, for example, for the production of biofuels. Other features and advantages will be apparent from the following detailed description and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows pathways for nitrogen assimilation in *Phaeodactylum tricornutum*. Putative assimilation pathways for urea, NH_4^+, and NO_3^- into amino acids are shown. NH_4^+, while shown as being assimilated in the plastid, may also be assimilated in the mitochondria. Numbers are for enzyme activity, not specific proteins: 1, Urease; 2, Glutamine synthase; 3, Glutamate synthase; 4, Glutamate dehydrogenase; 5, Carbamoyl phosphate synthase III; 6, Ornithine transcarbomylase; 7, Citrin; 8, Arginosuccinate

synthase; 9, Arginosuccinate lyase; 10, Arginase; 11, Arginine decarboxylase; 12, Agmatinase; 13, Nitrate reducatase; and 14, Nitrite reductase. Dashed lines indicate putative pathways. Carbon containing metabolites are not shown.

Figure 2 shows the amino acid sequence (**A**, SEQ ID NO: 1) and nucleic acid sequence (**B**, SEQ ID NO: 2) of *Phaeodactylum triconutum* nitrate reductase.

Figure 3 shows the amino acid sequence (**A,** SEQ ID NO: 3) and nucleic acid sequence (**B**, SEQ ID NO: 4) of *Thalassiosira pseudonana* nitrate reductase.

Figure 4 shows the amino acid sequence (**A,** SEQ ID NO: 5) and nucleic acid sequence (**B**, SEQ ID NO: 6) of *Phaeodactylum triconutum* pyr-decarboxylase.

Figure 5 shows the amino acid sequence (**A**, SEQ ID NO: 7) and nucleic acid sequence (**B**, SEQ ID NO: 8) of *Thalassiosira pseudonana* pyr-decarboxylase.

Figure 6A shows NO production in *P. tricornutum* cell extracts as monitored by direct measurement of spin trapped NO $(MGD)_2$-Fe(II)-NO in electron paramagnetic resonance spectra (EPR). Normalized NO production was calculated by finding the average peak-to-trough height for the three peaks in the EPR spectrum. Basal NO levels were measured without substrates in wild type (WT) and overexpressing NR *P. tricornutum* cell lines. **Figure 6B** and **6C** show confocal images of cytosolic and peroxisome localization of *P.tricornutum* NR-N'YFP respectively. **Figure 6D** shows an electron micrograph of unfixed *P. tricornutum* wild type cells. The peroxisome is denoted by the arrow. **Figure 6E** shows immunogold localization of *P.tricornutum* NR-N'YFP localized to the peroxisome.

Figure 7 shows an immunoblot analysis using primary antibody raised specifically on *P. tricornutum* NR. The *P. tricornutum* NR protein is predicted to be 890AA, MW 100.12 kDa. *P. tricornutum* cultures were grown on NH_4^+ (T_0), pelleted washed and resuspended in NO_3^- and assayed for NR protein content at 6 and 24 hrs after resuspension

in NO_3^-. Normalized 100 kDa band volumes are T_6 WT, 1.46 ug; T_6 31i, 0.849; T_{24} WT, 1.59 ug; T_{24} 31i, 0.57.

Figure 8 shows images taken using CARS confocal micriscopy imaging specific for lipids performed on exponential- and stationary phase-wild type and NR RNAi line *P. tricornutum* cells. 8A is a representative image of a wild type exponential *P. tricornutum* cell. 8B is a representative image of a wild type stationary *P. tricornutum* cell. 8C is a representative image of a NR RNAi exponential *P. tricornutum* cell.

Figure 9 shows chloroplast pyrenoid localization of *P. triconutum* overexpressing fluorescently tagged native Fructose Bisphosphate Aldolase (FBA) and β Carbonic Anydrase (CA). Confocal micrographs of 9A is C' YFP tagged FBA; 9B is C' CFP tagged β-CA; 9C is an overlay of C' YFP tagged FBA and C' CFP tagged β-CA; 9D and 9E are electron micrographs of immunogold localization of β-CA.

Figure 10 shows an analysis of CA activity in a β-CA over-expression *P. tricornutum* cell line. In separate analyses, equivalent numbers of wild type or β-CA overexpressing *P.tricornutum* cells were added to the MIMS chamber (at t=0) which contained ^{18}O enriched inorganic carbon. ^{18}O in CO_2 is exchanged with ^{16}O from water by cycles of hydration/dehydration, a process catalyzed by CA once cells are added.

Figure 11A shows a confocal micrograph of C' YFP tagged *P.tricornutum* protein 21592; a putative ornithine/arginine/diaminopimelate family decarboxylase. The autofluorescence in the center of the cell is the plastid. This location was recognized from previous experience to be consistent with pyrenoid localization. **Figure 11B** shows bootstrapped (100 replicates) maximum likelihood (ML) phylogenetic tree of an ornithine/arginine/diaminopimelate family decarboxylase proteins (*P. tricornutum* protein ID 21592) as inferred by the PROML program of PHYLIP 3.69, and midpoint rooted. The alignment (515 columns) was created by aligning the decarboxylase proteins with MUSCLE 3.8.31 and removing columns that contained more than 20% gaps.

Figure 12 shows SDS PAGE gel of the PtPyrDecar-C' GST fusion product and purification: lane 1) Molecular marker, 2) *E. coli* cell lysate, 3) Flow through containing most of the whole proteins, 4-6) Fractions 1-3 collected after wash and elution of GST protein

Figure 13 shows LipidTox green stains of (top) exponential phase-wild type cells and (bottom) exponential phase PtPyrDecar-over expression cell lines.

DETAILED DESCRIPTION

Described herein are engineered microalgae that accumulate large amounts of lipids while still maintaining exponential growth. The microalgae have been engineered so that the amount of carbon assimilated is increased relative to wildtype, the amount of nitrogen assimilated is decreased relative to wildtype or a combination of both. These microalgae produce high levels of lipid during exponential cell growth. Such microalgae are useful, for example, for the production of lipids that can serve as biofuel precursors.

1. Definitions

In order for the present invention to be more readily understood, certain terms and phrases are defined below and throughout the specification.

The articles "*a*" and "*an*" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

As used herein the term "*algae*" represents a large, heterogeneous group of primitive photosynthetic organisms which occur throughout all types of aquatic habitats and moist terrestrial environments. The term "*algae*" includes, for example, diatoms (bacillariophytes), green algae (chlorophytes), blue-green algae (cyanophytes), golden-brown algae (chrysophytes), haptophytes, freshwater algae, saltwater algae, Amphipleura,

Amphora, Chaetoceros, Cyclotella, Cymbella, Fragilaria, Hantzschia, Navicula, Nitzschia, Phaeodactylum, Thalassiosira Ankistrodesmus, Botryococcus, Chlorella, Chlorococcum, Dunaliella, Monoraphidium, Oocystis, Scenedesmus, Nanochloropsis, Tetraselmis, Chlorella, Dunaliella, Oscillatoria, Synechococcus, Boekelovia, Isochysis and Pleurochysis.

As used herein, the term "*carbon assimilation*" refers to the process by which microalgae concentrate CO_2 in chloroplast and the subsequent incorporation of carbon into biomass. Proteins that facilitate carbon assimilation include, but are not limited to, pyr-decarboxylase (*e.g.* GI:219122853, GI:223993801), Rubsico (*e.g.,* GI:118411023, GI:118411104), Beta CA (*e.g.,* GI:219109680) and Chloroplast HCO_3 transporter (*e.g.,* GI:219118294, GI:219111471, GI:223994025).

As used herein, the term "*diatom*" refers to unicellular microalgae that are encased within a silica cell wall. Diatoms include both centric diatoms (*e.g., Thalassiosira pseudonana* and pinnate diatoms (*e.g., Phaeodactylum triconutum*).

As used herein, a nucleic acid sequence is "*exogenous*" to a cell if it had been artificially introduced into the cell or a parent of the cell. The exogenous nucleic acid may be from a different species or from the same species relative to the cell to which it was introduced. In the case where the nucleic acid is from the same species of the cell to which it was introduced, the introduced nucleic acid occupies a different location in the genome of the cell relative to the endogenous copy of the nucleic acid or is operably linked to different nucleic acids than the endogenous gene. The exogenous nucleic acid may be present in more than one copy in the cell. The exogenous nucleic acid may be maintained in a cell as an insertion into the genome or as an episomal molecule.

An "*expression vector*" is a vector which is capable of promoting transcription of a nucleic acid incorporated therein. Typically, the nucleic acid to be expressed is "*operably linked*" to a transcription control element, such as a promoter and/or an enhancer, and is

therefore subject to transcription regulatory control by the transcription control element.

The term "*gene*" is used broadly to refer to any nucleic acid associated with a biological function. The term "gene" applies to a specific genomic sequence, as well as to a cDNA or an mRNA encoded by that genomic sequence.

The term "*introduced*" when referring to a heterologous or isolated nucleic acid refers to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell. The term includes such methods as "infection," "transfection," "transformation" and "transduction."

As used herein, the term "*isolated*" refers to the state in which substances (*e.g.,* polynucleotides) are free or substantially free of material with which they are naturally associated such as other polypeptides or polynucleotides with which they are found in their natural environment or the environment in which they are prepared (*e.g.,* cell culture).

As used herein, "*lipids*" are a class of molecules that are soluble in nonpolar solvents (such as ether and chloroform) and are relatively or completely insoluble in water. Lipid molecules consist predominantly of long hydrocarbon tails which are hydrophobic in nature. Examples of lipids include fatty acids (saturated and unsaturated); glycerides or glycerolipids (such as monoglycerides, diglycerides, triglycerides or neutral fats, and phosphoglycerides or glycerophospholipids); nonglycerides (sphingolipids, sterol lipids including cholesterol and steroid hormones, prenol lipids including terpenoids, fatty alcohols, waxes, and polyketides); and complex lipid derivatives (sugar-linked lipids, or glycolipids, and protein-linked lipids).

As used herein, the term "*microalgae*" includes all forms of microscopic, aquatic algae including, for example, phytoplankton and diatoms.

As used herein, the term "*nitrogen assimilation*" refers to the process by which microalgae reduce NO_3^- to NO_2^- and the subsequent incorporation of reduced nitrogen into biomass. Proteins that facilitate nitrogen assimilation include, but are not limited to, nitrate

reductase (*e.g.,* GI:21912672, GI:224010906), nitrate transporter (*e.g.,* GI:219115383, GI:223998258), NADPH Nitrate Reductase (*e.g.,* GI:219120092, GI:223995983), Ferredoxin Nitrate Reductase (*e.g.,* GI:219119472, GI:223999185), Nitrate Transporter (*e.g.,* GI:219120485, GI:223997254) and Glutamine Synthetase II (*e.g.,* GI:219123807, GI:224012585).

As used herein, the terms "*nucleic acid*," "*polynucleotide*," "*polynucleotide sequence*" and "*nucleic acid sequence*" refer to single-stranded or double-stranded deoxyribonucleotide or ribonucleotide polymers, or chimeras or analogues thereof. As used herein, the term optionally includes polymers of analogs of naturally occurring nucleotides having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (*e.g.,* peptide nucleic acids).

As used herein, "*operable linkage*" refers to a functional linkage between two nucleic acid sequences, such as a transcription control element (*e.g.,* a promoter) and the linked transcribed sequence. For example, a promoter is in operable linkage with a gene if it can mediate transcription of the gene.

The term "*vector*" refers to the means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophage, pro-viruses, phagemids, transposons, and artificial chromosomes, and the like, that may or may not be able to replicate autonomously or integrate into a chromosome of a host cell.

2. Inhibition of Nitrogen Assimilation

Nitrogen assimilation in microalgae, including diatoms, is the process by which NO_3^- is reduced to NO_2^- and subsequently incorporated into cellular biomass. Microalgae, like other photosynthetic eukaryotes, reduce NO_3^- to NO_2^- using an assimilatory nitrate

reductase enzyme (NR) NR catalyzed NO_3^- reduction appears to be the rate-limiting process in nitrogen acquisition (Campbell, *Annu. Rev. Plant Physiol. Plant Mol.* 50:277-303 (1999)).

A simplified diagram of the nitrogen assimilation pathways in the diatom *Phaeodactylum tricornutum* is provided in Figure 1, including putative assimilation pathways for urea, NH_4^+, and NO_3^- into amino acids. In the case of urea, the mitochondrial location of urease indicates that the resulting ammonia is assimilated by the mitochondria GS-GOGAT or urea cycle. NO_3^- derived ammonia is assimilated in the plastid, which is where nitrite reducatase is located. NH_4^+, while shown in Figure 1 as being assimilated in the plastid, might also be assimilated in the mitochondria.

In one aspect, the instant invention relates to engineered microalgae, including engineered diatoms that do not assimilate as much nitrogen as wild-type (i.e. the corresponding microalgae, which has not been engineered). The reduction in the rate of nitrogen assimilation can be by any amount. In certain embodiments, the rate of nitrogen assimilation is reduced by at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70% 80% or 90% relative to wild-type.

In some embodiments, the reduction in nitrogen assimilation can be achieved through the inhibition of the expression or activity of a protein that facilitates nitrogen assimilation. Inhibition of the expression or activity of a protein that facilitates nitrogen assimilation can be, but does not have to be, complete. For example, in certain embodiments, the expression or activity of the protein that facilitates nitrogen assimilation is reduced by at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70% 80% or 90%. In certain embodiments, the activity or expression of the protein that facilitates nitrogen assimilation is not completely inhibited. Thus in some embodiments, nitrogen assimilation is inhibited by no more than 20%, 25%, 30%, 40%, 50%, 60%, 70% 80% , 90% or 95%.

Any protein that facilitates nitrogen assimilation can be inhibited in the engineered microalgae of the instant invention. In certain embodiments, the inhibited protein is nitrate reductase, which is a protein that catalyzes the rate limiting step in nitrogen assimilation. The nucleic acid sequence that encodes *Phaeodactylum triconutum* nitrate reductase is provided in SEQ ID NO: 2 and Figure 2, while the nucleic acid sequence that encodes *Thalassiosira pseudonana* nitrate reductase is provided in SEQ ID NO: 4 and Figure 3.

In some embodiments the inhibited protein is a facilitator of nitrogen assimilation other than nitrate reductase. For example, in certain embodiments, the inhibited protein that facilitates nitrogen assimilation is selected from a group consisting of nitrate reductase (*e.g.,* GI:21912672, GI:224010906), Nitrate Transporter (*e.g.,* GI:219115383, GI:223998258), NADPH Nitrate Reductase (*e.g.,* GI:219120092, GI:223995983), Ferredoxin Nitrate Reductase (*e.g.,* GI:219119472, GI:223999185), Nitrate Transporter (*e.g.,* GI:219120485, GI:223997254) and Glutamine Synthetase II (*e.g.,* GI:219123807, GI:224012585).

The expression or activity of proteins that facilitate nitrogen assimilation can be inhibited using any technique known in the art. For example, in certain embodiments, the expression of a protein that facilitates nitrogen assimilation is inhibited by a nucleic acid inhibitor, such as an RNA interference (RNAi) molecule or an antisense molecule. Techniques that employ nucleic acid inhibitors, such as RNAi and antisense inhibition, can be used in microalgae, including in diatoms, to inhibit expression of any protein of interest (De Riso *et al., Nucleic Acids Research* 37:e96 (2009), incorporated by reference in its entirety). Such nucleic acid inhibitors can be directly contacted with a microalgae cell. Alternatively, expression vectors encoding such molecules may be contacted with or introduced into a microalgae cell using any technique known in the art. Such vectors may, for example, incorporate into the microalgae genome or may remain episomal.

The nucleic acid inhibitor can be operably linked to any transcription control element that can induce transcription of the nucleic acid inhibitor in the microalgae to which it has been added. In certain embodiments, the transcription control element is a constitutive promoter, while in other embodiments the transcription control element is an inducible promoter. In certain embodiments, the transcription control element is selected from a group consisting of the 400 base pair region upstream of the fucoxanthin binding protein (FcpB. Siaut, M. et al. (2007) *Gene 406*:23-35; De Riso, V. et al., (2009) *Nucl. Acids Res* epub May 31, 2009); the promoter of the Fucoxanthin chlorophyll a/c protein (NCBI accession no. GI:219112237), the promoter of the *P. tricornutum* gene (GI:219127450), the promoter for diatom specific cell wall protein alpha frustulin 3 (GI:219117241), the promoter for polyubiquitin (GI:219118861), the promoter for the *P. tricornutum* gene (GI:219123984), and the promoter for *P. tricornutum* trypsin-like serine protease (GI:219116468) (Allen, AE et al., *Proc. Natl. Acad. Sci. 105*:10438-10443; .Maheswari, U, et al., *Genome Biology 11*:R85).

In certain embodiments, the inhibitor is an RNAi molecule, for example, double-stranded (ds) RNA molecules of any length, siRNA molecules, shRNA molecules and amiRNA molecules. Such molecules are known in the art and the skilled artisan would be able to create oligonucleotide inhibitors of proteins that facilitate nitrogen assimilation using routine methods.

RNAi is the process through which dsRNA molecules inhibit the expression of mRNA to which the dsRNA has sequence homology. In plants, this process is also known as post-transcriptional gene silencing. Inhibition of mRNA expression can occur through a number of mechanisms, including epigenetic inhibition of the transcription of the mRNA molecule, targeted post-transcriptional degradation of the mRNA molecule, or inhibition of translation of the mRNA molecule. Because RNAi inhibition is primarily dependant on

sequence homology between the dsRNA molecule and the mRNA target, RNAi molecules can be routinely designed to inhibit any mRNA target.

In certain embodiments, long dsRNA molecules are used to inhibit expression of a protein that facilitates nitrogen assimilation. Such dsRNA molecules may comprise at least 30, 40, 50, 60, 70, 80, 90, 100, 150, 200 or 250 nucleotides of the sequence that is homologous to the mRNA sequence that encodes the protein that facilitates nitrogen assimilation. Notably, the sequence of the long dsRNA molecule need not be identical to the mRNA sequence it inhibits. Thus, in certain embodiments the long dsRNA molecule is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the mRNA that encodes a protein that facilitate nitrogen assimilation.

In certain embodiments, shorter RNAi molecules, such as small interfering RNA (siRNA) molecules, short hairpin (shRNA) molecules and artificial micro RNA (amiRNA) molecules, are used to inhibit expression of a protein that facilitates nitrogen assimilation. Such molecules typically have sequences that comprise at least 15, 19, 21, 22, or 23 nucleotides that are homologous to a target sequence. Like the long dsRNA molecules, the sequences of the shorter RNAi molecules do not have to be perfectly identical to the target. Thus, in certain embodiments, the sequence of the short RNAi molecule is at least 70%, 75%, 80%, 85%, 90% or 95% identical to the sequence of the mRNA that encodes a protein that facilitates nitrogen assimilation.

In certain embodiments, antisense oligonucleotides are used to interfere with expression of a protein that facilitates nitrogen assimilation. Typically an antisense molecule comprises at least 15, 17, 19, or 21 nucleotides of the complement of the mRNA sequence that encodes the protein that facilitates nitrogen assimilation. However, longer antisense RNA molecules can also be used. Thus, in certain embodiments, antisense RNA molecules can be up to 50, 100, 200, 300, 400, 500 or more nucleotides in length.

Furthermore, the sequence of antisense molecules need not be perfectly complementary to the mRNA sequence it inhibits. Thus, in certain embodiments, the antisense RNA molecule is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% complementary to the mRNA that encodes a protein that facilitate nitrogen assimilation.

In certain embodiments, the engineered microalgae of the instant invention contain a mutation that inhibits the expression or activity of a protein that facilitates nitrogen assimilation. In certain embodiments, the mutation is in the gene that encodes the protein that facilitates nitrogen assimilation. In some embodiments, the mutation is in an endogenous transcription control element operably linked to a gene encoding a protein that facilitates nitrogen assimilation. The mutation can: (i) completely eliminate the activity or expression of the protein, (*e.g.,* through a complete knock-out mutation), (ii) reduce the activity of the protein (*e.g.* through a point mutation to the protein-coding region of the protein), or (iii) reduce the expression of the protein (*e.g.*, through a mutation to the transcription control element operably linked to the protein).

Mutations that inhibit the expression or production of a nitrogen assimilating protein can be introduced into a microalgae using any method known in the art. Because of the small size and rapid growth of microalgae, a desired mutation can be generated through techniques such as random mutagenesis and screening. Certain microalgae, including the diatoms *Phaeodactylum triconutum* and *Thalassiosira pseudonana*, are particularly suitable for mutagenesis, because their genomes are relatively small and have been completely sequenced. Mutagenesis can be performed using methods known in the art, including radiation or chemically induced mutations. Screens for a desired mutation can be performed, for example, using whole genome oligonucleotide tilling arrays (*see, e.g.,* Mock *et al., PNAS,* 5:1579-1584 (2008), incorporated by reference in its entirety), through direct sequencing of the gene of interest, by monitoring expression of the mRNA or protein of

interest, or by phenotype screening for microalgae that produce high levels of lipid during exponential growth.

3. Enhancement of Carbon Assimilation

Carbon assimilation in microalgae, including diatoms, is the process by which CO_2 is concentrated in chloroplasts and subsequently incorporated into cellular biomass. In diatoms and other microalgae, much of carbon assimilation occurs in pyrenoids. The pyrenoid is a subcellular crystalline structure within the chloroplast of unicellular algae, but is absent in plants. This "suborganelle" contains mostly rubisco, fructose bisphopsphate aldloase (FBA) and a β-carbonic anhydrase (CA) and it is the location where photosynthetically driven carbon fixation occurs.

The existence of pyrenoids facilitates CO_2 concentrating mechanisms that are essential to carbon assimilation in microalgae. Due to the low affinity of rubisco for CO_2, interference from O_2, and the low concentrations of CO_2 in aquatic systems, microalgae must deliver large amounts of CO_2 to rubisco in order for carbon fixation to occur. This can be achieved either biophysically or biochemically. The biophysical approach involves bicarbonate uptake and then delivery to the pyrenoid with transporters, and subsequent conversion to CO_2 by a carbonic anhydrase that is located close to the active site of carbon fixation.

CO_2 may be biochemically concentrated by carboxylating an organic acid outside of the chloroplast, delivering to the chloroplast by a transporter and decarboxylating at the site of rubisco. Inhibitors of the anaplerotic carboxylase phosphoenolpyruvate carboxylase (PEPC) have been shown to reduce carbon fixation in certain microalgae, including *P. tricornutum*, indicating the presence of a biochemical CO_2 concentrating mechanism (McGinn and Morel, *Plant Physiology,* 146:300-309 (2008)). Pyrenoid-localized decarboxylase, ("pyr-decarboxylase"), a highly conserved protein present in all of the

currently sequenced marine phytoplankton genomes, but not in cyanobacteria or plants, is involved in biochemical carbon fixation. The pyr-decarboxylase gene in *P. tricornautam* is shown in Figure 4 and SEQ ID NOs: 5 and 6. The pyr-decarboxylase gene in *T. pseudonana* is shown in Figure 5 and SEQ ID NOs: 7 and 8.

In another aspect, the instant invention relates to engineered microalgae, including engineered diatoms, that have an increased level of carbon assimilation. The increase in the rate of carbon assimilation can be by any amount. In certain embodiments, the rate of carbon assimilation is increased by at least 25%, 50%, 75%, 100% or 200%.

In some embodiments, the elevation in carbon assimilation can be achieved through an increase in the expression or activity of a protein that facilitates carbon assimilation. Increase in the expression or activity of a protein that facilitates carbon assimilation can be by any amount. For example, in certain embodiments, the expression or activity of the protein that facilitates carbon assimilation is increased by at least 25%, 50%, 75%, 100% or 200%.

The expression or activity of any protein that facilitates carbon assimilation can be increased in the engineered microalgae of the instant invention. In certain embodiments, the protein is pyr-decarboxylase. The nucleic acid sequence that encodes *Phaeodactylum triconutum* pyr-decarboxylase is provided in SEQ ID NO: 6 and Figure 4, while the nucleic acid sequence that encodes *Thalassiosira pseudonana* pyr-decarboxylase is provided in SEQ ID NO: 8 and Figure 5. In certain embodiments, the invention relates to an isolated nucleic acid molecule or vector that comprises a sequence encoding pyr-decarboxylase operably linked to a transcription control element.

In some embodiments the protein is a facilitator of carbon assimilation other than pyr-decarboxylase. For example, in certain embodiments, the protein that facilitates carbon assimilation is selected from a group consisting of pyr-decarboxylase (*e.g.* GI:219122853,

GI:223993801), Rubsico (*e.g.,* GI:118411023, GI:118411104), Beta CA (*e.g.,* GI:219109680) and Chloroplast HCO_3 transporter (*e.g.,* GI:219118294, GI:219111471, GI:223994025).

The expression or activity of proteins that facilitate carbon assimilation can be increased using any technique known in the art. For example, in certain embodiments, the expression of a protein that facilitates carbon assimilation is increased in microalgae through the introduction into the microalgae of a nucleic acid that encodes a protein that facilitates carbon assimilation operably linked to a transcription control element that drives transcription of the nucleic acid in the microalgae. For example, expression vectors encoding such molecules may be introduced into a microalgae cell using any technique known in the art. Such vectors may, for example, incorporate into the microalgae genome or may remain episomal. In other embodiments, the expression of an endogenous gene that encodes a protein that facilitates carbon assimilation is increased, for example, by operably linking it to an exogenous transcription control element.

Any transcription control element that drives expression of an operably linked nucleic acid in microalgae of interest can be used. In certain embodiments, the transcription control element is a constitutive promoter, while in other embodiments the transcription control element is an inducible promoter. In certain embodiments, the transcription control element is selected from a group consisting of is selected from a group consisting of the promoter of the 400 base pair region upstream of the fucoxanthin binding protein (FcpB. Siaut, M. et al. (2007) *Gene 406*:23-35; De Riso, V. et al., (2009) *Nucl. Acids Res* epub May 31, 2009); the promoter of the Fucoxanthin chlorophyll a/c protein (NCBI accession no. GI:219112237), the promoter of the *P. tricornutum* gene (GI:219127450), the promoter for diatom specific cell wall protein alpha frustulin 3 (GI:219117241), the promoter for polyubiquitin (GI:219118861), the promoter for the *P.*

tricornutum gene (GI:219123984), and the promoter for *P. tricornutum* trypsin-like serine protease (GI:219116468) (Allen, AE et al., *Proc. Natl. Acad. Sci. 105*:10438-10443; .Maheswari, U, et al., *Genome Biology 11*:R85).

4. Engineered Microalgae

In certain embodiments, the instant invention relates to engineered microalgae with enhanced lipid expression during exponential growth. In some embodiments, the microalgae are engineered such that their rate of nitrogen assimilation is reduced relative to wild-type. In some embodiments, the microalgae are engineered such that their rate of carbon assimilation is increased relative to wild-type.

The microalgae to be engineered can be any species in which inhibition of nitrogen assimilation or elevation of carbon assimilation leads to enhanced lipid production during exponential growth. In certain embodiments the species of the engineered microalgae of the instant invention is selected from the group consisting of *Phaeodactylum tricornutum, Thalassiosira pseudonana, Dunaliella tertiolecta, Chlamydomonas reinhardtii, Volvox varteri, Aureococcos anophagefferens and Nanochloropsis sp.*

In certain embodiments, the engineered microalgae of the instant invention are a diatom. Diatoms are unicellular microalgae that are encased within a silica cell wall. Diatoms include both centric diatoms and pinnate diatoms. In some embodiments, the species of the microalgae is *Phaeodactylum triconutum* or *Thalassiosira pseudonana*. The genomes of *P. triconutum* and *T. pseudonana* have been completely sequenced and are publically available.

The engineered microalgae can be grown under any condition appropriate for their particular species. Exemplary growth conditions for microalgae can be found in, for example, U.S. Patent Application Publication Nos. 2009/0215140, 2009/0274736, 2010/0151339, 2010/0170144, 2010/0184197 and 2010/0261918. For example, if the

engineered microalgae is a diatom, it can be grown as a batch culture under continuous fluorescent light at 18° C in 0.2 μm filtered and autoclaved coastal seawater amended with f/2 nutrients using NO_3^- as the sole nitrogen source, as described in Guillard, *Culture of Marine Invertebrate Animals*. Plenum Press, New York, pp. 29-60 (1978) and Allen *et al., J. Phycol.* 41:95-105 (2005), each of which is incorporated by reference in its entirety.

Certain microalgae, such as *P. triconutum*, are capable of being cultured as a biofilm. Methods of inducing microalgae growth as a biofilm are known in the art. For example, exemplary methods can be found in Stanley and Callow, *European Journal of Phycology* 42:191-197 (2007) and Vardi *et al., Current Biology* 18:895-899 (2008). The growth of microalgae in a biofilm can be useful because it facilitates the harvesting of lipids secreted into the growth media. Thus, in certain embodiments, the invention relates to the growth of the engineered microalgae of the invention in a biofilm.

5. Methods of Lipid Production

In certain embodiments, the instant invention relates to methods for producing lipids using the engineered microalgae of the instant invention. In certain embodiments, this method comprises the steps of: (1) growing a culture of the engineered microalgae for a sufficient period of time and under appropriate conditions such that lipid is produced during exponential growth and (2) harvesting lipids from the microalgae culture.

Engineered microalgae can be grown under condition, which are well known in the art including, but not limited to, those described herein. In certain embodiments, the engineered microalgae are grown under conditions in which they are not nutrient (*e.g.,* nitrogen or silicon) deprived. In certain embodiments, the engineered microalgae are grown under conditions such that a nucleic acid encoding a RNAi molecule that inhibits expression of a protein that facilitates nitrogen assimilation is transcribed. In some

embodiments, the engineered microalgae are grown under conditions such that a nucleic acid encoding a protein that facilitates carbon assimilation is transcribed.

Any of a variety of methods known in the art can be used for harvesting lipids from microalgae (See, for example, U.S. Patent Application Publication Nos. 2010/0151339, 2010/0170144, 2010/0184197 and 2010/0261918.

In certain embodiments, microalgae may be separated from the medium and various components, including lipids, may be extracted using any method known in the art. For example, microalgae may be partially separated from the medium using a standing whirlpool circulation, harvesting vortex and/or sipper tubes or other methods known in the art. Alternatively, industrial scale commercial centrifuges of large volume capacity may be used. Such centrifuges may be obtained from known commercial sources (*e.g.,* Cimbria Sket or IBG Monforts, Germany; Alfa Laval A/S, Denmark). Centrifugation, sedimentation and/or filtering may also be of use to purify lipids from other microalgal components. Separation of microalgae from the aqueous medium may be facilitated by addition of flocculants, such as clay (*e.g.,* particle size less than 2 microns), aluminum sulfate, FeC13 at pH about pH 9-10, polyacrylamide or the like. In the presence of flocculants, microalgae may be separated by simple gravitational settling, or flotation, or may be more easily separated by centrifugation. Flocculant-based separation of microalgae is disclosed, for example, in U.S. Patent Application Publication No. 2002/0079270, incorporated herein by reference in its entirety.

In certain embodiments, microalgae may be disrupted to facilitate separation of lipids. Any method known for microalgae disruption may be utilized, such as ultrasonication, French press, osmotic shock, mechanical shear force, cold press, thermal shock, rotor-stator disruptors, valve-type processors, fixed geometry processors, nitrogen decompression or any other known method.

The invention now having been generally described will be more readily understood by reference to the following examples, which are included merely for purposes of illustration and are not intended to be limiting in any way.

EXAMPLES

Example 1: Generation of Engineered Microalgae having Reduced Nitrogen Assimilation

Both *P. triconutum* and *T. pseudonana* encode a single copy of nitrate reductase (NR), the enzyme that controls the rate-limiting step of nitrogen assimilation in all known eukaryotic marine algae (Allen *et al., J. Phycol.* 41:95-104 (2005)). Transgenic diatoms that expressed a NR-yellow fluorescence protein (YFP) N'-terminus fusion revealed that NR is localized in both the cytoplasm and the peroxisome. This localization was further verified using immuno-gold labeling transmission electron microscopy (TEM) (Figure 6).

To generate an engineered diatom having reduced nitrogen assimilation, vectors encoding an RNAi molecule that inhibited NR were developed. Using relative growth rates on nitrate and ammonium as a preliminary screen, several putative NR-RNAi lines were identified. These engineered diatoms exhibit reduced growth rates on nitrate relative to ammonium, over 50% reduced NR activity in crude protein extracts, and reductions by 40-60% in NR protein relative to wild type as determined through immunoblotting (Figure 7). These engineered cell lines have been stable for over 18 months.

Example 2: Engineered Microalgae having Reduced Nitrogen Assimilation Exhibit Enhanced Lipid Production During Exponential Growth.

Engineered diatoms having reduced nitrogen assimilation were generated as described above. Observations with light microscopy revealed the presence of numerous lipid droplets in the engineered diatom culture, even when the diatoms were experiencing

exponential growth. Further light microscopy and epifluorescence experiments using LipidTox neutral green confirmed that these were lipid-rich droplets, which further confirmed using confocal microscopy was performed on a CARS (Coherent Anti-Stokes Raman Scattering) platform verified that these droplets are in fact composed of lipid (Figure 8).

Notably, the exponentially growing engineered microalgae exhibit a similar lipid distribution to nitrogen-limited stationary phase wild type microalgae

Example 3: Generation of Engineered Microalgae having Increased Carbon Assimilation

As described above, the pyrenoid is a subcellular crystalline structure within the chloroplast of microalgae but not plants. This "suborganelle" contains mostly rubisco, fructose bisphopsphate aldloase (FBA) and a -carbonic anhydrase (CA) and it is the location where photosynthetically driven carbon fixation occurs (Figure 9).

Pyrenoid localization facilitates CO_2 concentrating mechanisms that are essential to microalgae. Because of the low affinity of rubisco for CO_2, interference from O_2, and the low concentrations of CO_2 in aquatic systems, microalgae must deliver large amounts of CO_2 to rubisco. This can be achieved biophysically or biochemically.

The biophysical approach involves bicarbonate uptake and then delivery to the pyrenoid with transporters, and subsequent conversion to CO_2 by a carbonic anhydrase that is located closely to the active site of carbon fixation. Using inlet membrane mass spectrometry (MIMS) we have found that overexpression of the pyrenoid localized CA in *P. tricornutum* results in increased delivery of bicarbonate and carbon fixation; these experiments provide evidence for a potential for a biophysical CCM (Figure 10).

The biochemical approach involves carboxylation of an organic acid outside of the chloroplast, followed by delivery and decarboxylation at the site of rubisco. Inhibitors of

the anaplerotic carboxylase phosphoenolpyruvate carboxylase (PEPC) have been shown to reduce carbon fixation in *P. tricornutum*, indicating that it plays a role in biochemical CCM.

To generate an engineered microalgae with enhanced carbon assimilation, a putative ornithine/arginine/diaminopimelate family decarboxylase (protein id 21592) was fused with C' YFP and overexpressed. Confocal microscopy revealed that the decarboxylase localized to the pyrenoid (Figure 11a). Based on its localization, the decarboxylase was called "pyr-decarboxylase". Manual examination of the decarboxylase amino acid sequence revealed a canonical TAAFAP amino acid motif used for targeting and import to complex multi-membrane diatom plastids (Vugrinec *et al., Journal of Cell* Biology 88:81-81 (2009)). The localization of the *P. tricornutum* pyr-decarboxylase (PtPyrDecarboxylase) indicates that it may be the missing link for a biochemical CCM in *P. tricornutum*. Furthermore, pyr-decarboxylase is conserved in all marine phytoplankton genomes sequenced to date (Figure 11b), but absent in other major green lineages such as cyanobacteria and plants.

PtPyrDecarboxylase was fused with a C'terminus glutathione S-transferase and cloned into an *Escherichia coli* expression vector with an L-arabinose induction system. After induction, the PtPyrDecarboxylase -C'GST was purified using glutathione affinity chromatography (Figure 12).

Example 4: Engineered Microalgae having Increased Carbon Assimilation Exhibit Enhanced Lipid Production During Exponential Growth.

P. tricornutum overexpresssing PtPyrDecarboxylase-YFP exhibit wild-type growth rates in typical media. As with the NR-RNAi engineered microalgae, the PtPyrDecarboxylase-YFP microalgae contain a large number of visible lipid droplets while in exponential growth. Preliminary epifluorescence studies with LipidTox neutral green

confirm that droplets are packed with lipids, and the cells are also more lipid rich than exponentially growing wild type cells (Figure 13).

EQUIVALENTS

While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The appended claims are not intended to claim all such embodiments and variations, and the full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

We claim:

1. An engineered microalgae that produces lipid during exponential growth, wherein the microalgae has been engineered to enhance the expression level or activity of a protein that facilitates carbon assimilation or reduce the expression level or activity of a protein that facilitates nitrogen assimilation relative to the wild-type microalgae.

2. The engineered microalgae of claim 1, which is a diatom.

3. The engineered microalgae of claim 2, which is *Phaeodactylum triconutum* or *Thalassiosira pseudonana*.

4. The engineered microalgae of claim 1, which contains a nucleic acid, which encodes a protein that facilitates carbon assimilation operably linked to an exogenous transcription control element.

5. The engineered microalgae of claim 4, wherein the protein that facilitates carbon assimilation is selected from a group consisting of pyr-decarboxylase, rubsico, beta CA and chloroplast HCO_3 transporter.

6. The engineered microalgae of claim 5, wherein the protein that facilitates carbon assimilation is pyr-decarboxylase.

7. The engineered microalgae of claim 4, wherein the nucleic acid and the transcription control element are both exogenous.

8. The engineered microalgae of claim 4, wherein the transcription control element is the fucoxanthin binding protein promoter.

9. The engineered microalgae of claim 1, which contains a nucleic acid encoding an RNAi molecule that is an inhibitor of the expression of the protein that facilitates nitrogen assimilation.

10. The engineered microalgae of claim 9, wherein the protein that facilitates nitrogen assimilation is selected from the group consisting of nitrate reductase, nitrate transporter, NADPH nitrate reductase, ferredoxin nitrate reductase and glutamine synthetase II.

11. The engineered microalgae of claim 9, wherein the nucleic acid is operably linked to a transcription control element.

12. The engineered microalgae of claim 11, wherein the transcription control element is the fucoxanthin binding protein promoter.

13. The engineered microalgae of claim 1, wherein a gene encoding a nitrogen assimilation protein contains a mutation that reduces the expression or activity of the protein.

14. The engineered microalgae of claim 13, wherein the protein that facilitates nitrogen assimilation is selected from the group consisting of nitrate reductase, nitrate transporter, NADPH nitrate reductase, ferredoxin nitrate reductase and glutamine synthetase II.

15. The engineered microalgae of claim 14, wherein the protein that facilitates nitrogen assimilation is nitrate reductase.

16. A method of producing lipids from microalgae comprising:

a) growing a culture of the engineered microalgae of claim 1 for a sufficient period of time and under appropriate conditions such that lipid production is enhanced during exponential growth; and

b) harvesting lipids from the microalgae culture.

17. The method of claim 16, wherein the microalgae is a diatom.

18. The method of claim 17, wherein the diatom is grown in a biofilm.

19. An expression vector comprising a nucleic acid sequence encoding pyr-
5 decarboxylase operably linked to a transcription control element.

20. The expression vector of claim 19, wherein the transcription control element is the fucoxanthin binding protein promoter.

Figure 1

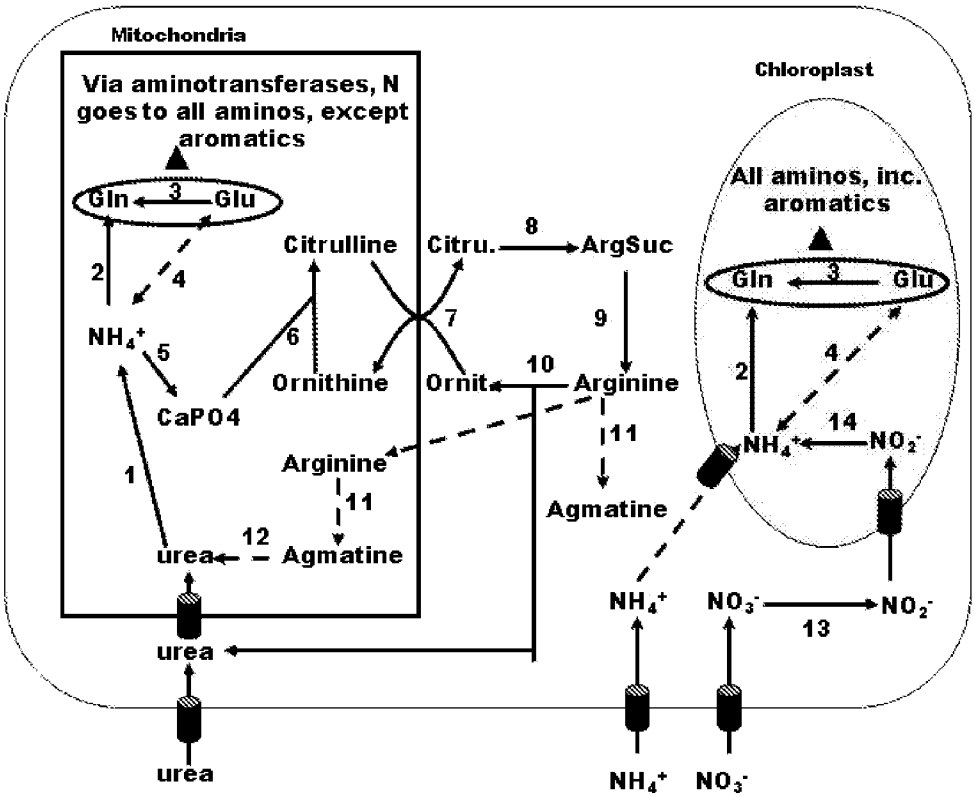
Mitochondria
Via aminotransferases, N goes to all aminos, except aromatics
Gln
Glu
3
2
4
NH4+
5
CaPO4
6
Citrulline
Ornithine
7
Citru.
8
ArgSuc
9
10
Ornit
Arginine
11
Agmatine
1
12
urea
urea
Chloroplast
All aminos, inc. aromatics
Gln
Glu
3
2
4
NH4+
14
NO2-
NH4+
NO3-
13
NO2-
NH4+
NO3-

Figure 2 *Phaeodactylum tricornutum* Nitrate Reductase

A (SEQ ID NO: 1)

MVPKPEDPTVKAENNAAMDQLSLLDKDDISSASRSCRELYGPYPKAIPVPFLNSRNEAREGDTPAASVIAQAK
TIFDVPADYRDVGTPDEWVPRDGRLVRLTGKHPFNVEPPLAILKQHRFITPSSLHYVRNHGACPKLSWKEHTV
CVGGKLVPNALELSMDEIVAMEPRELPVTLVCAGNRRKEQNMIRQTIGFNWGPSGVSTSVWKGVLLRDLLLRA
GVSEKNMAGKHVEFIGVEDLPNKVGPGPFQEEPWGKLVKYGTSVPLARAMNPAYDILIAYEQNGEVLQPDHGY
PVRLIIPGYIGGRMIKWLKYINVIPHETKNHYHYHDNRILPGGWWYKPEYIFNELNINSAIAAPDHNETLSIA
KNIAKTYDVTGYAYTGGGRLITRVEISVDGGIHWELAKLERKEQPTDYGMYWCWTWWNYEVKVADLVGAKEII
CRAWDESNNPQPVVPTWNLMGMGNNQAFRVKVHMDKTASGEHVFRFEHPTQPGQQTGGWMTKVATKPESAGFG
RLLEVQGESKEDAAPAPPPKENTKIFTMEEIEKHNTEEDCWIVVKDRVYDCTEYLELHPGGIDSIVINGGADS
TEDFVAIHSTKATKMLEKYYIGQLDKSSVAEEKKQEDEPLVDADGNALALNPKKKTPFRLQNKITLSRDSYLL
DFALPSPKHVLGLPTGKHMFISALINGEMVLRRYTPISSNYDIGCVKFVVKAYRPCERFPDGGKMSQYLDQIN
VGDYVDMRGPVGEFEYSANGSFTIDAEPCFATRFNMLAGGTGITPVMQIAAEILRNPQDPTQMSLIFACREEG
DLLMRSTLDEWAANFPHKFKIHYILSDSWSSDWKYSTGFVDKALFSEYLYEAGDDVYSLMCGPPIMLEKGCRP
NLESLGHKKDKIFSF

B (SEQ ID NO: 2)

ATGGTACCGAAACCTGAAGATCCCACAGTCAAGGCAGAGAATAATGCGGCGATGGATCAACTTAGTCTCCTCG
ACAAAGATGATATATCGTCGGCTTCTCGCTCGTGCCGAGAACTCTACGGACCTTACCCCAAAGCTATTCCTGT
GCCGTTCTTGAATTCTCGTAACGAAGCTCGCGAAGGTGACACTCCCGCCGCCAGCGTCATCGCGCAAGCCAAA
ACCATCTTTGACGTACCGGCGGACTATCGTGACGTGGGAACACCGGATGAATGGGTTCCCCGCGATGGACGCC
TCGTGCGTCTGACGGGTAAGCATCCCTTCAACGTCGAACCACCGCTGGCGATTCTGAAGCAGCATCGATTTAT
TACGCCGTCCTCGTTGCATTACGTACGCAACCACGGAGCGTGCCCGAAGCTGTCTTGGAAAGAACACACTGTT
TGTGTGGGAGGAAAACTGGTACCGAATGCCTTGGAGCTCTCGATGGACGAAATCGTAGCGATGGAACCGCGAG
AGCTGCCCGTCACGTTGGTCTGTGCCGGAAATCGTCGGAAGGAACAAAACATGATCCGTCAAACAATCGGCTT
CAACTGGGGCCCGAGCGGCGTCTCAACCAGCGTTTGGAAGGGAGTGCTCCTACGCGATTTGTTGCTCCGCGCA
GGGGTTTCGGAAAAGAACATGGCAGGGAAGCACGTCGAATTTATTGGTGTCGAAGACTTGCCGAACAAGGTGG
GACCCGGGCCGTTCCAGGAGGAACCATGGGGCAAACTTGTCAAGTACGGAACCAGTGTCCCGCTCGCTCGGGC
TATGAATCCAGCGTACGACATCCTCATTGCCTATGAGCAGAACGGCGAAGTCTTGCAGCCCGATCACGGCTAC
CCCGTCCGTCTCATCATTCCTGGTTATATTGGAGGACGGATGATTAAATGGCTTAAATACATCAACGTGATTC
CGCACGAAACCAAGAATCACTATCATTACCACGACAATCGCATTTTACCGGGAGGTTGGTGGTACAAACCGGA
GTACATTTTCAATGAACTCAACATCAATTCGGCCATCGCTGCTCCTGATCACAATGAAACGCTTTCGATCGCC
AAGAATATTGCCAAGACGTATGACGTTACGGGTTACGCATATACTGGTGGTGGTCGTCTCATCACCAGGGTCG
AAATTTCAGTTGATGGCGGTATCCATTGGGAACTTGCCAAACTTGAACGCAAGGAGCAGCCAACGGACTACGG
AATGTACTGGTGCTGGACTTGGTGGAACTACGAAGTAAAGGTGGCCGACTTGGTGGGAGCCAAGGAAATTATA
TGCCGCGCCTGGGATGAGTCCAACAACCCTCAGCCAGTTGTTCCAACATGGAATCTGATGGGTATGGGAAATA
ATCAAGCCTTTCGTGTCAAGGTACACATGGACAAGACAGCTAGCGGCGAGCATGTGTTTCGGTTTGAGCATCC
AACTCAGCCTGGTCAACAAACTGGTGGGTGGATGACAAAGGTCGCCACCAAGCCTGAGTCGGCTGGGTTCGGA
CGGTTGCTGGAAGTGCAGGGTGAGTCCAAAGAAGACGCGGCCCCGGCTCCACCTCCGAAGGAAAATACCAAAA
TTTTCACGATGGAAGAGATTGAAAAGCACAACACTGAAGAAGACTGTTGGATTGTGGTGAAGGATCGTGTCTA
CGACTGTACCGAGTATCTAGAGCTGCACCCTGGCGGCATTGACTCGATTGTTATCAACGGCGGCGCAGATTCC
ACGGAAGACTTTGTGGCAATCCACTCTACCAAGGCTACAAAGATGCTCGAGAAGTACTACATTGGCCAGCTCG
ACAAAAAGTAGTGTGGCCGAGGAGAAAAAACAAGAAGACGAACCTCTCGTCGATGCCGATGGCAATGCTCTTGC
CTTGAACCCAAAGAAGAAGACGCCATTTCGTCTACAAAACAAAATCACACTTAGTCGAGACAGCTACCTATTG
GATTTTGCTTTGCCAAGCCCAAAGCATGTTTTGGGGCTACCCACGGGAAAGCACATGTTTATTTCGGCCCTCA
TTAATGGAGAGATGGTACTCCGCCGCTACACTCCTATCTCATCCAATTACGACATTGGATGTGTAAAGTTTGT
TGTCAAGGCATACCGTCCGTGTGAACGCTTTCCAGACGGTGGCAAGATGAGCCAATACCTAGACCAGATCAAT
GTTGGCGACTATGTTGATATGCGCGGACCAGTTGGGGAATTTGAGTACTCGGCCAACGGCAGTTTTACAATCG
ACGCCGAACCTTGTTTTGCCACCAGGTTCAACATGCTTGCTGGGGGGACCGGCATAACGCCCGTAATGCAGAT
TGCTGCGGAAATTTTGCGAAACCCACAAGACCCTACACAAATGTCCCTTATTTTTGCATGCCGCGAGGAAGGC
GATCTCTTGATGCGAAGCACTTTGGACGAATGGGCTGCTAACTTTCCTCACAAGTTCAAGATTCACTACATCC
TATCTGACAGCTGGTCTTCCGACTGGAAGTATTCCACAGGATTCGTAGACAAAGCGCTATTTTCCGAGTACTT
GTACGAAGCAGGCGATGATGTTTACAGCCTCATGTGCGGCCCACCAATTATGTTAGAGAAAGGCTGCCGTCCA
AACTTGGAGAGCCTTGGTCACAAAAAGGACAAAATTTTTTCCTTTTAA

Figure 3 *Thalassiosira pseudonana* Nitrate reductase

A (SEQ ID NO: 3)

MAPINGMKRADTSESSVDLTTLIKPSTSIQNFKSISSSQPTKEQTCVDLYGPYPSSIPVPTISKDGSVPPTDV
TSKAKTMWDVQSYPDHRDVGTPDEWIPRDGKLVRLTGRHPFNVEPPLSVHQEHKFITPTCLHYVRNHGACPNI
KWEEHRVRVGGLVDTPLDLGMDEIVAMEPRELPVTLVCAGNRRKEQNMIRQTIGFNWGAGGVSTNVWKGVTLR
DLLLKAGVSEKNMAGKHVEFIGAEDLPNKVGPGPFKDEPWGKLVKYGTSVPLARAMNPAYDILIAYEANGEVL
QPDHGYPIRLIIPGYIGGRMIKWLTDINVLEHETKNHYHYHDNRILPPHITAEESLTGGWWYKPEYIFNELNI
NSAMTAPDHNETIDLASSIGSSYEVGGYAYTGGGRRISRVEVSTDGGVHWEIANINQIEKPTDYGMYWCWIWW
TFDLKVADLVGTKELWCRAWDESNNVQPNDPTWNLMGMGNNQVFRIKVHLDKDVNGKHVFRFEHPTKPGQQEG
GWMTTLAGKPDSAGFGRLLEQGQPAKEAAPAAAPAKTSGSKLIKMEEVRKHNKEEDVWIVVNNKVYDCTEYLD
LHPGGADSILINAGEDATEDFVAIHSTKATKMLDKFYVGDLDTTSVAVVSDAEEERLCPKTGRKVALDPKHKH
AFKLQTKTVLSRDSFELDFALQTPEHVLGLPTGKHVFLSADINGEMVMRRYTPTTSDHDIGQIKFVIKAYPPC
ERFPLGGKMSQYLDSLKVGDTIDMRGPVGEFDYHGNGKFLKEHDECYATHFNMIAGGTGITPVMQIASEILRN
PDDKTTMSLVFGCREEGDLLLRSTLDEWAVKHADRFKVHYVLSENAPPGWTHSIGFVSKELFEKELFPAGDNC
YNLMCGPPIMLERGCTPNLKALGHKEDNIFSF

B (SEQ ID NO: 4)

ATGGCACCCATCAACGGCATGAAACGAGCCGACACATCCGAGTCCTCCGTGGACCTCACCACCCTCATCAAAC
CGTCCACCTCCATCCAAAACTTTAAATCCATCTCCTCCTCCCAACCCACCAAAGAACAAACATGCGTCGACCT
CTACGGCCCCTACCCCTCCTCCATCCCCGTCCCCACCATCTCCAAAGACGGTAGCGTCCCACCCACCGACGTC
ACCTCCAAAGCCAAGACCATGTGGGACGTCCAATCCTACCCCGACCATCGTGACGTCGGTACACCCGACGAAT
GGATTCCACGCGATGGCAAATTAGTGAGGTTAACTGGACGTCATCCCTTCAACGTCGAACCTCCCTTGAGTGT
CCATCAAGAACACAAGTTCATCACTCCCACCTGTCTTCACTACGTTCGTAATCACGGAGCATGTCCCAATATC
AAGTGGGAGGAGCATCGTGTTCGTGTGGGTGGACTGGTGGATACGCCGCTTGATTTGGGGATGGATGAGATTG
TGGCGATGGAACCGAGGGAGCTTCCGGTGACGTTGGTGTGTGCTGGGAATAGGAGGAAGGAGCAGAATATGAT
TAGGCAGACGATTGGGTTTAATTGGGGTGCGGGAGGTGTTAGCACGAACGTTTGGAAGGGCGTTACTTTGAGG
GATTTGTTGTTGAAGGCTGGTGTTAGCGAGAAGAACATGGCGGGCAAACACGTTGAATTCATCGGAGCCGAAG
ACCTCCCCAACAAGGTAGGCCCCGGTCCATTCAAGGATGAACCATGGGGCAAACTCGTCAAATACGGTACTTC
CGTCCCTCTCGCACGTGCCATGAACCCCGCCTATGACATTCTCATCGCCTACGAAGCCAACGGAGAGGTACTT
CAACCGGATCACGGATATCCTATCCGTCTCATCATTCCTGGATACATTGGTGGAAGGATGATCAAGTGGCTTA
CGGATATCAATGTGTTGGAGCACGAGACGAAGAACCATTATCACTATCATGATAATCGTATCTTGCCTCCTCA
TATTACGGCCGAAGAGAGTTTGACAGGTGGATGGTGGTACAAGCCCGAGTACATCTTTAATGAGTTGAACATT
AACTCTGCCATGACTGCTCCTGATCACAATGAAACGATTGATCTGGCTTCCAGTATTGGAAGTTCCTACGAGG
TGGGAGGATATGCCTACACGGGAGGTGGACGTCGCATCTCCCGTGTCGAGGTATCTACCGATGGAGGAGTGCA
TTGGGAGATTGCCAATATTAACCAGATTGAGAAGCCAACCGATTATGGAATGTACTGGTGTTGGATCTGGTGG
ACGTTTGATCTCAAGGTTGCTGATTTAGTTGGAACGAAGGAACTCTGGTGTCGTGCTTGGGATGAATCCAACA
ACGTTCAGCCCAACGATCCTACCTGGAATCTCATGGGAATGGGAAACAACCAAGTCTTCCGTATCAAGGTCCA
TCTCGATAAGGATGTGAACGGAAAGCATGTCTTTAGGTTTGAGCATCCTACCAAGCCTGGACAGCAGGAGGGT
GGATGGATGACTACTCTCGCTGGAAAGCCGGATAGTGCTGGGTTCGGAAGGTTGTTGGAGCAAGGACAGCCTG
CGAAGGAGGCAGCACCTGCTGCGGCTCCTGCCAAGACTTCTGGTAGCAAGCTGATCAAGATGGAGGAGGTGAG
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CTTCATCCTGGTGGAGCTGATTCCATCCTCATCAACGCTGGAGAGGATGCCACCGAAGATTTCGTCGCCATCC
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TTCTGATGCTGAGGAGGAACGTCTCTGCCCCAAGACAGGAAGGAAGGTGGCGTTGGATCCCAAGCACAAGCAT
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AGCACGTCCTTGGTTTGCCAACAGGAAAGCACGTCTTCCTGTCTGCGGATATCAACGGTGAGATGGTGATGCG
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GAACGTTTCCCACTCGGAGGTAAGATGTCACAATACCTCGACTCTCTAAAGGTTGGAGATACCATCGATATGA
GAGGACCGGTCGGAGAGTTTGACTACCACGGCAACGGAAAGTTCCTCAAGGAGCACGACGAGTGTTACGCCAC
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CCCGATGACAAGACTACCATGTCATTGGTCTTTGGATGTCGTGAGGAGGGTGATTTGCTTTTGAGATCAACTC
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TACAATCTCATGTGTGGACCTCCAATCATGTTGGAGAGGGGGTGTACTCCCAACTTGAAGGCTCTTGGGCACA
AGGAGGATAACATCTTCTCATTCTAA

Figure 4 *Phaeodactylum tricornutum* Pyrenoid Decarboxylase

A (SEQ ID NO: 5)

MRMSTAALLCSVYTAGSTAAFAPALLTRRYSSSSSTLSATTNPLQSIFLTPETAKACIDAAGGTPLYAYSIDK
LEEAADACLAFPNAYGLTVRYAMKACPNASILKYFHSKNIHVDASSGFEVRRAMDAGVPAENISLSTQELPED
FAALVDMGVKLNACSVSQLERFGEHYAGKGAKVGVRVNPGVGSGGFSASTTGFSKTNVGGPSSSFGIWHELVT
DGTVPDIVERYGLEVERIHTHIGSGSDPEIWQQVATKSLSFCKVFPTVKTMNLGGGYKVGRNKGEVTTDLQKI
GKPVADAFKKFAEKEGRELQMEIEPGTYLVAMAGALVSKVQDKVHTTGENSHTFLKLDAGMTDVLRPSLYGAV
HPITILPGSGNSADVGDETESVVVVGHCCESGDLMTPAPGEPEQLAEQELRAAAVGDILVMDGSGAYCSGMST
KNYNSFPEAPEVLVDKAGKAHLIRKRQTLSQIYENEISVEGV

B (SEQ ID NO: 6)

ATGAGAATGTCTACTGCTGCTCTGCTCTGCTCAGTTTACACAGCCGGAAGCACTGCCGCGTTTGCTCCCGCTT
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GACGCCCGAAACTGCCAAGGCCTGCATTGATGCCGCCGGTGGGACACCTCTGTACGCGTACAGTATCGACAAG
CTGGAGGAAGCCGCCGATGCCTGTCTAGCTTTTCCCAACGCGTACGGACTGACGGTACGCTACGCCATGAAAG
CCTGTCCCAACGCGTCCATTCTCAAGTATTTTCACTCGAAAAATATTCACGTTGACGCATCTTCCGGTTTCGA
AGTACGCCGGGCGATGGATGCCGGTGTTCCGGCCGAAAATATCTCCCTGAGTACGCAGGAGTTGCCCGAAGAC
TTTGCGGCACTGGTTGATATGGGTGTCAAGCTCAATGCTTGTTCCGTCTCGCAGCTAGAGCGGTTCGGTGAGC
ACTATGCTGGAAAAGGTGCGAAGGTGGGCGTCCGAGTGAATCCGGGAGTGGGGTCGGGAGGCTTCTCCGCGAG
TACCACTGGATTCAGTAAAACTAATGTCGGCGGACCGAGCAGTTCGTTCGGGATTTGGCACGAACTCGTCACC
GATGGAACCGTCCCAGATATCGTGGAAAGGTACGGTTTGGAAGTGGAACGTATTCATACACATATTGGATCAG
GTAGTGATCCGGAGATTTGGCAGCAAGTTGCCACCAAATCCTTGTCCTTTTGCAAGGTGTTTCCCACCGTCAA
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GGGAAGCCTGTGGCGGATGCCTTTAAAAAGTTCGCGGAAAAGGAAGGCCGGGAATTGCAAATGGAAATTGAGC
CCGGAACTTATCTCGTGGCCATGGCTGGAGCACTCGTCTCCAAGGTCCAAGACAAGGTTCACACCACCGGAGA
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CATCCTATTACGATTCTGCCCGGGTCGGGAAATTCTGCCGACGTTGGCGATGAAACCGAATCTGTAGTGGTGG
TTGGACATTGTTGTGAATCAGGGGACCTCATGACTCCGGCCCCGGGTGAGCCGGAACAACTAGCGGAACAAGA
ACTTCGTGCGGCAGCGGTAGGTGATATTCTAGTGATGGATGGCTCTGGGGCGTACTGCTCCGGCATGTCGACG
AAGAACTACAACAGCTTTCCCGAAGCACCAGAAGTGTTGGTGGACAAGGCAGGCAAGGCACACTTGATCCGTA
AACGACAAACCCTGAGTCAAATTTACGAGAACGAAATCTCCGTAGAAGGCGTGTTTTAA

Figure 5 *Thalassiosira pseudonana* Pyrenoid Decarboxylase

A (SEQ ID NO: 7)

MKVSSLAIVTALFATSTNAFSPIQPHSTTPIITTSQLHATPPSQSNFLTPELATTCIATAQGTPLYAYSLSQL
AAAATATLAFPNAFGLTVRYAMKACPNGSILKYFLSRGICIDASSGYEVRRAMSMGVPAEKISLSSQELPADF
DELIGLGVKINACSVSQLERIGKAFPNTSQKVGIRINPGVGSGGFSSSTTGFSKTNVGGPSSSFGIWHELVTD
GTVPDIVTKYGLEVERIHTHIGSGSDPAIWQSVATRSLSFCKLWDTITTLNLGGGYKVGRNPGEKTTDLNEIG
APVADAFRDFAKETGRELQMEIEPGTYLVANAGALVTTIQDKVSTKSASSDEGHIYLKMDAGMTDVLRPSLYG
AIHPITILPASGKSSDIGTATESVVVVGHCCESGDLMTPKPGEPEALEERVLRTAEIGDIAVMDGSGAYCAGM
STKNYNSFPEAPEVLVDLEGKVHLIRKRQSLQQIYENECDVPSGLF

B (SEQ ID NO: 8)

ATGAAGGTCTCCAGCCTCGCCATCGTAACGGCCCTATTCGCGACCTCCACCAATGCCTTCTCTCCGATCCAGC
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CCCCGAACTAGCCACCACCTGCATCGCCACCGCCCAAGGCACGCCCCTCTACGCCTACTCCCTCTCCCAACTA
GCCGCCGCCGCCACAGCCACCCTCGCCTTCCCCAACGCATTCGGTCTTACTGTACGATATGCCATGAAGGCGT
GTCCCAATGGAAGTATTTTGAAGTACTTTTTGAGTAGGGGGATTTGTATCGATGCGAGTTCCGGGTATGAAGT
GAGGAGAGCAATGAGTATGGGTGTTCCGGCCGAGAAGATTAGTTTGAGTTCGCAGGAGTTGCCGGCGGATTTT
GATGAGTTGATTGGTTTGGGGGTCAAGATTAATGCTTGCTCCGTATCCCAACTCGAACGCATCGGCAAAGCCT
TCCCCAACACCTCTCAAAAGGTAGGCATCCGCATCAACCCCGGCGTAGGATCCGGTGGATTCTCCTCCTCCAC
AACCGGCTTTTCCAAAACCAACGTCGGCGGTCCTTCCTCCTCCTTTGGTATCTGGCACGAACTCGTTACCGAT
GGAACCGTACCTGACATTGTAACCAAGTATGGATTGGAGGTGGAGAGGATTCACACCCACATCGGTTCGGGGT
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GCCCCTGTTGCCGATGCGTTTAGGGACTTTGCAAAGGAGACGGGGAGGGAGTTGCAGATGGAGATCGAGCCGG
GCACGTATTTGGTTGCGAATGCTGGAGCTCTCGTGACGACGATTCAGGATAAGGTATCGACCAAATCGGCATC
CTCCGACGAAGGACACATTTACCTCAAAATGGATGCTGGTATGACGGATGTCCTTCGTCCTTCCCTCTACGGA
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ACGTGTTCTCCGTACTGCCGAGATTGGTGATATTGCCGTGATGGATGGAAGTGGAGCTTACTGTGCTGGAATG
TCGACTAAGAATTACAATAGTTTCCCCGAGGCGCCGGAGGTGTTGGTTGATTTGGAAGGAAAGGTTCATTTGA
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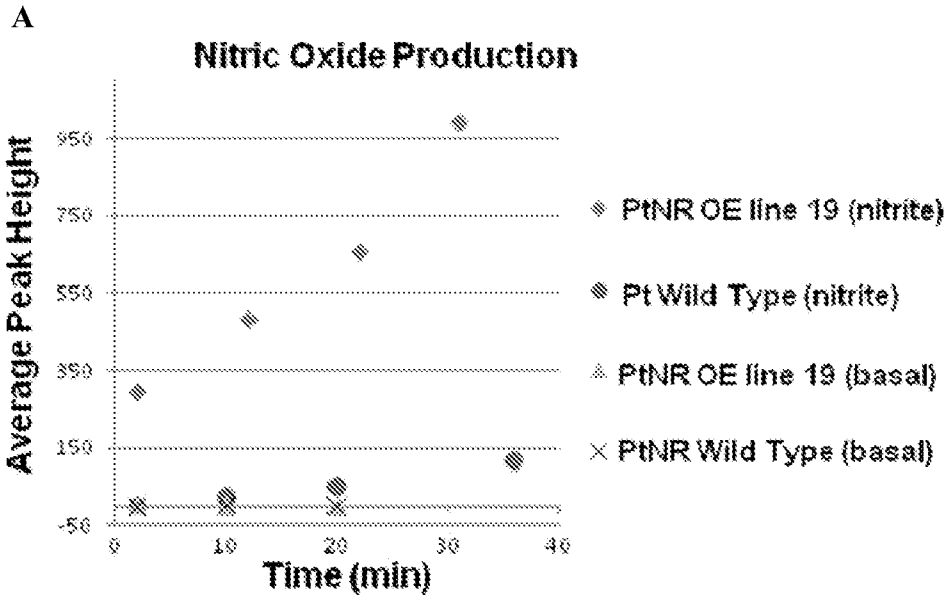
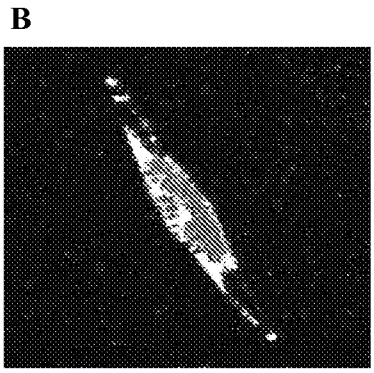
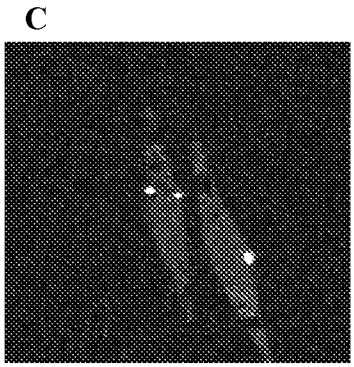
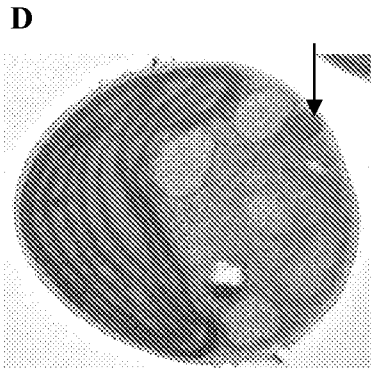
Figure 6


A
Nitric Oxide Production
Average Peak Height
950
750
550
350
150
-50
0
10
20
30
40
Time (min)
PtNR OE line 19 (nitrite)
Pt Wild Type (nitrite)
PtNR OE line 19 (basal)
PtNR Wild Type (basal)


B


C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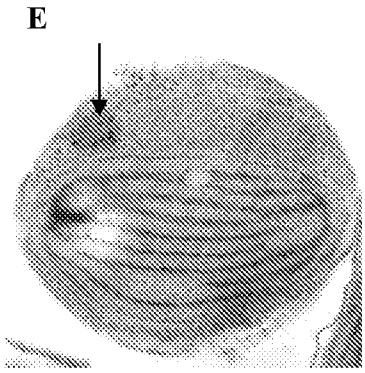

E

Figure 7

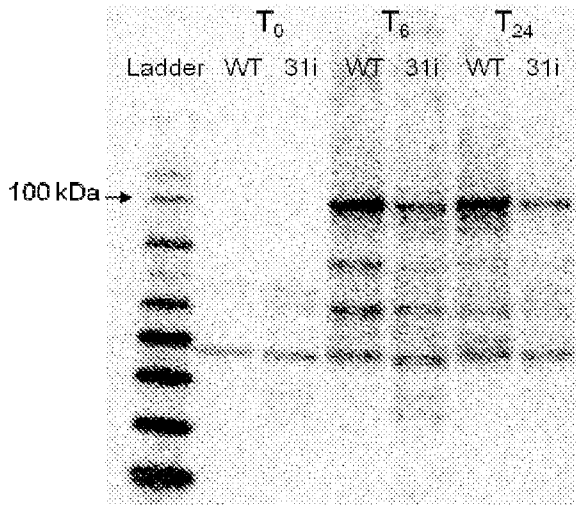
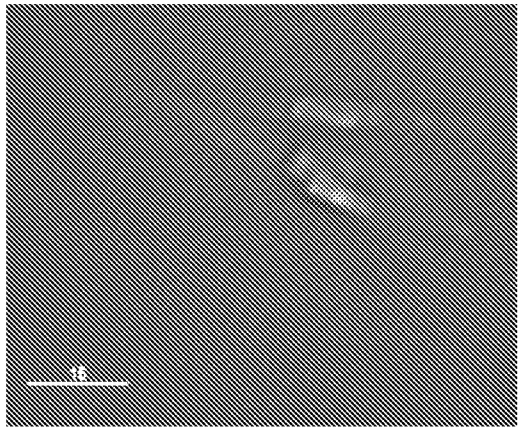
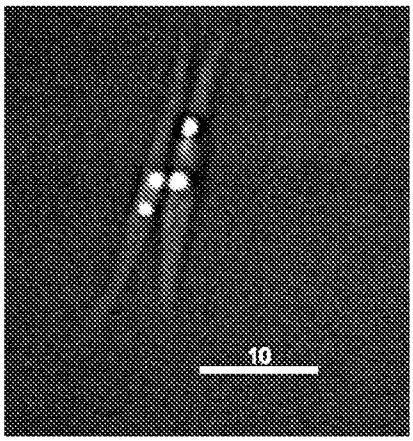


Figure 8

A



B



C

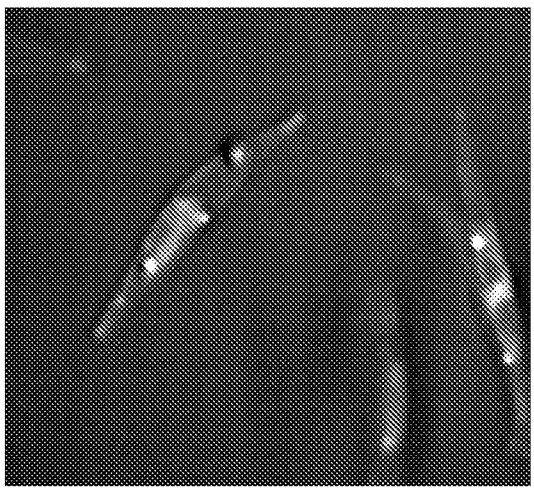


Figure 9

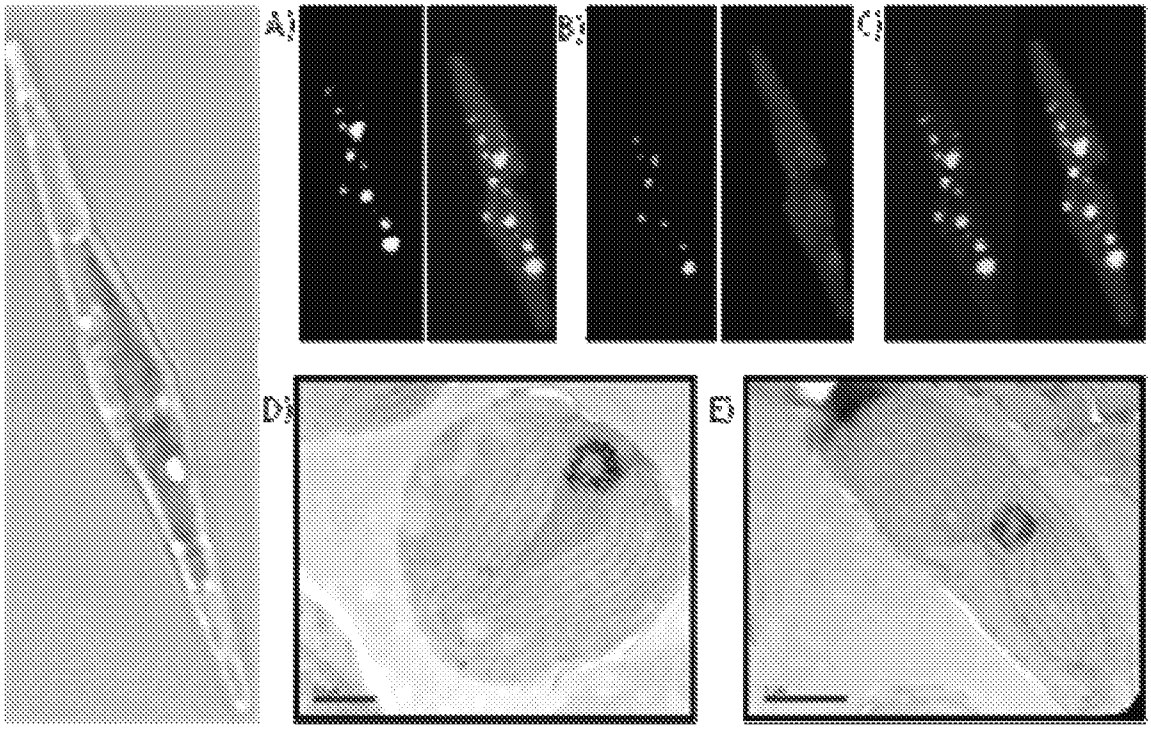


Figure 10

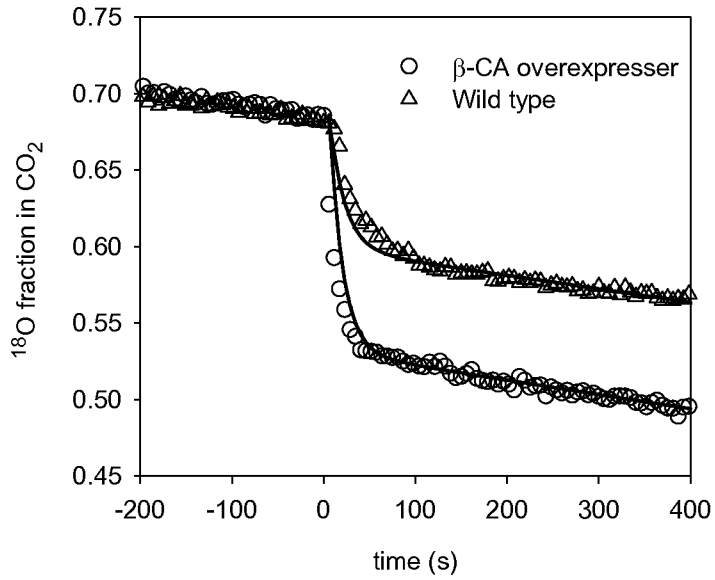
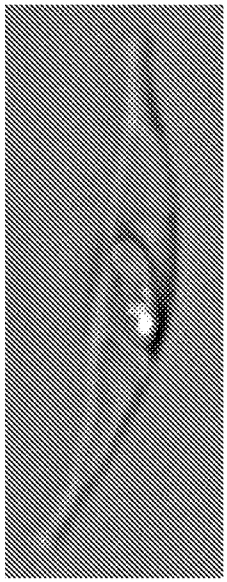

0.75
0.70
0.65
0.60
0.55
0.50
0.45
^{18}O fraction in CO_2
-200
-100
0
100
200
300
400
time (s)
β-CA overexpresser
Wild type

Figure 11

A



B

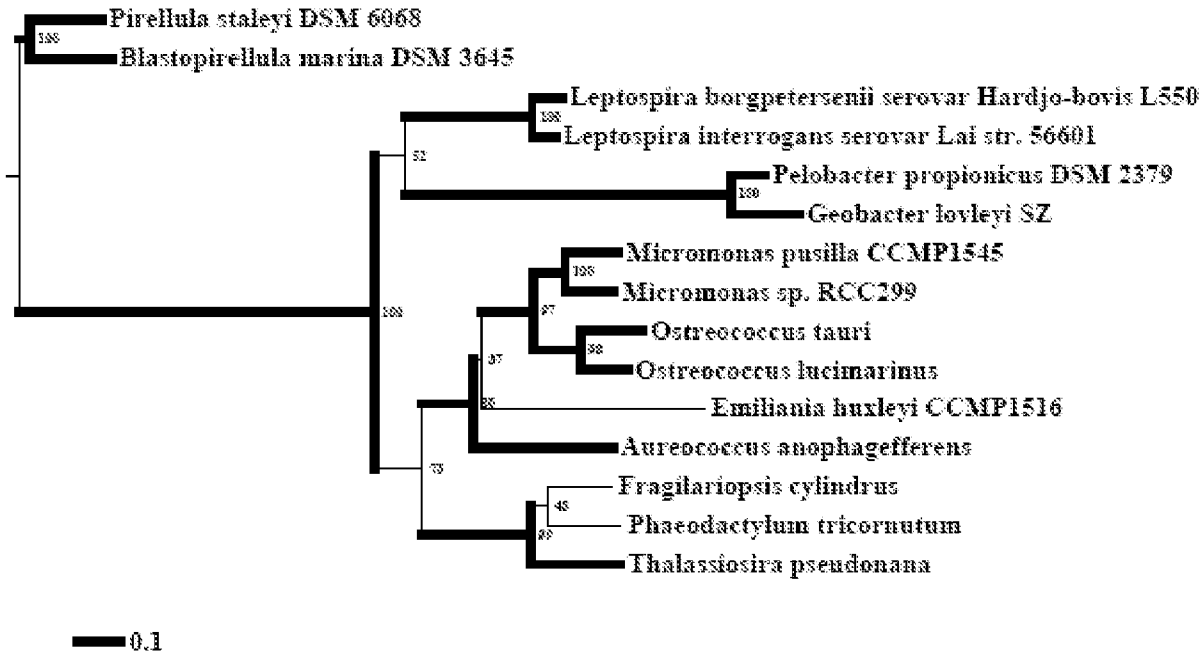

Pirellula staleyi DSM 6068
Blastopirellula marina DSM 3645
Leptospira borgpetersenii serovar Hardjo-bovis L550
Leptospira interrogans serovar Lai str. 56601
Pelobacter propionicus DSM 2379
Geobacter lovleyi SZ
Micromonas pusilla CCMP1545
Micromonas sp. RCC299
Ostreococcus tauri
Ostreococcus lucimarinus
Emiliania huxleyi CCMP1516
Aureococcus anophagefferens
Fragilariopsis cylindrus
Phaeodactylum tricornutum
Thalassiosira pseudonana
0.1

Figure 12

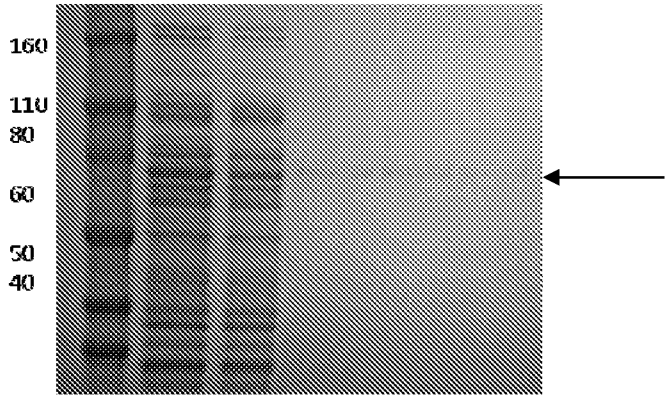


Figure 13