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[Continued on next page]

(54) Title: CHLOROPHYLL DEFICIENT ALGAL CELL WITH IMPROVED GROWTH AND PRODUCTION

(57) Abstract: Chlorophyll deficient algal strains, methods of making chlorophyll deficient algal strains, and methods of their use are provided.

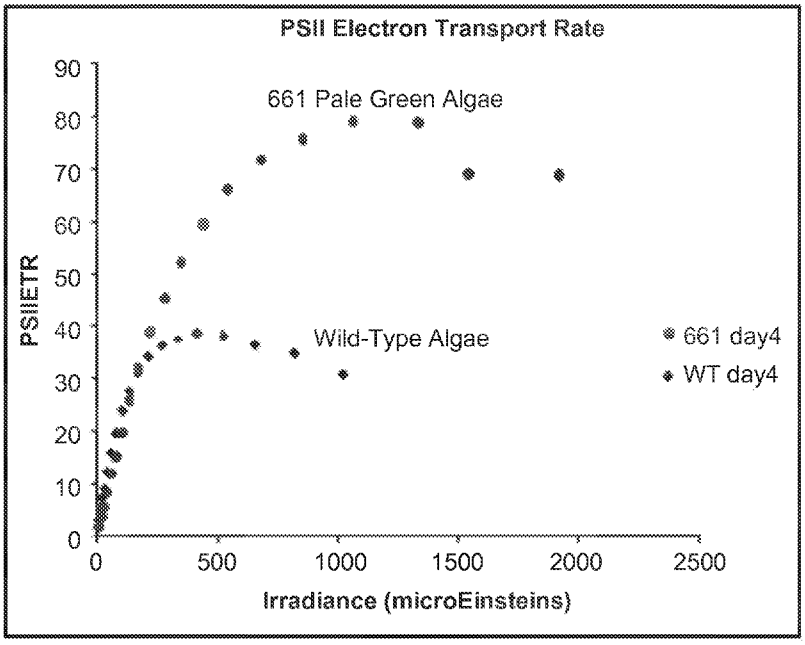

FIG. 4

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Chlorophyll Deficient Algal Cell with Improved Growth and Production

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 61/800,029, filed March 15, 2013, and U.S. Provisional Application No. 61/800,114, filed March 15, 2013, the contents of which are hereby incorporated by reference in the entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] Algae, *e.g.*, microalgae, are photosynthetic organisms that convert light energy and carbon dioxide into biomass including lipids, carbohydrates, and proteins. Marine algae strains can have an oil content of 10-50%, (w/w) and produce a high percentage of total lipids (up to 30-70% of dry weight) (Ward OP and Singh A, *Process Biochem*, 2005, 40(12):3627-3652). Various algal strains can produce omega-3 polyunsaturated fatty acids, *e.g.*, eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6-n3). For example, algae of the genus *Nannachloropsis* are abundant in EPA as well as the omega-7 fatty acid, palmitoleic acid (C16:1n-7), and the ω-7 fatty acid, arachidonic acid (C20:4n-6) Algae are becoming an increasing important source of nutritionally important omega-3 polyunsaturated fatty acids.

[0003] The composition of the fatty acids varies among the different algal strains. For instance, it has been reported that *Nannochloropsis spp.* contains 26.7% (percentage from total fatty acids) EPA and DHA (Hu H and Gao K, *Biotechnol Lett*, 2003, 25(5):421-425), while *Nannochloropsis oceania* has 23.4% EPA (Patil *et al.*, Aquac Int, 2007, 15(1):1-9) and *Nannochloropsis salina* contains about 28% EPA (Van Wagenen *et al.*, *Energies*, 2012, 5(3):731-740).

[0004] The consumption of omega-3 fatty acids has been shown to prevent cardiovascular disease, enhance brain function, and diminish symptoms of inflammatory conditions such as rheumatoid arthritis, Crohn's disease, and ulcerative colitis. For instance, the EPA only therapeutic formulation Vascepa® is approved for the treatment of hypertriglyceridemia. There is a growing demand for algal-derived omega-3 EPA only compositions in the nutraceutical and

pharmaceutical industry. Thus, it is desirable to cultivate algal strains such as those of the genus *Nannochloropsis* for production of EPA and other compositions.

[0005] However, it can be difficult to grow such microalgae at sufficient scale and with sufficient productivity. For example, the biochemical steps of harvesting light energy and fixing carbon dioxide into biomass can be saturated at high photon flux. Additionally, the light harvesting pigments of an organism can absorb excess photons, thereby shading other microalgae and preventing their light harvesting. Excess photon absorption may further limit photosynthesis by causing photo-inhibition. The present invention addresses these and other related needs by providing a novel algal cell.

BRIEF SUMMARY OF THE INVENTION

[0006] The invention relates to chlorophyll deficient microalgae of the genus *Nannochloropsis*, methods of generating such chlorophyll deficient algae, and methods of their use.

[0007] In a first aspect, the present invention provides an isolated algal cell of the genus *Nannochloropsis*, the algal cell having less chlorophyll content than a wild-type cell, wherein the algal cell comprises at least one polymorphism in comparison to a wild-type cell. In some embodiments of the first aspect, the at least one polymorphism is selected from the group consisting of the polymorphisms recited in SEQ ID NOS:1-51. In some embodiments of the first aspect, the at least one polymorphism is at or near (*e.g.*, within less than about 1 kb, 500 bp, 250 bp, 100 bp, 50 bp, 25 bp, 20 bp, 10 bp, 5 bp, 4 bp, 3 bp, 2 bp, or at) a genomic region selected from the group consisting of SEQ ID NOS:69-119. In some embodiments of the first aspect, the at least one polymorphism is at or near (*e.g.*, within less than about 1 kb, 500 bp, 250 bp, 100 bp, 50 bp, 25 bp, 20 bp, 10 bp, 5 bp, 4 bp, 3 bp, 2 bp, or at) a genomic location selected from the group consisting of any the mutation coordinates of Table 1. In some cases, the at least one polymorphism is selected from the group consisting of SEQ ID NOS:1-11. In some embodiments of the first aspect, the isolated algal cell has less than about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 80%, or 90% of the chlorophyll content of a wild-type cell.

[0008] In some embodiments of the first aspect, the mRNA expression of 1, 2, 3, 4, or more (*e.g.*, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17) mRNAs selected from the group consisting of

mRNAs at least partially encoded by SEQ ID NOS:52-68 is less than about ½, 1/10th, 1/50th, 1/100th, or 1/1000th of the expression of the mRNA in a wild-type cell. In some cases, the mRNA expression compared to a wild-type algal cell is mRNA expression of log-phase cultures in F2N2 media (*e.g.*, mRNA expression of log-phase cultures in F2N2 media of wild-type compared to an algal cell of the invention).

[0009] In some embodiments of the first aspect, the algal cell exhibits a higher biomass productivity as measured by grams of dry weight algal cells per square meter per day when grown in outdoor cultivation ponds as compared to a wild-type cell. In some cases, the algal cell exhibits at least a 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 65%, or more higher biomass productivity as measured by grams of dry weight algal cells per square meter per day when grown in outdoor cultivation ponds as compared to a wild-type cell. In some cases, the increased productivity is provided in log-phase cultures in F2N2 media at between about 25-28 °C under about 300 µmol photons/m^2/s light intensity.

[0010] In some embodiments of the first aspect, the algal cell contains at least about 3.9-5% EPA per gram dry weight. The concentration of EPA as a proportion of total fatty acids can be at least 5-15%, or 15-30%, higher than the wild-type cell. In some cases, the concentration of EPA as a proportion of total fatty acids can be at least about 30%. In some cases, the algal cell contains no, or substantially no, or less than about 0.5% Docosahexaenoic acid (DHA) per gram dry weight. The algal cell can contain less than about 0.61% ARA per gram dry weight. In some cases, the algal cell has an EPA/ARA mass ratio of greater than about 5:1. In some cases, the algal cell has a Pmax at least 25%, 50%, 75%, 100%, 150%, 200% or more higher than the Pmax of a wild-type algal cell grown under the same conditions.

[0011] In a second aspect, the present invention provides a culture of chlorophyll deficient *Nannochloropsis* algal cells, wherein the culture comprises a plurality of any one or more of the foregoing algal cells.

[0012] In a third aspect, the present invention provides a composition comprising dry whole *Nannochloropsis* biomass, wherein the biomass comprises an algal cell of any one of the preceding claims. In some embodiments of the second aspect, the composition has an EPA/ARA ratio of greater than about 5:1.

[0013] In a fourth aspect, the present invention provides a process for obtaining a total algal oil rich in EPA, the process comprising: cultivating any one of the foregoing algal cells and isolating the oil.

[0014] In a fifth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a polymorphism recited in any one of SEQ ID NOS:1-11. In some embodiments, the isolated chlorophyll deficient algal cell has any one of the polymorphisms recited in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, or SEQ ID NO:11. In some embodiments, the isolated chlorophyll deficient algal cell has any two of the polymorphisms recited in SEQ ID NOS:1-11. In some embodiments, the isolated chlorophyll deficient algal cell has any three of the polymorphisms recited in SEQ ID NOS:1-11. In some embodiments, the isolated chlorophyll deficient algal cell has any four of the polymorphisms recited in SEQ ID NOS:1-11. In some embodiments, the isolated chlorophyll deficient algal cell has any five of the polymorphisms recited in SEQ ID NOS:1-11. In some embodiments, the isolated chlorophyll deficient algal cell has any six of the polymorphisms encoded by SEQ ID NOS:1-11. In some embodiments, the isolated chlorophyll deficient algal cell has any seven of the polymorphisms recited in SEQ ID NOS:1-11. In some embodiments, the isolated chlorophyll deficient algal cell has any eight of the polymorphisms recited in SEQ ID NOS:1-11. In some embodiments, the isolated chlorophyll deficient algal cell has any nine of the polymorphisms recited in SEQ ID NOS:1-11. In some embodiments, the isolated chlorophyll deficient algal cell has any ten of the polymorphisms recited in SEQ ID NOS:1-11. In some embodiments, the isolated chlorophyll deficient algal cell has all eleven of the polymorphisms recited in SEQ ID NOS:1-11.

[0015] In a sixth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:1. In a first embodiment of this aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:2. In any of the first embodiment or the sixth aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:3. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:4. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:5. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown

in SEQ ID NO:6. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:7. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:8. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:9. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:10. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:11.

[0016] In a seventh aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:2. In a first embodiment of this aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:3. In any of the first embodiment or the seventh aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:4. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:5. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:6. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:7. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:8. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:9. In one any of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:10. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:11.

[0017] In an eighth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:3. In a first embodiment of this aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:4. In any of the first embodiment or the eighth aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:5. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:6. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:7. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:8. In any one of the foregoing, the isolated chlorophyll deficient

algal cell can also have a mutation shown in SEQ ID NO:9. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:10. In any of one the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:11.

[0018] In a ninth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:4. In a first embodiment of this aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:5. In any of the first embodiment or the ninth aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:6. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:7. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:8. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:9. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:10. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:11.

[0019] In a tenth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:5. In a first embodiment of this aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:6. In any of the first embodiment or the tenth aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:7. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:8. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:9. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:10. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:11.

[0020] In an twelfth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:6. In a first embodiment of this aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:7. In any one of the first embodiment or the twelfth aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:8. In any one of the foregoing, the isolated

chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:9. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:10. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:11.

[0021] In a thirteenth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:7. In a first embodiment of this aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:8. In any of one of thirteenth aspect or the first embodiment, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:9. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:10. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:11.

[0022] In a fourteenth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:8. In a first embodiment of this aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:9. In any one of the fourteenth aspect or the first embodiment, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:10. In any one of the foregoing, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:11.

[0023] In a fifteenth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:9. In a first embodiment of this aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:10. In any one of the fifteenth aspect or the first embodiment, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:11.

[0024] In a sixteenth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:10. In a first embodiment of this aspect, the isolated chlorophyll deficient algal cell can also have a mutation shown in SEQ ID NO:11.

[0025] In a seventeenth aspect, the present invention provides an isolated chlorophyll deficient algal cell having a mutation shown in SEQ ID NO:11.

[0026] In an eighteenth aspect, the present invention provides a method of making any one of the foregoing algal cells, the method comprising:

modifying the genome of an algal cell from a parent algal cell line to introduce a polymorphism selected from the group consisting of the polymorphisms recited in SEQ ID NOS:1-51;

modifying the genome of an algal cell from a parent algal cell line to introduce a polymorphism at or near (*e.g.*, within less than about 1 kb, 500 bp, 250 bp, 100 bp, 50 bp, 25 bp, 20 bp, 10 bp, 5 bp, 4 bp, 3 bp, 2 bp, or at) a genomic region selected from the group consisting of SEQ ID NOS:69-119; or

reducing the expression of an mRNA transcript in an algal cell, wherein the mRNA transcript is selected from the group consisting of the transcripts encoded by, or partially encoded by, SEQ ID NOS:52-68, in any combination (*e.g.*, in any of the combinations described in Table 3).

DEFINITIONS

[0027] The term "algae" or "algal cell" refers to a marine algal cell or algae, including algae or algal cells of the kingdom *chromista*, algae or algal cells of the class *Chrysophyceae*, *Cryptophyceae*, *Prasinophyceae*, *Rhodophyceae*, *Xanthophyceae*, *Bacillariophyceae*, *Glaucophyceae*, and *Eustignatophyceae*. In some embodiments, the algae is of the genus *Nannochloropsis*. In preferred embodiments, the *Nannochloropsis* is *Nannochloropsis gaditana*, *Nannochloropsis granulate*, *Nannochloropsis limnetica*, *Nannochloropsis oceanica*, *Nannochloropsis oculata* or *Nannochloropsis salina*. In some cases, the algal cell is a *Nannochloropsis oceanica* algal cell.

[0028] The term "biomass" refers to a harvested mass of organisms. The organisms can be living or dead. In certain embodiments, the biomass is algal biomass. The algal biomass can be dry, substantially dry, or wet. Typically, the biomass refers to organisms that have not been extracted with a solvent or otherwise substantially altered in composition. Accordingly, harvested biomass can retain at least the approximate, or in some cases the same, lipid, protein,

and/or carbohydrate composition of the cultivated organisms from which the biomass is harvested.

[0029] The term "productivity" refers to the amount of product an organism can produce under defined conditions. Productivity can be measured in grams of dry weight algal cells produced per m^2 per day of culture. Alternatively, productivity can be measured in terms of algal cells produced per unit area or unit volume per unit time. As another alternative, productivity can be measured in terms of moles of carbon fixed per m^2 per day of culture. As yet another alternative, productivity can be measured in terms of moles of O_2 evolved per m^2 per day of culture. As yet one more alternative, productivity can be measured in terms of the rate of production of a desirable product or product(s) such as lipid (*e.g.*, total lipid or EPA), protein, and/or carbohydrate. Moreover, any one of the productivity measurements can, *e.g.*, be determined with respect to culture volume (*e.g.*, grams of algal cells/ m^3/ day) or number of cells, including an average (*e.g.*, mean or median) per cell. For example, productivity can be measured in terms of nmol of O_2 evolved per algal cell per day.

[0030] The term "non photon saturating conditions" refers to conditions in which an algal cell does not exhibit photon saturation. Photon saturation can depend on a variety of factors such as temperature, wavelength of incident light, density of culture, nutrient availability, composition of dissolved gases, *etc.*. In general, photon saturation occurs in laboratory conditions under incident light of greater than about 400 µmol photons/m^2/s light intensity (*e.g.*, at least about 400, 450, 500, 600, 750, 1000, 2000, or more µmol photons/m^2/s). Non photon saturating conditions generally occur in the laboratory under incident light of less than about 400 µmol photons/m^2/s light intensity (*e.g.*, less than about 400, 350, 300, 250, 200, 150, 100, or fewer µmol photons/m^2/s).

[0031] The term "eicosapentaenoic acid" or "EPA" refers an omega-3 fatty acid polyunsaturated fatty acid with the following connotation C20:5-n3. It is a carboxylic acid with a 20-carbon chain and five *cis* double bonds; the first double bond is located at the third carbon from the omega end.

[0032] The term "docosahexaenoic acid" or "DHA" refers an omega-3 fatty acid PUFA. It is a carboxylic acid with a 22-carbon chain and six *cis* double bonds; the first double bond is located at the third carbon from the omega end. DHA is also denoted as C22:6-n3.

[0033] The term "arachidonic acid" or "ARA" refers an omega-6 PUFA with a 20-carbon chain and four *cis*-double bonds; the first double bond is located at the sixth carbon from the omega end. ARA is also denoted as C20:4-n6.

[0034] The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* **19**:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* **260**:2605-2608 (1985); and Rossolini *et al.*, *Mol. Cell. Probes* **8**:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

[0035] The term "gene" means the segment of DNA involved in producing a polypeptide chain. It may include regions preceding and following the coding region (*e.g.*, 5' and 3' untranslated regions, promoters, *etc.*) as well as intervening sequences (introns) between individual coding segments (exons).

[0036] A "promoter" is defined as an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription.

[0037] An "expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular polynucleotide sequence in a host cell. An expression cassette may be part of a

plasmid, viral genome, or nucleic acid fragment. Typically, an expression cassette includes a polynucleotide to be transcribed, operably linked to a promoter.

[0038] The term "polymorphism" refers to a polynucleotide sequence variant. The polymorphism can be a knock-out mutation, in which the function of an encoded gene is abolished or reduced below an effective level. The polymorphism can arise due to substitution, deletion, or insertion of one or more nucleotides at a polymorphic site. Polymorphisms can give rise to variant protein sequences, result in defective proteins, or result in up or downregulation of a gene as compared to wild-type.

[0039] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. "Amino acid mimetics" refers to chemical compounds having a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0040] Amino acids may be referred to herein by either the commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0041] "Polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. All three terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length,

including full-length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] FIG. 1: is a table showing growth data for chlorophyll deficient *Nannochloropsis* when grown in 2.6 m^2 outdoor ponds, which have an approximate volume of 650 liters.

[0043] FIG. 2: is two photographs, one photograph showing a testing pond comprising chlorophyll deficient *Nannochloropsis*, and the other photograph showing a testing pond comprising the wild-type *Nannochloropsis* parent strain from which the chlorophyll deficient strain in the opposite pond was derived. The biomass density in both ponds is approximately identical.

[0044] FIG. 3: is a table showing eicosapentaenoic acid (EPA) levels as measured in approximately one-acre open raceway ponds in Australia.

[0045] FIG. 4: is a graph comparing growth of a T661 strain versus growth of a wild-type *Nannochloropsis* strain.

[0046] Fig. 5 depicts polymorphisms detected in chlorophyll deficient algal strain T661 in comparison to the reference genome sequence of *Nannochlorpsis oceanica*. Category B polymorphism SNP-B-1-11 correspond to SEQ ID NOS:1-11 respectively. Category C polymorphisms SNP-C-1-40 correspond to SEQ ID NOS:12-51 respectively.

[0047] FIG. 6: is a graphical representation of mRNA expression in a wild-type *Nannochloropsis* strain versus a chlorophyll deficient strain (IC171/T661). Datapoints above the line represent mRNA transcripts that are over-expressed in T661 relative to wild-type. Datapoints below the line represent mRNA transcripts that are under-expressed in T661 relative to wild-type.

DETAILED DESCRIPTION

I. Chlorophyll Deficient Algal Cells

[0048] Described herein is a chlorophyll deficient algal cell. The chlorophyll deficient algal cell has a pale green phenotype as compared to a wild-type algal cell and, as further described below, can exhibit increased productivity and/or grow to a higher cell density as compared to a wild-type algal cell under the same lighting conditions. Generally, the chlorophyll deficient phenotype is stable through multiple generations without selection. Moreover, the chlorophyll deficient algal cell is capable of robust growth under standard conditions.

[0049] For example, the chlorophyll deficient algal cell can exhibit productivity that is at least about 25%, 50%, 75%, 80%, 85%, 90%, 95%, or 99% of the productivity of a wild-type algal cell under non-photon saturating conditions. In some cases, the chlorophyll deficient algal cell can exhibit the same, or substantially the same, productivity as a wild-type algal cell under non-photon saturating conditions. In some cases, the chlorophyll deficient algal cell exhibits higher growth or productivity as compared to a wild-type algal cell under non-photon saturating conditions. As another example, the chlorophyll deficient algal cell can exhibit productivity that is at least about 25%, 50%, 75%, 80%, 85%, 90%, 95%, or 99% of the productivity of a wild-type algal cell under conditions that would saturate photosynthesis of wild-type algal cells. In some cases, the conditions that saturate photosynthesis of wild-type algal cells do not saturate photosynthesis of the chlorophyll deficient algal cell.

[0050] In some embodiments, a chlorophyll deficient algal cell of the present invention has one or more mutations as compared to a wild-type algal cell. The one or more mutations can be generated through directed mutagenesis or may be derived through random mutagenesis. In some cases the chlorophyll deficient algal cell comprises a mutation at or near (*e.g.*, within less than about 1 kb, 500 bp, 250 bp, 100 bp, 50 bp, 25 bp, 20 bp, 10 bp, 5 bp, 4 bp, 3 bp, 2 bp, or at) one or more of the genomic locations of Table 1, in any combination:

Table 1: Exemplary Genomic Locations of Mutations Providing Pale Green Algae (Coordinates Relative to *Nannochloropsis Oceanica* genome CCMP1779 *available at* bmb.msu.edu/Nannochloropsis.html)

SEQ ID NO: (SNP ID)	Contig	Start Coordinate	End Coordinate	Mutation Coordinate
SEQ ID NO:1 (SNP-B-1)	nanno_1467	563	464	506
SEQ ID NO:2 (SNP-B-2)	nanno_6752	606	705	645
SEQ ID NO:3 (SNP-B-3)	nanno_842	7451	7351	7426, 7436, 7377, 7378
SEQ ID NO:4 (SNP-B-4)	nanno_687	2795	2696	2758
SEQ ID NO:5 (SNP-B-5)	nanno_885	49095	48990	49045
SEQ ID NO:6 (SNP-B-6)	nanno_1334	1469	1367	1428
SEQ ID NO:7 (SNP-B-7)	nanno_3629	22817	22718	22801
SEQ ID NO:8 (SNP-B-8)	nanno_6293	2209	2308	2298
SEQ ID NO:9 (SNP-B-9)	nanno_66	5544	5446	5500
SEQ ID NO:10 (SNP-B-10)	nanno_751	10787	10688	10752
SEQ ID NO:11 (SNP-B-11)	nanno_84	17825	17924	17873

SEQ ID NO:14 (SNP-C-3)	nanno_4111	1	93	41
SEQ ID NO:31 (SNP-C-20)	nanno_104	1811	1909	1891
SEQ ID NO:33 (SNP-C-22)	nanno_402	25079	24977	25072, 25071
SEQ ID NO:43 (SNP-C-32)	nanno_727	34764	34865	34856, 34833

[0051] In some cases, the chlorophyll deficient algal cell comprises one or more of the polymorphisms encoded by SEQ ID NOS:1-11, in any combination. As an example, the chlorophyll deficient algal cell can comprise any one of, any two of, any three of, any four of, any five of, any six of, any seven of, any eight of, any nine of, any ten of, or all eleven of the polymorphisms encoded by SEQ ID NOS:1-11.

[0052] In some cases, the chlorophyll deficient algal cell comprises one or more of the polymorphisms encoded by SEQ ID NOS:12-51, in any combination. As an example, the chlorophyll deficient algal cell can comprise any 1 of, 2 of, 3 of, 4 of, 5 of, 6 of, 7 of, 8 of, 9 of, 10 of, 11 of, 12 of, 13 of, 14 of, 15 of, 16 of, 17 of, 18 of, 19 of, 20 of, 21 of, 22 of, 23 of, 24 of, 25 of, 26 of, 27 of, 28 of, 29 of, 30 of, 31 of, 32 of, 33 of, 34 of, 35 of, 36 of, 37 of, 38 of, 39 of, or all 40 of the polymorphisms encoded by SEQ ID NOS:12-51.

[0053] In some cases, the chlorophyll deficient algal cell comprises one or more of the polymorphisms encoded by SEQ ID NOS:1-51, in any combination. As an example, the chlorophyll deficient algal cell can comprise any 1 of, 2 of, 3 of, 4 of, 5 of, 6 of, 7 of, 8 of, 9 of, 10 of, 11 of, 12 of, 13 of, 14 of, 15 of, 16 of, 17 of, 18 of, 19 of, 20 of, 21 of, 22 of, 23 of, 24 of, 25 of, 26 of, 27 of, 28 of, 29 of, 30 of, 31 of, 32 of, 33 of, 34 of, 35 of, 36 of, 37 of, 38 of, 39 of, 40 of, 41 of, 42 of, 43 of, 44 of, 45 of, 46 of, 47 of, 48 of, 49 of, 50 of, or all 51 of the polymorphisms encoded by SEQ ID NOS:1-51.

[0054] In some embodiments, the chlorophyll deficient algal cell is a cell of the strain T661, or an isolate of a T661 culture. Chlorophyll deficient algal cells of the strain T661, or isolates thereof, are characterized by at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 , 37, 38, 39, 40, 41, 42, 43, 44, 45, 4, 47, 48, 49, 50, or all 51 of the polymorphisms encoded by SEQ ID NOS:1-51. The chlorophyll deficient algal cell of the strain T661 are *Nannochloropsis oceanica* algal cells having a pale green phenotype.

[0055] In some embodiments, the chlorophyll deficient algal cell comprises one or more of the polymorphisms encoded by SEQ ID NOS:1-51, in any combination, wherein the polymorphism lies in a protein encoding region, or a putative promoter region, of the genome. For example, the chlorophyll deficient algal cell can comprise any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15, of the mutations disclosed in Table 2. As another example, the chlorophyll deficient algal cell can comprise a mutation at or near (*e.g.*, within less than about 1 kb, 500 bp, 250 bp, 100 bp, 50 bp, 25 bp, 20 bp, 10 bp, 5 bp, 4 bp, 3 bp, 2 bp, or at) one or more of the mutations provided in Table 2, in any combination.

Table 2: Exemplary Pale Green Mutations found in Coding Regions (Locus Classification as defined for *Nannochloropsis Oceanica* genome CCMP1779 *available at* bmb.msu.edu/Nannochloropsis.html)

SEQ ID NO: (SNP)	Locus	Class
SEQ ID NO:1 (SNP-B-1)	nanno_1467	coding region SNP
SEQ ID NO:2 (SNP-B-2)	nanno_6752	coding region SNP
SEQ ID NO:3 (SNP-B-3)	nanno_842	coding region SNP
SEQ ID NO:4 (SNP-B-4)	nanno_687	coding region SNP
SEQ ID NO:5 (SNP-B-5)	nanno_885	coding region SNP
SEQ ID NO:6 (SNP-B-6)	nanno_1334	coding region SNP
SEQ ID NO:7 (SNP-B-7)	nanno_3629	coding region SNP
SEQ ID NO:8 (SNP-B-8)	nanno_6293	coding region SNP
SEQ ID NO:9	nanno_66	coding region SNP

(SNP-B-9)		
SEQ ID NO:10 (SNP-B-10)	nanno_751	coding region SNP
SEQ ID NO:11 (SNP-B-11)	nanno_84	coding region SNP
SEQ ID NO:14 (SNP-B-14)	nanno_4111	Promoter region SNP
SEQ ID NO:31 (SNP-C-20)	nanno_104	Promoter region SNP
SEQ ID NO:33 (SNP-C-22)	nanno_402	Promoter region SNP
SEQ ID NO:43 (SNP-C-32)	nanno_727	Promoter region SNP

[0056] In some embodiments, the chlorophyll deficient algal cell of the present invention has one or more mutations in a gene involved in photosynthesis as compared to a wild-type algal cell. For example, the mutation can be in a component of a light harvesting complex or a photosystem complex. In some cases, the algal cell can have a mutation of a component of the Chl *a* or Chl *b* light harvesting complex. In some cases, the chlorophyll deficient algal cell has a mutation in a gene involved in assembly of, or regulation of, the Chl *a* or Chl *b* light harvesting complex. For example, the mutation can be in a gene identical to, homologous to, or orthologous to ALB3.1, TLA1, TLA2, or NAB1 of a microalgal cell. As another example, the mutation can be in a gene encoding a component of the signal recognition particle or a gene involved in the assembly or regulation of the signal recognition particle. In some cases, the mutation can be in a gene identical to, homologous to, or orthologous to a CpFTSY gene.

[0057] In some embodiments, the chlorophyll deficient algal cell is characterized by down-regulation of one or more mRNA transcripts as compared to a wild-type cell. For example, the chlorophyll deficient algal cell can exhibit a reduction in mRNA expression of one or more of the mRNA transcripts encoded by SEQ ID NOS:52-68, in any combination. As an example, the chlorophyll deficient algal cell can exhibit a reduction in mRNA expression of any one 1 of, 2 of, 3 of, 4 of, 5 of, 6 of, 7 of, 8 of, 9 of, 10 of, 11 of, 12 of, 13 of, 14 of, 15 of, 16 of, or all 17 of the mRNA transcripts encoded by SEQ ID NOS:52-68.

[0058] In some embodiments, the chlorophyll deficient algal cell is characterized by down-regulation of one or more mRNA transcripts encoding a protein involved in chloroplast function.

For example, the chlorophyll deficient algal cell can be down-regulated in one or more mRNA transcripts encoded by SEQ ID NOS:54, or 55. The chlorophyll deficient algal cell can, in some cases, exhibit down-regulation of the mRNA transcript encoded by SEQ ID NO:54, exhibit down-regulation of the mRNA transcript encoded by SEQ ID NO:55, or exhibit down-regulation of the mRNA transcripts encoded by SEQ ID NOS:54 and 55. In some embodiments, the down-regulation is in comparison to a wild-type algal cell during log-phase growth in F2N2 media.

[0059] In some embodiments, the chlorophyll deficient algal cell is characterized by down-regulation of one or more mRNA transcripts encoding a transcription factor that affects (*e.g.*, activates or represses) transcription of a protein involved in chloroplast function. For example, the chlorophyll deficient algal cell can be down-regulated in one or more mRNA transcripts encoded by SEQ ID NOS:56, 57, 60, 63, 64, or 65. The down-regulation can be in comparison to a wild-type algal cell during log-phase growth in F2N2 media. For example, the chlorophyll deficient algal cell can be down regulated in any one or more of the mRNA transcripts encoded by the SEQ ID NO: combinations listed in Table 3.

Table 3: Exemplary Combinations of Down-regulated Transcripts of Pale Green Algae

	Down-Regualted mRNA Transcripts Encoded By:					
Combination	SEQ ID NO:56	SEQ ID NO:57	SEQ ID NO:60	SEQ ID NO:63	SEQ ID NO:64	SEQ ID NO:65
1	X					
2		X				
3			X			
4				X		
5					X	
6						X
7	X	X				
8	X		X			
9	X			X		
10	X				X	
11	X					X
12		X	X			
13		X		X		
14		X			X	
15		X				X
16			X	X		
17			X		X	

18			X			X
19				X	X	
20				X		X
21					X	X
22	X	X	X			
23	X	X		X		
24	X	X			X	
25	X	X				X
26	X		X	X		
27	X		X		X	
28	X		X			X
29	X			X	X	
30	X			X		X
31	X				X	X
32		X	X	X		
33		X	X		X	
34		X	X			X
35		X		X	X	
36		X		X		X
37		X			X	X
38			X	X	X	
39			X	X		X
40			X		X	X
41				X	X	X
42	X	X	X	X		
43	X	X	X		X	
44	X	X	X			X
45	X	X		X	X	
46	X	X		X		X
47	X	X			X	X
48	X		X	X	X	
49	X		X	X		X
50	X		X		X	X
51	X			X	X	X
52		X	X	X	X	
53		X	X	X		X
54		X	X		X	X
55		X		X	X	X
56			X	X	X	X
57	X	X	X	X	X	
58	X	X	X	X		X

59	X	X	X		X	X
60	X	X		X	X	X
61	X		X	X	X	X
62		X	X	X	X	X
63	X	X	X	X	X	X
64						

[0060] In some embodiments, the chlorophyll deficient algal cell is characterized by down-regulation of one or more mRNA transcripts encoded by SEQ ID NOS:56, 57, 60, 63, 64, or 65 and/or one or more of the combinations described in Table 3. For example, the chlorophyll deficient algal cell can exhibit down-regulation of the mRNA transcript encoded by SEQ ID NO:54 (*e.g.*, and/or any of the combinations described in Table 3), exhibit down-regulation of the mRNA transcript encoded by SEQ ID NO:55 (*e.g.*, and/or any of the combinations described in Table 3), or exhibit down-regulation of the mRNA transcripts encoded by SEQ ID NOS:54 and 55 (*e.g.*, and/or any of the combinations described in Table 3). In some embodiments, the down-regulation is in comparison to a wild-type algal cell during log-phase growth in F2N2 media.

[0061] The mRNA expression of the one or more transcripts encoded by SEQ ID NOS:52-68 in the chlorophyll deficient algal cell can be less than about ½, $1/10^{th}$, $1/50^{th}$, $1/100^{th}$, or $1/1000^{th}$ of the expression of the mRNA in a wild-type cell. For example, the mRNA expression of any one of the foregoing combinations of transcripts can be less than about ½, $1/10^{th}$, $1/50^{th}$, $1/100^{th}$, or $1/1000^{th}$ of the expression of the mRNA in a wild-type cell. In some cases, the down-regulation of one or more mRNA transcripts is obtained, measured, or detected during log-phase growth of the algal cells. For example, a chlorophyll deficient algal cell in log-phase growth in F2N2 media can exhibit down-regulation of one or more mRNA transcripts encoded by SEQ ID NOS:52-68, in any combination in comparison to a wild-type algal cell during log-phase growth in F2N2 media.

[0062] In some embodiments, the photosynthesis of the chlorophyll deficient algal cell is saturated or nearly saturated at higher irradiance levels as compared to a wild-type algal cell. For example, the photon flux that saturates photosynthesis can be higher for the chlorophyll deficient algal cell as compared to a wild-type algal cell. In some cases, the rate of photosynthesis in the wild-type algal cell reaches a maximum level, and is therefore saturated, at

less than about 1000, 600, 550, 500, 450, 400, 350, 300, or 250 μmol photons/m^2/s of irradiance, whereas the chlorophyll deficient algal cell is saturated at an irradiance level that is at least approximately 10% higher, 15% higher, 20% higher, 25% higher, 30% higher, 35% higher, 40% higher, 50% higher, 75% higher, 100% higher, 150% higher, 200% higher, or more. In some cases, the chlorophyll deficient algal cell can exhibit saturation of photosynthesis at irradiance levels that are at least 1.2-fold, 1.5-fold, 1.75-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold or more higher than the photosynthesis saturating irradiance level of a wild-type algal cell. In some cases, saturation of photosynethsis is determined in algal cells grown in F2N2 medium (*e.g.*, F2N2/50% seawater medium) at log phase under approximately 200 μmol photons/m^2/s irradiance.

[0063] In some embodiments, the chlorophyll deficient algal cell exhibits a significantly higher maximum photosynthetic rate as compared to a wild-type algal cell. For example, the chlorophyll deficient algal cell can exhibit a maximum photosynthetic rate that is at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, or 300% of the maximum photosynthetic rate of a wild-type algal cell. In some cases, the chlorophyll deficient algal cell can exhibit a maximum photosynthetic rate that is increased by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, or 300% of the maximum photosynthetic rate of a wild-type algal cell. In some cases, the maximum photosynthetic rate is measured as the maximum electron transport rate (ETR)of photosystem II (PSII) or the maximum relative ETR (rETR) of PSII. In some cases, the maximum photosynthetic rate is measured in cells grown under non-nutrient limiting conditions. In some cases, saturation of photosynethsis is determined in algal cells grown in F2N2 medium (*e.g.*, F2N2/50% seawater medium) at log phase under approximately 200 μmol photons/m^2/s irradiance.

[0064] In some embodiments, the chlorophyll deficient algal cell exhibits a greater resistance to photo-inhibition as compared to a wild-type cell. For example, the chlorophyll deficient algal cell can exhibit less photo-inhibition at high irradiance levels than a wild-type cell. In some cases, the chlorophyll deficient algal cell can exhibit no, or substantially no, photo-inhibition at irradiance levels of at least about 300, 350, 400, 450, 500, 600, 750, 1000, 1500, 2000 μmol photons/m^2/s or more.

[0065] The chlorophyll deficient algal cell can have less than about 90%, 80%, 70%, 60%, 50%, 40%, 39%, 38%, 37%, 35%, 34%, 33%, 32%, 31%, 30%, 20%, or less than about 10% of the chlorophyll content than a wild-type algal cell. In some cases, the chlorophyll deficient algal cell can have about 0.11 pg Chl *a* per cell, 0.10 pg Chl *a* per cell, 0.09 pg Chl *a* per cell, 0.08 pg Chl *a* per cell, 0.07 pg Chl *a* per cell, 0.06 pg Chl *a* per cell, 0.05 pg Chl *a* per cell, or less. For example, the chlorophyll deficient algal cell can have any one of the foregoing Chl *a* amounts when grown at, *e.g.*, log phase, in F2N2 media under standard growth conditions (*e.g.*, F2N2 media, 20 ppt salinity, 3% CO_2, and 250 µmol photons/m^2/s).

[0066] In some embodiments, the chlorophyll deficient algal cell has a reduced photosystem I or photosystem II antenna size as compared to a wild-type algal cell. For example, the average antenna size of photosystem I or photosystem II can be less than about 99%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, or 10%, of the antenna size in a wild-type algal cell.

[0067] The chlorophyll deficient algal cell can be useful for enhanced growth or productivity as compared to a wild-type algal cell. In some cases, a chlorophyll deficient algal cell can exhibit enhanced growth or productivity when photons are not evenly distributed throughout a culture. For example, the chlorophyll deficient algal cell can exhibit enhanced growth or productivity in an open pond format. In an open pond, or other similar format, a culture receives incident photons from the sun. Therefore, wild-type algal cells near the surface of the pond will absorb the vast majority of photosynthetically active photons. Consequently, algal cells distal from the surface will be photon limited. In contrast, a culture of chlorophyll deficient algal cells can allow a greater proportion of photosynthetically active photons to penetrate, and/or penetrate deeper into, the culture volume. Thus, more algal cells will have access to light and fewer algal cells in the culture will be photon limited. Moreover, in some cases, the chlorophyll deficient algal cells can have a higher maximum photosynthesis rate at saturating light intensities, therefore utilizing a greater percentage of the incident light. As a result, the population of chlorophyll deficient algal cells in the culture can exhibit greater productivity as compared to wild-type.

[0068] In some embodiments, the chlorophyll deficient algal cell can be useful for enhanced growth or productivity as compared to a wild-type algal cell even when photons are evenly, or

substantially evenly, distributed throughout a culture. In some cases, chlorophyll deficient algal cells that exhibit a higher maximum rate of photosynthesis can provide enhanced growth or productivity even when photons are evenly, or substantially evenly distributed throughout a culture. Examples of culture geometries providing even, or substantially even, distribution of photons include thin tubes, flat membranes, planar modules, flexible plastic film bioreactors, helical bioreactors, *etc*.

[0069] In some cases, the chlorophyll deficient algal cell can exhibit productivity of at least about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 80, 90, 100, or more grams of dry algal biomass/ m^2/ day.

[0070] The chlorophyll deficient algal cell can be cultured in conditions suitable for marine algae. Such conditions include a wide range of salinity and temperature. For example, the chlorophyll deficient algal cell can survive and/or grow at temperatures of between about 4 °C–40 °C and a salinity range between about 8 parts per thousand (ppt) to over 50 ppt. In some cases, the chlorophyll deficient algal cell exhibits the same, or approximately the same, ideal growth temperature and salinity conditions as a wild-type algal cell. In other cases, the chlorophyll deficient algal cell exhibits enhanced heat tolerance, enhanced salinity tolerance, enhanced low salinity tolerance, enhanced cold tolerance, or enhanced tolerance to osmotic pressure (*e.g.*, enhanced growth under low salinity conditions) as compared to a wild-type cell. In some cases, the chlorophyll deficient algal cell exhibits enhanced tolerance to ambient heat because fewer cells are exposed to potentially damaging light intensities, which are typically higher saturating light intensities. Cells exhibiting less damage by excessive light irradiation might thus endure more damage by higher ambient temperatures.

[0071] In some embodiments, the chlorophyll deficient algal cell is acclimated to a low salinity growth environment. In some cases, the chlorophyll deficient algal cell that is acclimated to a low salinity growth environment is able to grow in medium having a total dissolved solids concentration of less than about 20 ppt, less than about 18 ppt, less than about 16 ppt, less than about 15 ppt, less than about 14 ppt, less than about 12 ppt, less than about 10 ppt, less than about 8 ppt, less than about 6 ppt, less than about 4 ppt, or lower.

[0072] In some cases, the low salinity acclimated chlorophyll deficient algal cell can grow in a low salinity medium in which a wild-type algal cell exhibits growth inhibition. For example, the low salinity acclimated chlorophyll deficient algal cell can exhibit growth and/or productivity in a low salinity growth medium that is at least about 10%, 20%, 50%, 75%, 100%, 150%, 200%, 300%, or more higher than the growth of a wild-type algal cell under the same conditions. In an exemplary embodiment, the low salinity acclimated chlorophyll deficient algal cell exhibits growth inhibition of 5% in medium containing 10 ppt dissolved solids as compared to medium containing 20 ppt dissolved solids. In contrast, a wild-type algal cell, for example, can exhibit 50%, 75%, 80%, 90%, 95%, 99%, about 100%, or 100% growth inhibition in growth medium containing 10 ppt dissolved solids.

[0073] Similarly, the chlorophyll deficient algal cell can be acclimated to a high salinity growth environment. In some cases, the chlorophyll deficient algal cell that is acclimated to a high salinity growth environment is able to grow in medium having a total dissolved solids concentration of greater than about 20 ppt, greater than about 25 ppt, greater than about 30 ppt, greater than about 35 ppt, greater than about 40 ppt, greater than about 45 ppt, greater than about 50 ppt, or higher. In some cases, the chlorophyll deficient algal cell that is acclimated to a high salinity growth environment can exhibit growth and/or productivity in a high salinity growth medium that is at least about 10%, 20%, 50%, 75%, 100%, 150%, 200%, 300%, or more higher than the growth of a wild-type algal cell under the same conditions.

[0074] In some embodiments, the chlorophyll deficient algal cell produces a high proportion of lipids. For example, the chlorophyll deficient algal cell can produce up to about 20%, 30%, 40%, 50%, 60%, 70%, or more lipid by dry weight. In some cases, the chlorophyll deficient algal cell produces the same, or substantially the same lipid amount as a wild-type algal cell. In some cases, the chlorophyll deficient algal cell produces at least about 10%, 20%, 25%, 30%, 40%, 50%, 70%, 80%, or 100% more lipid than a wild-type algal cell, *e.g.*, when cultured under the same conditions. In some cases, the chlorophyll deficient algal cell has a lipid profile that is the same, or substantially the same as a wild-type algal cell. In other cases, the chlorophyll deficient algal cell has an improved lipid profile in comparison to a wild-type algal cell. For example, in some cases, the chlorophyll deficient algal cell has a higher percentage of EPA or a greater amount of EPA as compared to a wild-type algal cell. In some cases, the chlorophyll

deficient algal cell has a lower percentage of ARA or DHA or a lower amount of ARA or DHA as compared to a wild-type algal cell. Percentage of a particular lipid can be measured as, *e.g.*, a percentage of dry algal weight or a percentage of total lipid.

[0075] In some cases, the chlorophyll deficient algal cell has at leasta bout 3.9-5% or more EPA per gram dry weight. In some cases, the EPA content of the chlorophyll deficient algal cell is at least about 5-15% higher than the EPA content in a wild-type algal cell. In some cases, the chlorophyll deficient algal cell has an EPA concentration as a proportion of total fatty acids of at least about 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, or more. In some cases, the chlorophyll deficient algal cell produces no, or substantially no (*e.g.*, less than about 0.5%) DHA per gram dry weight or as a percentage of total fatty acids. In some cases, the chlorophyll deficient algal cell has an ARA content of less than about 1%, 0.9%, 0.8%, 0.7%, 0.61%, 0.6%, 0.5%, 0.4%, 0.3%, or less than about 0.2%. per gram dry weight, or as a percentage of total fatty acids. In some cases, the chlorophyll deficient algal cell has an EPA/ARA mass ration of greater than about 30:1, 25:1, 20:1, 15:1, 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, or 2:1.

[0076] The chlorophyll deficient algal cell can provide easier extraction of one or more desired compounds. In some cases, the easier extraction is provided by a higher content of the one or more desired compounds. In some cases, the easier extraction is provided by a greater production of the desired compound. In some cases, the easier extraction is provided by a reduced cell wall integrity of the algal cell. For example, the algal cell can be more susceptible to disruption by heating, mechanical means, sonication, solvent extraction, or a combination thereof. In some cases, the easier extraction is provided by a lower aqueous content of the algal cell. In some cases, the reduced amount of chlorophyll per algal cell affords a greater efficiency of oil extraction and/or purification.

II. Methods

A. Random Mutagenesis

[0077] A chlorophyll deficient algal cell of the present invention can be generated by random mutagenesis, directed mutagenesis, or by interfering with the transcription or translation of one or more genes or gene products. In one embodiment, the chlorophyll deficient algal cell is generated by random mutagenesis. Random mutagenesis can be useful for generating strains of algal cells that exhibit a desired phenotype but are not subject to restrictions generally placed on

so-called genetically modified organisms ("gmo"). Methods of generating a chlorophyll deficient algal cell by random mutagenesis are provided herein.

[0078] Wild-type algal cells can be contacted with a mutagen to generate a library of random mutant algal cells. Suitable mutagens include but are not limited to one or more of the following chemical mutagens: an acridine mutagen (*e.g.*, ICR-191), ethylmethane sulphonate (EMS), methyl methane sulfonate, 2-aminopurine, ethylene imine (EI), nitrosoethyl urea, nitrosoethyl urethane, N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG), or sodium azide. Suitable mutagens further include irradiation (*e.g.*, fast neutron bombardment, X-ray, ultraviolet, or gamma ray irradiation). Mutagenesis can also be performed by mobilization of transposable elements or random insertion of transposable elements (or other suitable nucleic acid) into the genome of the algal cells.

[0079] The library of random mutant algal cells can be selected for a chlorophyll deficient phenotype. Suitable methods for selecting chlorophyll deficient algal cells include plating the algal cell to generate colonies and selecting colonies that exhibit a pale green phenotype as compared to wild-type algal cells. Alternatively, the cells can be inoculated by limiting dilution into individual wells, cultured, and selected for cultures that are pale-green as compared to wild-type algal cultures. The selection can be performed manually or in an automated fashion. Pale green colonies can be resuspended and replated one, two, three, or more times to ensure stability of the pale green phenotype. Similarly, pale green cultures can be selected, diluted, and re-cultured to ensure stability of the pale green phenotype. In some cases, stability is defined as the absence of normally pigmented colonies or cultures, or the absence of sectored colonies (*e.g.*, colonies having sectors with varying degrees of pigmentation), after re-plating or re-culturing.

[0080] In some embodiments, the chlorophyll deficient algal cell is generated by random mutagenesis and then one or more algal cells containing a chlorophyll deficient mutation is identified and/or selected based upon the presence or absence of a known genetic element. For example, the chlorophyll deficient mutation can be identified and/or selected by TILLING (described at tilling.ucdavis.edu/index.php/Main_Page) or EcoTILLING. In some cases, the TILLING or EcoTILLING is performed to identify algal cells having a mutation in one or more of the genomic sequences encoded by SEQ ID NOS: 69-119.

[0081] A specific method of generating a chlorophyll deficient algal cell of the invention by random mutagenesis is described in Example 1.

B. Directed Mutagenesis

[0082] In some embodiments, the chlorophyll deficient algal cell is generated by directed mutagenesis. Directed mutagenesis can be performed by homologous recombination to introduce a nucleic acid at or near the gene to be mutated. Directed mutagenesis can additionally, or alternatively, be performed using a Zinc-Finger nuclease, TALEN, or CRSPR-Cas system to cleave or nick genomic DNA at or near the gene to be mutated. The cleavage or nick-site can then be repaired by non homologous end joining or homologous recombination to introduce one or more mutations. In some cases, the algal cell can simultaneously or sequentially be contacted with a second nucleic acid containing homology to one or more regions flanking the cleavage or nick site. The second nucleic acid can then be introduced into the cleavage or nick site to disrupt the target sequence. Suitable targets for generating a chlorophyll deficient algal cell by directed mutagenesis include, but are not limited to, one or more of the following genomic sequences: SEQ ID NOS: 69-119.

[0083] In some embodiments, recombinant DNA vectors containing isolated nucleic acid sequences suitable for transformation, and directed mutagenesis, of algal cells are prepared. Techniques for transforming algal cells are known in the art. *See, e.g.*, Kilian, *et al.*, *PNAS* 108(52), p. 21265-69 (2011). A DNA sequence coding for the desired RNA or polypeptide, for example a cDNA sequence encoding a full length protein, will preferably be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the introduced polynucleotide.

[0084] For example, for overexpression, a promoter fragment may be employed which will direct expression of the gene in the algal cell. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include, without limitation, the promoter region of: translation elongation factor alpha (TEF-alpha) or acyl carrier protein (ACP). *See, e.g.*, U.S. Patent Publication No: 2013/0289262. Exemplary constitutive promoters can further include a heat shock protein promoter, or a violaxanthin chlorophyll A

binding protein promoter (*e.g.*, VCP1 or VCP2). *See*, *e.g.*, U.S. Patent Application No: 13/685,659, filed 2/6/2014 and U.S. Patent Publication No: 2009/0317904.

[0085] Alternatively, the promoter may direct expression of the polynucleotide of the invention under more precise environmental control (inducible promoters). Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, nutrient conditions, elevated temperature, or the presence or absence of light. As an example, promoters driving genes associated with nitrogen metabolism are highly regulated. The promoter of the ammonium transporter, *e.g.*, can be used to drive expression of a heterologous gene during nitrogen limiting conditions. As another example, the nitrate reductase or nitrate transporter promoters can be used to repress expression in the presence of ammonium.

[0086] If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural gene, or from a variety of other algal genes.

[0087] The vector comprising the sequences (e.g., promoters or coding regions) from genes of the invention can optionally comprise a marker gene that confers a selectable phenotype on algal cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosluforon or Basta. The expression vector can be designed to facilitate random insertion into the genome of the algal cell, or targeted insertion into the genome of the algal cell, *e.g.*, by homologous recombination. Alternatively, the vector can propagate extrachromosomally.

C. Transcriptional or Translational Interference

[0088] Chlorophyll deficient algal cells of the present invention can be generated by interfering with the transcription or translation of one or more genes encoded by SEQ ID NOS:52-68. For example, an algal cell can be contacted with a polynucleotide encoding an antisense RNA, an shRNA, or an siRNA that binds to one or more of the mRNA transcripts encoded by SEQ ID NOS:52-68. As another example, CRSPR-interference can be utilized to bind a sequence upstream from one or more of the genes encoded by SEQ ID NOS:52-68 and inhibit their transcription. In some cases, CRSPR can be used to localize an additional protein that represses transcription upstream of the one or more genes encoded by SEQ ID NOS:52-68.

For example, the transcriptional repressor can be fused to Cas9, or a mutant thereof (*e.g.*, a nuclease deficient Cas 9).

[0089] One or more expression vectors encoding a nucleic acid for transcription or translation can be prepared. For example, an expression vector encoding a CRSPR-Cas system or a portion thereof, *e.g.*, a sgRNA or dCas9 can be prepared and introduced into a cell. Similarly, one or more expression vectors encoding an antisense RNA (*e.g.*, shRNA) can be prepared and introduced into a cell. As described above, such expression vectors can include a constitutive or inducible promoter operably linked to the encoded RNA or protein. The expression vector can further encode polyadenylation sequences and/or selectable markers. The expression vector can be designed to facilitate insertion into the genome of the algal cell, *e.g.*, by homologous recombination. Alternatively, the vector can propagate extrachromosomally.

D. Selection of Suitable Chlorophyll Deficient Algal Cells

[0090] Algal cells exhibiting a stable pale green phenotype after random or directed mutagenesis, or after transcriptional or translational inhibition of one or more mRNA transcripts, can be assayed to ensure that the pale green phenotype is stable and is not due to poor health of the cells. For example, assays can be performed to rule out chlorosis or a defect in thylakoid, chloroplast, or photosystem assembly, such assays include measurement of photosystem II electron transport. For example, the algal cells exhibiting a stable pale green phenotype can be assayed to determine a Pmax value. Pale-green algal cells exhibiting a Pmax that is higher than wild-type algal cells and/or a Pmax value that is saturated at a higher irradiance level as compared to wild-type can be selected for use in the methods of the present invention. In some cases, pale green algal cells exhibiting wild-type, near wild-type, or supra wild-type growth and/or productivity, pale-green algal cells exhibiting a greater lipid content compared to a wild-type algal cell, or pale-green algal cells exhibiting a desirable lipid profile, including, but not limited to, a higher EPA content as compared to a wild-type cell can be selected.

[0091] In an exemplary embodiment, pale green algal cells of the present invention are selected using one or more of the following selections after alteration of the genome or inhibition of transcription or translation of one or more genes. Colonies can be analyzed for chlorophyll content, *e.g.*, using a spectrophotometer or a spectrofluorometer to verify that the pale green phenotype is present. Re-plating and/or re-culturing can be performed to identify stable algal

cell lines. Stable algal cell lines can be characterized to identify cells that exhibit at least wild-type growth under high light intensity on solid medium. Stable algal cell lines can be characterized to identify cultures that exhibit at least wild-type growth under medium and high light-intensity in liquid culture at lab scale. Chlorophyll deficient algal cells selected according to any one or more of the foregoing criteria can be further assessed in large scale cultures (*e.g.*, open ponds).

E. Culturing Algal Cells

[0092] Described herein is a method of culturing a chlorophyll deficient algal cell. The chlorophyll deficient algal cell can be cultured at a wide range of temperatures and culture media. For example, the chlorophyll deficient algal cell can be cultured between about 4 °C and about 40 °C. In some cases, the chlorophyll deficient algal cell is cultured between about 15 °C and about 35 °C. In some cases, the chlorophyll deficient algal cell is cultured at an average temperature of about 10, 12, 14, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 °C. In some cases, the chlorophyll deficient algal cell is cultured at a temperature that is about 30 °C, or less than about 30 °C (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29 °C).

[0093] The chlorophyll deficient algal cell can be cultured in culture media having a wide range of salinity. For example, the salinity can range from 8 parts per thousand total dissolved solids or less to about 52 parts per thousand total dissolved solids or more. In some cases, the chlorophyll deficient algal cell is cultured in F2N2 media. In some cases, the chlorophyll deficient algal cell is cultured in F2N2 media at between about 25-28 °C under about 300 µmol photons/m^2/s light intensity.

[0094] Algae can be cultured under conditions to promote the accumulation of algal oil high in EPA. In some cases, algae can be cultured under conditions to promote the accumulation of algal oil low in ARA. In some cases, algae can be cultured under conditions to promote the accumulation of algal oil that is free, or substantially free, of DHA. For instance, the lipid content and compositions can be modulated by varying growth conditions such as light intensity, light-dark cycles, temperature, nutrient content, nutrient availability, salinity, pH, culture density, culture temperature, and other environmental conditions. Descriptions of growth conditions for *Nannochloropsis* are found in, *e.g.*, Sukenik, A. "Chapter 3: Production of eicosapentaenoic acid by the marine Eustigmatophyte *Nannochloropsis*," Chemical from Microalgae., ed. Zvi Cohen,

CRC Press, 1999, and Pal *et al., Appl Microbiol Biotechnol,* 2011, 90:1429-1441. Standard culture systems such as open ponds, *e.g.*, open race way ponds, and photobioreactors can be used to grow algae.

[0095] To generate an algal biomass, standard methods, *e.g.*, flocculation, centrifugation, and filtration (dead end filtration, microfiltration, ultrafiltration, pressure filtration, and tangential flow filtration) can be used for dewatering algae. For instance, cationic chemical flocculants, such as $Al_2(SO_4)_3$, $FeCl_3$, and $Fe_2(SO_4)_3$, can be used to coagulate harvested algae into a biomass.

[0096] The dried (dewatered) algal biomass may include at least about 10% lipids, *e.g.*, about 10%, 20%, 30%, 40%, 50%, 60%, 70% or more lipid; at least about 15% carbohydrates, *e.g.*, about 15%, 16%, 17%, 185, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30% or more carbohydrates; at least about 25% protein, *e.g.*, about 25%, 26%, 27%, 28%, 29%, 20%, 21%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40% or more protein; at least about 3% moisture, *e.g.*, about 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20% or more water; and at least about 5% ash, about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20% or more ash.

E. Preparing Crude Algal Oil from the Algal Biomass

[0097] The algae can be processed to obtain algal products such as one or more of algal oil, algal protein, or algal carbohydrates. The products can separated from the algal biomass by disruption methods that do not degrade the desired product. For instance, the algal cells of the biomass can be disrupted by, *e.g.*, high-pressure homogenization, bead milling, expression/expeller press, sonication, ultrasonication, microwave irradiation, osmotic shock, electromagnetic pulsing, chemical lysis or grinding of dried algal biomass, to release the algal products (*e.g.*, algal oil). Optionally, one or more products can be separated from the algal cell debris by, *e.g.*, centrifugation. For example, centrifugation produces an oil layer and an aqueous layer containing the cell debris. The oil layer can be collected and further processed.

[0098] Other useful methods for extracting fatty acids from algae include, but are not limited to: Bligh and Dyer's solvent extraction method; solvent extraction with a mixture of ionic liquids and methanol; hexane solvent extraction; ethanol solvent extraction; methanol solvent extraction; soxhlet extraction; supercritical fluid/CO_2 extraction; and organic solvent (*e.g.*, benzene,

cyclohexane, hexane, acetone, chloroform) extraction. See, *e.g.*, Ratledge *et al.* "Chapter 13: Down-Stream Processing, Extraction, and Purification of Single Cell Oils," Single Cell Oils, ed. Zvi Cohen and Colin Ratledge, AOCS Press, Champaign, IL, 2005. The extraction method may affect the fatty acid composition recovered from the algal biomass. For instance, the concentration, volume, purity and type of fatty acid may be affected.

[0099] In some embodiments, a polar solvent such as methanol or ethanol is used to extract the crude algal oil from an algal biomass, such as dried algal biomass. In some cases, it has been shown that ethanol extraction of fatty acids from algae can generate relatively high yields compared to other solvent-based methods.

[0100] After ethanol extraction, the extracted product can be further processed to remove water and polar components from the lipids. For example, as described in Fajardo *et al.*, (*Eur. J. Lipid Sci. Technol.*, 109 (2007) 120-126), the ethanol extract can be subjected to an apolar solvent extraction, *e.g.*, hexane extraction to generate a biphasic extract containing a hexanic phase and a hydroalcoholic phase.

[0101] The extracted products may be processed using separation methods such as, but not limited to, distilling, decanting, and centrifuging. For example, the wet solids may be separated from the liquid fraction containing the crude algal oil. Alternatively, the ethanol extract can undergo an isolation step such as chromatography to produce a lipid-enriched composition. Additional methods and compositions for culturing algal cells and obtaining algal products are described in U.S. Patent Appl. No: __/_____, entitled, COMPOSITIONS OF CRUDE ALGAL OIL, Attorney Docket No.:95844-902550-005200US, filed on March 18, 2014, the contents of each are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

[0102] The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: F2N2 Growth Media

[0103] The following stock solutions were made:

B solution buffer:

[0104] 2M Tris, pH 7.6

Fe Solution

[0105] In a final total volume of 1 L of H_2O, add 6.5 g $FeCl_3*6H_2O$, 45 g EDTA, pH 4.5

T-Solution: Trace metals

[0106] In a final total volume of 1 L of H_2O, add:

- 5.0 ml $CuSO_4*5H_2O$ 980 mg / 100 ml dH_2O,
- 5.0 ml $Na_2MoO_4*2H_2O$ 630 mg / 100 ml dH_2O
- 5.0 ml $ZnSO_4*7H_2O$ 2.2 g / 100 ml dH_2O
- 5.0 ml $CoCl_2*6H_2O$ 1.0 g / 100 ml dH_2O
- 5.0 ml $MnCl_2*4H_2O$ 18.0 g / 100 ml dH_2O
- 10 ml 500 mM EDTA
- Adjust to pH 4.5

P-Solution: Phosphorus source

[0107] 100g $NaH_2PO_4*H_2O$ in 1 L H_2O

N-Solution: Nitrogen source

[0108] 1 M NH_4Cl in H_2O

V-Solution: Vitamins

[0109] In a final total volume of 1 L of H_2O add:

- 5 mg Biotin
- 5 mg Vit B_{12}
- 1 g Thiamin HCl

[0110] Add the following stock solutions to 1 L of sea water (can use different salinities), mix well, and filter sterilize:

- 10ml of B Solution
- 2ml of Fe Solution
- 2ml of T Solution

- 2ml of P Solution
- 8ml of N Solution
- 2ml of V Solution

Example 2: Generation of a Chlorophyll Deficient Algal Cell (T661)

[0111] The chlorophyll deficient algal strain T661 was generated according to the following random mutagenesis protocol.

[0112] Acridine mutagen ICR-191 was prepared as a stock solution of 1.0 mg/mL in 0.1 N HCL (filter sterilized). *Nannochloropsis* cells were grown to log phase. At a cell density of approximately 1 x 10^6 cells/ mL, 20 mL of *Nannochloropsis* culture was placed into a 125 mL flask and the stock ICR-191 solution was added to a final concentration of 2 µg/mL. The flask was cultured under approximately 50 µmol photons/m^2/s of irradiance in an orbital shaker at room temperature for 7 days. After the 7 day incubation period, the cells were washed 2x in F/2 media, spread onto solid F/2 agar, and incubated under 50 µmol photons/m^2/s at 27 °C.

[0113] After approximately three weeks, adequately sized colonies were observed and chlorophyll deficient colonies were selected based on their pale green color as compared to other colonies not exhibiting a pale green phenotype. The selected chlorophyll deficient colonies were re-suspended in F/2 media and re-plated onto fresh solid agar plates as described above. Selection of pale green colonies, re-suspension, and re-plating was repeated for at least two rounds to obtain stable clones that exhibit an absence of normally pigmented colonies or sectors after re-plating.

[0114] Stable, chlorophyll deficient mutants were tested in fluorescence assays to ensure that pigmentation deficiency was not due to poor health. For instance, the pale green (*e.g.*, chlorophyll deficient) phenotype can be caused by various mutations involving nitrogen assimilation, chlorophyll biosynthesis, *etc.* Specifically, the inventors probed PSII electron transport essentially as described in Suggett, *et al.* "Estimating aquatic productivity from active fluorescence measurements." Chlorophyll *a* Fluorescence in Aquatic Sciences: Methods and Applications. Springer Netherlands, 2010. 103-127. *See*, also, Genty, *et. al.* Biochimica et Biophysica Acta (BBA)-General Subjects, 990.1 (1989): 87-92.

[0115] In brief, algal cells are grown to log phase in N2F2, 50% seawater medium under an irradiance of approximately 200 µmol photons/m^2/s. Samples are assayed with a Pulse Amplitude Modulated (PAM) Fluorometer. The relative electron transport rate (rETR) is calculated as rETR = PAR*ETR-Factor*P_{PS2}/P_{PPS}*Y(II), where PAR is flux of incident photosynthetically active radiation, ETR-Factor is fraction of incident photons absorbed by photosynthetic pigments, P_{PS2}/P_{PPS} is the fraction of photons absorbed by PSII relative to photons absorbed by photosynthetic pigments, and Y(II) is the effective photochemical quantum yield of PSII. Suitable values for ETR-Factor, and P_{PS2}/P_{PPS} are known in the art, or readily determined.

[0116] As shown in Figure 4, as irradiance increases, the wild-type *Nannochloropsis* PSII electron transport rate (rETR) quickly saturates. In some cases, chlorophyll deficient strains displayed rETR curves substantially identical to the wild-type ETR curve. In other cases, chlorophyll deficient strains displayed even lower saturation levels and/or earlier photo-inhibition under low-irradiance conditions as compared to wild-type algal cells. In contrast, the chlorophyll deficient strain T661 saturates at a much higher level of irradiance and a much higher level of PSII rETR (Figure 4).

Example 3: Growth of a Chlorophyll Deficient Algal Cell (T661)

[0117] T661 was grown in 2.6 m^2 outdoor ponds having an approximate volume of 650 liters in Northwestern Australia. Growth data was collected (Figure 1). The chlorophyll deficient algal cells exhibited a productivity of up to 30-40 grams of dry weight algal cells per m^2/day or higher.

[0118] T661 and wild-type algal cells were also grown in smaller ponds in Mexico (Figure 2). In general, Mexico has less favorable culture conditions than observed in Australia. Nevertheless, over a period of 10 days, T661 averaged approximately 18 g/m^2/d, while the wild-type parent *Nannochloropsis* algae averaged approximately 10 g/m^2/d.

[0119] T661 algae cells were grown in approximately 1 acre open raceway ponds in Australia. EPA levels were determined by flame ion detection gas chromatography. The average EPA content across six one acre open raceway ponds was about 4.18% of the total biomass (Figure 3).

Example 4: Genetic Characterization of a Chlorophyll Deficient Algal Cell (T661)

[0120] Strain T661 was sequenced using a Genome Analyzer II platform. Polymorphism analysis was performed comparing T661 to two independent sequence data sets for the wild-type parent strain as well as the published genome of *Nannochloropsis oceanica*. In addition, the transcriptome of T661 was sequenced and used as a secondary reference for T661 polymorphisms.

[0121] Polymorphisms were categorized as follows:

a) Polymorphisms observed in the T661 genome that are polymorphic in comparison to the consensus sequence from three reference genomes.

b) Polymorphisms from category A that also occur in T661 transcripts (11).

c) Polymorphisms from category A that occur within 1 kb of an observed T661 transcript (40).

[0122] Polymorphisms from category B and C are more likely to lead to changes in protein expression that result in the pale-green phenotype. The polymorphisms from category B are provided as SEQ ID NOS:1-11. The polymorphisms from category C are provided as SEQ ID NOS:12-51.

Example 5: Transcriptional Analysis of a Chlorophyll Deficient Algal Cell (T661)

[0123] Wild-type *Nannochloropsis* (W2) and the chlorophyll deficient strain T661 (IC171) were grown to log-phase in F2N2 media and the mRNA was isolated. Next-generation sequencing and mRNA analysis were performed to identify transcripts that are selectively down-regulated in the chlorophyll deficient strain. Raw sequence reads of the wild-type and T661 datasets were assigned to a common reference sequence to quantify gene expression differences between the two strains (Figure 5).

[0124] The top 10% differentially down-regulated genes in the chlorophyll deficient strain were categorized as Class 1 or Class 2 by homology *via* BLASTx. Class 1 genes are homologs clearly associated with chloroplast function. Class 2 genes are putative transcription factors that could directly impact the expression of one or more chloroplast genes. DNA encoding the down-regulated mRNA transcripts are provided by SEQ ID NOS:52-68. Down-regulation of the

transcription, or translation of any one or more of SEQ ID NOS:52-68, in any combination can provide a chlorophyll deficient algal strain. Similarly, inhibition of the activity of a protein encoded by any one or more of SEQ ID NOS:52-68 can provide a chlorophyll deficient algal strain.

[0125] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, nucleic acid accession identifiers, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

WHAT IS CLAIMED IS:

1. An isolated algal cell of the genus *Nannochloropsis*, the algal cell having less chlorophyll content than a wild-type cell,

wherein the algal cell comprises at least one polymorphism in comparison to a wild-type cell, and

wherein the at least one polymorphism is selected from the group consisting of the polymorphisms encoded by: SEQ ID NOS:1-51.

2. The isolated algal cell of claim 1, wherein the at least one polymorphism is selected from the group consisting of SEQ ID NOS:1-11.

3. The isolated algal cell of claim 1, wherein the algal cell has less than about 50% of the chlorophyll content of a wild-type cell.

4. The isolated algal cell of any one of the preceding claims, wherein the mRNA expression of 1, 2, 3, 4 or more mRNAs selected from the group consisting of mRNAs at least partially encoded by SEQ ID NOS:52-68 is less than about ½, $1/10^{th}$, $1/50^{th}$, $1/100^{th}$, or $1/1000^{th}$ of the expression of the mRNA in a wild-type cell.

5. The isolated algal cell of claim 4, wherein the mRNA expression compared to a wild-type algal cell is mRNA expression of log-phase cultures in F2N2 media.

6. The isolated algal cell of claim 1, wherein the algal cell exhibits a higher growth productivity as measured by grams of dry weight algal cells per square meter per day when grown in outdoor cultivation ponds as compared to a wild-type cell.

7. The isolated algal cell of claim 6, wherein the algal cell exhibits at least a 10% higher growth productivity as measured by grams of dry weight algal cells per square meter per day when grown in outdoor cultivation ponds as compared to a wild-type cell.

8. The isolated algal cell of claim 1, wherein the algal cell contains at least about 3.9-5% EPA per gram dry weight.

9. The isolated algal cell of claim 1, wherein the concentration of EPA as a proportion of total fatty acids is at least 5-15%, or 15-30%, higher than the wild-type cell.

10. The isolated algal cell of claim 9, wherein the concentration of EPA as a proportion of total fatty acids is at least about 30%.

11. The isolated algal cell of claim 1, wherein the algal cell contains no, or substantially no, or less than about 0.5% Docosahexaenoic acid (DHA) per gram dry weight.

12. The isolated algal cell of claim 1, wherein the algal cell contains less than about 0.61% ARA per gram dry weight.

13. The isolated algal cell of claim 1 or 12, wherein the algal cell has an EPA/ARA mass ratio of greater than about 5:1.

14. A composition comprising dry whole *Nannochloropsis* biomass, wherein the biomass comprises an algal cell of any one of the preceding claims.

15. A process for obtaining a total algal oil rich in EPA, the process comprising:

cultivating any one of the algal cells of claims 1-13; and

isolating the oil.

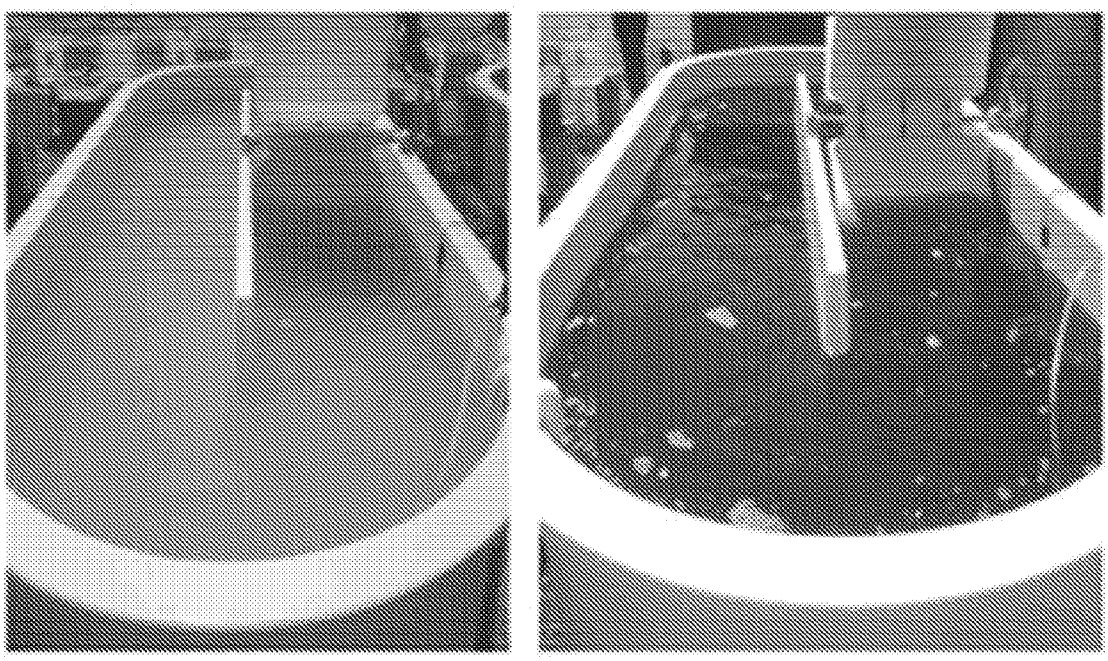
16. A method of making any one of the algal cells of claims 1-13, the method comprising:

modifying the genome of an algal cell from a parent algal cell line to introduce a polymorphism selected from the group consisting of the polymorphisms encoded by SEQ ID NOS:1-51; or

reducing the expression of an mRNA transcript in an algal cell, wherein the mRNA transcript is selected from the group consisting of the transcripts encoded by, or partially encoded by, SEQ ID NOS:52-68.

Date	T661 pond #1 Productivity (gms/m^2/d)	Date	T661 pond #2 Productivity (gms/m^2/d)
25-Oct	40	25-Oct	16
26-Oct	15	26-Oct	5
27-Oct	21	27-Oct	21
28-Oct	22	28-Oct	14
29-Oct	22	29-Oct	18
30-Oct	9	30-Oct	12
31-Oct	43	31-Oct	19
1-Nov	25	1-Nov	30
2-Nov	25	2-Nov	17
3-Nov	39	3-Nov	26
4-Nov	22	4-Nov	13
5-Nov	36	5-Nov	12
6-Nov	30	6-Nov	37
7-Nov	48	7-Nov	19
8-Nov	39	8-Nov	32

FIG. 1



661 in Mexico WT in Mexico

FIG. 2

Date	Pond	EPA content (% of total biomass)
Oct 26th, 2011	A1	4.0
Oct 26th, 2011	A2	5.0
Oct 26th, 2011	A3	3.9
Oct 26th, 2011	B1	4.1
Oct 26th, 2011	B2	3.9
Oct 26th, 2011	B3	4.2

FIG. 3

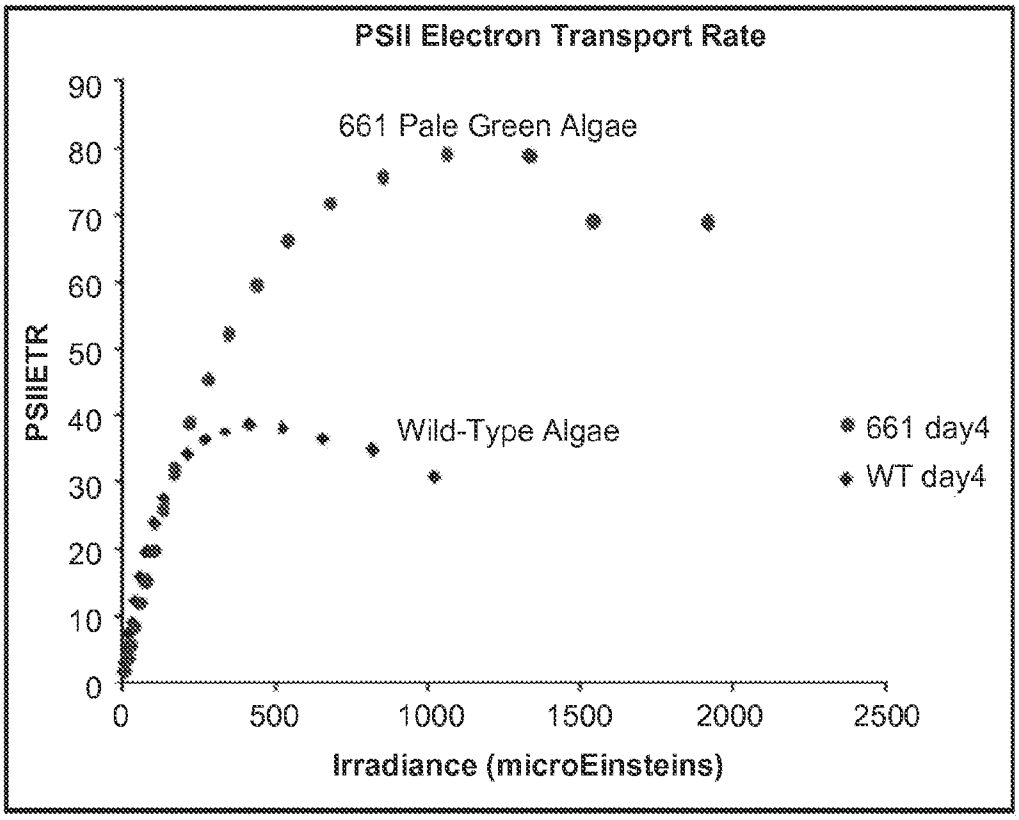

PSII Electron Transport Rate
661 Pale Green Algae
Wild-Type Algae
661 day4
WT day4
PSIIETR
Irradiance (microEinsteins)
0
10
20
30
40
50
60
70
80
90
0
500
1000
1500
2000
2500

FIG. 4

SNP-B-1

ATGATCTGCTCGAGGCTATGGACGGGAAAGGAGTCCAGTTTGAAGTGTATGGCCAGC(G>A)GGGACTC
ATCAGCAGCGTAGTCTTCGAGGGCAAAAATTGTCG

SNP-B-2

TCCTCTATCCTCCCCTCCTTCCCTCCCTCCTTCCCTCCC(T>C)CATCATCCATCTGTCCTCGTGCTACCCTTGC
CGCCTCTTCCGCCACCACCCTCAGGGGCA

SNP-B-3

ACGGGGAGAGTAAGA(+A)TAATGAGTA(ΔG)TATTTTTGGGGGGGGGGG(A>G)GGGGG(+G)CGGTGCTC
GATTTCGATTTCTATAGATATGTCGTCACAGTCTTTCTCCTTT

SNP-B-4

AAGTGATAGTAGCAGCAGCAACAGCAGCAACAGCAGCA(ΔA)CAGCAGCAGCAGCAGCGGCAGCACTAT
CACAACTACCAGCAGCATCAGCAGTAGCGGCAGC

SNP-B-5

TGGAGTACTCCTCCCCGCAGCAGCAGCAGCAGCAGCAGCAGCTT(ΔC)TACAGTTGGACGTGCGCTACGC
GACTTTAGCTTCTGCTTTAGGAGAAGCAGGAGC

SNP-B-6

ACAAGAAGACGAAGAAGAAGAAGAAGAAGAAGAAGAAA(ΔT)AGAAGCAGAAGAAGGACGAGAAAAG
GAAAAAAGAACATGAATGACACGAGCAAGAATGTTG

SNP-B-7

TCTCGGTAGATGTGTTAGGCCTTTTATTTTCATTGGTGTTCATATATATATATATATATATGTGTGTGT(A>
G)TCTGGCTGTCTGTCTGTCTGCGTGTGTCAG

SNP-B-8

TGATCAATATCAAAGAAGAGGCAGCGTCCCCTCGCTCTGCCACGCCATGACCCATCCCTTCCTCCCTCCCT
TCCTTCCTCCCTCCCTCC(C>T)TCCAGGCTCA

SNP-B-9

CCATCCCCTCCCCCCGGCATCATCATCATCCCTCCCTCCCT(+C)CCCGCCTCCCACCTCATCATCATCGTCAT
CATCGCCTTCCTCCGCGAGCAAAGCCGGG

SNP-B-10

TCCCACCCCAGATTCAGTCCCATTCCCATTTCCCC(G>A)TCATTCTCCCCTTCCTTGGGATTCACATTTCCCA
CCTCATCCTTCTCCTCCTTCTTCTTCTCT

SNP-B-11

GCCCTCCTTCTCCAGCACCTCGGCCGCCTTGATCCCCTCCCAGGTCGA(G>A)GCAATCTTAATCAGAATCC
GGCTTTTGTCGATTCCAAGTTCTTTATACATT

FIG. 5

Category C SNPS:

SNP-C-1

ACAACCCTACGTTCTCTTTTTCTCTGACTTATAATTACCTCACACTTCCATGTCCTC(+T)TTTTTTTTTCTTTTT TTTTTACCCTTCTATTTTTACATTCAA

SNP-C-2

CTTCATGGAGCCC(+C)TCTCTAATTCGGTGCATTTTTTTTGTGATTTCTGACTCACGTCAACCCCTCCACAAC ACTCATATATATATCCAAATCCTCATACAC

SNP-C-3

GAGGGAGGGAGGGAGGGAGAGGTGGGACGTACCTGAAGTGGCGTCCC(ΔC)GGTGATGAGCAGGCGG AAGGTGCTCTTGAACATACGGACCTGTGAGGGAAAA

SNP-C-4

AGGAGGAGGAGGAAGGGG(ΔG)CATTGAAAGAGGACATGGGCGAGGAAGCTGTCGATCGAATGGCAA CGGGTGGAAGCATGCTTCTGTCTTGATAAGGGGGGA

SNP-C-5

AAAAGGAGGGCCGACGATATATATATAGAGAGAGAGA(ΔC)GGAAGGAAGAGAGGGATGGAT(ΔC)GG AGGGACGGACGGACGAACAGAAGAAGGGAAGGAGGGAG

SNP-C-6

ACCCACCCCCCTTCCCTCCAGACCCTCATCCTCCCCCTCGGCCCTCCCTCTCCACCTT(+C)CCCCCCCTTTTC CTCCTCTTTCACGACCTCCTCCCTAAGCT

SNP-C-7

CCTCCTATCCCCTCACTTTCTCTCTTTTCCCC(+C)TACTCCTGTATCCGCTCATCCACCATCGTTCCCTCCCTC CTTCTCGTCCTCTTTCTTTTCCCTCCCA

SNP-C-8

AAAAGGAGAAAGAGGAGGTGGGGGGAGAAGCA(+C)GCCCCCAAGTAAAGCCCAATGAGGCGGCTGCG ATGGAAGTCATATGCTTGTCAGACGACGACTAGGT

SNP-C-9

TTCAAGAGAAGAGAATGTAGAGGTGGCAGACGAGGAGGAAGGGAAGGA(ΔG)GGGGGGAAACCTTCCT TTGCCGGGCGCGTCGTCAACGACCACGTCCACCGC

SNP-C-10

GTTTACCTTCTTTCACCTTCCCTTTTCTCCCCCC(ΔC)TCCTTCGTCTCTTCCCGCGGGCACTAACTCGTGCCC CTCATCCTCCCCCCTCCCTCTCTTCTCCC

FIG. 5 (cont'd)

SNP-C-11

AGAAGGCTTGGCCATTGCAAGCCTGCGGATGGCAAGACAATCGGCAAAGTAAACT(ΔC)CCCCCCACCCT CTCACCCTCCCTCCCTCTCACCCTTCCTCCCTC

SNP-C-12

AAACGACCTTTTGGCAAAGGAAGGGGACAGAAGGAGGAGGAAA(+A)GCAGGAGGAGGAGGAGGTCA CGTGCAAGCCATCCGAGGACTTCGTTGGTGTTAGAC

SNP-C-13

GAGAGAAAGATAGGATGGGAAAGAGGGAGGGAGGG(+AGG)GGTGTCTCACCCGCATCGCATGGAAG GTCCGTGGGCACTGCTGGACGCGATGGAGACCCTGGCCG

SNP-C-14

GTATTTACTAGTGAGGCCGTTCTGACAGCCCATGGCCAGGGCACACGCCCACAAAAACTCGTGGCGCCT GAGCAGAGGGAAGTAATGGAG(A>G)AGGAATGAGA

SNP-C-15

CCGTCTTGATCGAATATATCAGCGCTGTTGGGGGTGGAGGTCGTGGTAGAGGGGGAGGCGAGGAGGG GGCGGACGGGGGAACGGCGGGG(A>G)GGG(A>G)AAAA

SNP-C-16

GCGGGGCCCGTTAAGCCTGATCTTGGACCCCGCATACCTT(+G)GGGGGGGAGAGGGGGAAGGGAAAG AGAGACGGGCAGATTAAGAAGGAGGAGCGGAGGAG

SNP-C-17

GCTGAAGCCGACCCCGATTCGCACATTGACATTCATGAAGGGGGGGG(+G)GAAGAGGGTGAGTGATGT GGGCGTGAGCGACCTGTTGCGGTACATCCAAAGC

SNP-C-18

GCGCAAACTTCTTGCAAGCCTACAGGGAGGAAGGAAAGGAGAAAAGGAGAAAA(+G)GGGGGAGGGAA AGAGGGGAGTTGCCCGAGAGGTTAATCAGTCAGGG

SNP-C-19

CCCTTGGTAGACAAATTA(T>C)GTGCTAATATTGTTTCAAGGGCATCAAATTTTGGGGTATCAGTATCAGA AAAATAAAATGTATGTACTACTACACACCCACG

SNP-C-20

CACAAAACCAGCTCCCCCGCCCCACGAAGACACTCCTCAATGTCCCTCACATTTGGCTTCCTCTTCCCTCCC CCCCTTCCT(ΔC)CCCCCCCATCCGGCACCT

SNP-C-21

GGATTTAACCGGCTGTCCCTTATCACGTGAGGCAGAAATTTGTCTTCCTCTAATTGTTTTCTCTCTCTC(ΔT) TTTCACACACACACACACACACACACACAC

FIG. 5 (cont'd)

SNP-C-22

GGGGGGG(AA>GG)GGGGGGGGAGAAAGAGAGCGGGGGAGAGGGAGGAGAGAGGAAGGGGAGAGG AGGACCGGGAGACGATGGACGAGCGGTCGGTTGGCAAGAATA

SNP-C-23

TTCCCTCCCTCCCT(+T)CCTCCCTTCTCTCCCTCACCTTGACTCCATGAAGGTCTCCAATTCCGCGCACAGTC TTTTTCGTTTCCTCCTTCCTCCTCCTCTT

SNP-C-24

GCGTTCGTTCTGGGGAGTCAGGCACGTGAGATGTGGGTTGTGGAAGACGAGAGATGGAAGCAGGGAA GAAGCCCGTTTGACGGGTGTG(G>A)GCCGTTGAGT

SNP-C-25

TTGCTGCTAACTTTGATTTAGGCAAAATTATTCTCAGGGGGGG(ΔG)CAGGCGAGTCTGGGGGTAGTGGG GGAGGGGAAGGAGGGAGAAAGAAAGGGGATTAG

SNP-C-26

ATGAGCAGTTTTGCAGGAAGCGTAACAGCAGCTTGACACCGCCCTCTCTTCCCCCCTCTT(+C)CCCCCCCC AATCCCTACTTCCCTTCCTTCCTACCGGGTG

SNP-C-27

TGCTTGTGTATGGCTCGTATGAAGTCTACAAGGACATGATCGTGGAAGGGTGAGGAGGGAGGGAGAGA (ΔG)GGGAAGGAAGGAGACAGGGAAGGAGGGAGAA

SNP-C-28

CCGC(T>C)CCCCC(G>C)CCCCCCCCCCCCCGTTGGCAAGTTCCCGGTCGCAAACCACAGATACTCCCCCAAC CGCCTCATCCAGTCCAATCCCCCACCTTCCTTT

SNP-C-29

GGGGATGGATATGTCGAAGACGCTATAGTCAAAGCATAAAAAGTACACTATGCGCAAAATTTATATCAT(T>C)TTTTATCGTGATCGCGCGCTTGTCTCGTC

SNP-C-30

GCAATGGGAGCGAATTCACCCTCCTTCCACTTCTCCTGAGTACCAACCCTC(ΔT)TTTTGCGAGAACCGAAA AATTTTCCTTCATTCGTTCCCTCCCTCCCCC

SNP-C-31

ATTCGCACCCTTCCCTCCCTTCCTCCCTCCCTCCCTC(ΔC)TCACCGCATTGTACTCCGCCATGAGTGAGGAG ACTGTCTGTCCGTCCTCCATCATCTGGTTC

SNP-C-32

CGTCAGTTCCATTCGTCATCCTCCTCTTTTTCCCCTCCATCGCCTCCTTCGATCGTTCATTTCCCACCATCTCC CCCTTTTCCTCCTCTT(+C)CTCCCCCCC

FIG. 5 (cont'd)

SNP-C-33

CCAGATGGGTGCTGCCCCTCCTCGCCGCAGAGGAAACTGTAGGAATGCGCGTCTAGGAGGGAGGGAAG GAGGGAGGGAGGGA(ΔAGG)AGGGAGGGAAAGAGG

SNP-C-34

AGGTCGTTCAAGGTCTCGAAAGGCGGGGCCTGAAACTGATCCACCGAAAGGTGCTGAGGAGGGAGGGA GGGAGGGAGGGTGTGAGG(G>A)AAGGAGGGAAGGGA

SNP-C-35

CTTGACATTTGTTTCCCCTCTGATAGGTGGAAGTGTAGATTGACTGCCCCCCC(CT>TC)TTCCTCGCCTTCG TCCTTCTCGTCGTCGTCCTCCTCCTCGCCGTC

SNP-C-36

TGCATGCGATAGTTCT(+T)AGAGGGATGTAGGAACGGCAAAAGAACTAGTAATTTCCTTTCTTGTTATGT GAATGTCTTACTTGTGTACATGGCCTCAGCTT

SNP-C-37

GAAGAAGACAAAGTAAACTTCCGAAAAGAAAATAAGTAAAATGAAAATATAGAAGGATAGTCACAC(T> C)TT(T>A)AAAAAAGACGCAA(T>C)CATTCCTCATTTA

SNP-C-38

CCTATTCAAGGACGAGACGAAGGGAGGGAGGCAGGGAGGAAGGAGAAGAGGGAGAGGAAGAA(+G)G GAGAGAAAGGAGGGAGGGAGGGAGGGAAGGTACCTC

SNP-C-39

GGCGGTGATGTGACCCTCCCC(ΔC)GGGGTGGATGATGAGGCTGCGGTCTTTGCGGCGCGGATGCGGGC CAGGTTGCAACAGCGGAACAAGCAGCACAAAACG

SNP-C-40

TGGGGGAGGAAAGTGTCAAGTAAAGGGGTGTAGACGCTTGCATCAGGGGAAGCA(+G)GGGGTTGTCG GATCTGTTCTTTTGTCGGGCACATCGGAACGTGTA

FIG. 5 (cont'd)

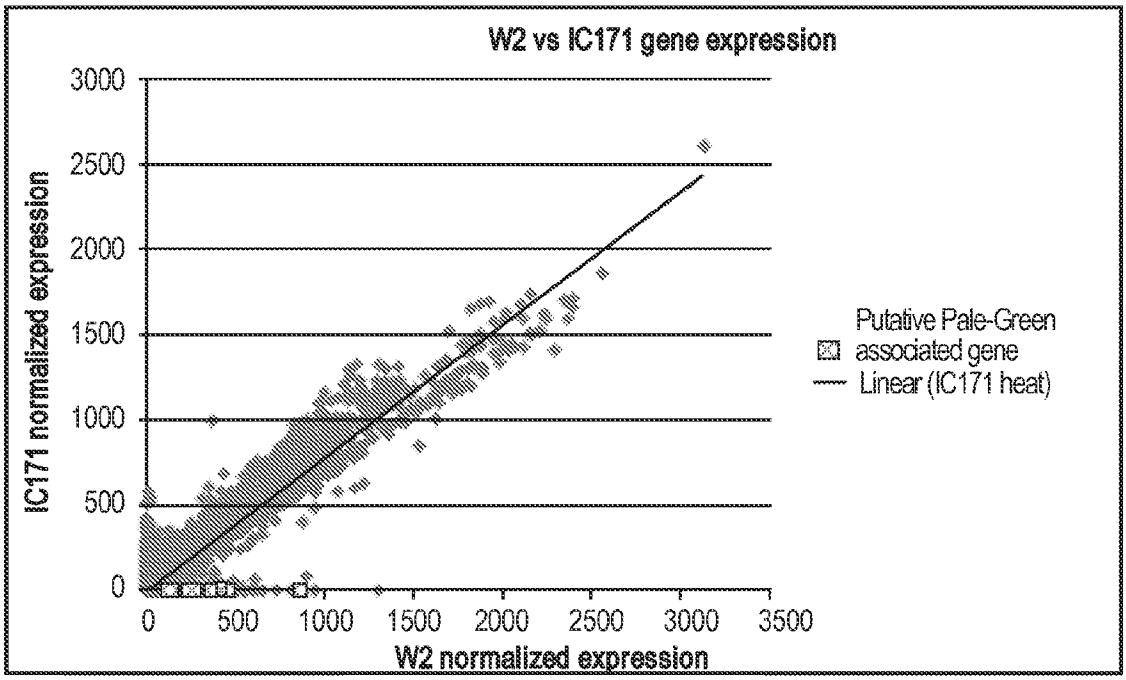

W2 vs IC171 gene expression
IC171 normalized expression
3000
2500
2000
1500
1000
500
0
0
500
1000
1500
2000
2500
3000
3500
W2 normalized expression
Putative Pale-Green associated gene
Linear (IC171 heat)

FIG. 6