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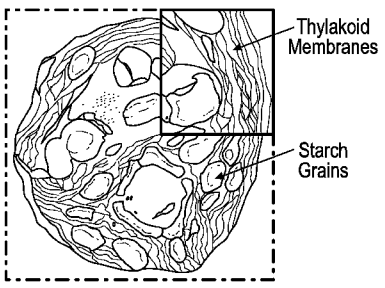
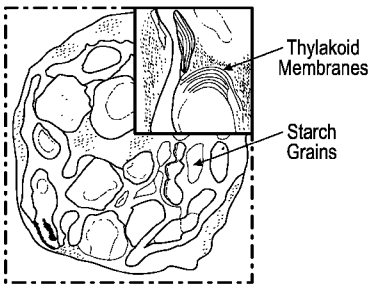
(54) Title: IMPROVED PRODUCTIVITY AND BIOPRODUCT FORMATION IN PHOTOTROPIN KNOCK/OUT MUTANTS IN MICROALGAE



WT Parent

FIG. 11A



PHOT K/O

FIG. 11B

(57) Abstract: Phototropin is a blue light receptor, which mediates a variety of blue-light elicited physiological processes in plants and algae. In higher plants these processes include phototropism, chloroplast movement and stomatal opening. In the green alga *Chlamydomonas reinhardtii*, phototropin plays a vital role in progression of the sexual life cycle and in the control of the eye spot size and light sensitivity. Phototropin is also involved in blue-light mediated changes in the synthesis of chlorophylls, carotenoids, chlorophyll binding proteins. We compared the transcriptome of phototropin knock out (PHOT KO) mutant and wild-type parent to analyze differences in gene expression in high light grown cultures {500 µmol photons m^{-2} s^{-1}). Our results indicate the up-regulation of genes involved in photosynthetic electron transport chain, carbon fixation pathway, starch, lipid, and cell cycle control genes. With respect to photosynthetic electron transport genes, genes encoding proteins of the cytochrome b6f and ATP synthase complex were up regulated potentially facilitating proton-coupled electron transfer. In addition genes involved in limiting steps in the Calvin cycle Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), Sidoheptulose 1,7 bisphosphatase (SBPase), Glyceraldehyde-3-phosphate dehydrogenase (3PGDH) and that mediate cell-cycle control (CDK) were also up regulated along with starch synthase and fatty acid biosynthesis genes involved in starch and lipid synthesis. In addition, transmission electron micrographs show increased accumulation of starch granules in PHOT mutant compared to wild type, which is consistent with the higher expression of starch synthase genes. Collectively, the altered patterns of gene expression in the PHOT mutants were associated with a two-fold increase in growth and biomass accumulation compared to wild type when grown in environmental photobioreactors (Phenometrics) that simulate a pond environment. In conclusion, our studies suggest that phototropin may be a master gene regulator that suppresses rapid cell growth and promotes gametogenesis and sexual recombination in wild type strains.

WO 2016/197136 A2

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PROVISIONAL PATENT APPLICATION

IMPROVED PRODUCTIVITY AND BIOPRODUCT FORMATION IN PHOTOTROPIN KNOCK/OUT MUTANTS IN MICROALGAE

TECHNICAL FIELD

[0001] The disclosed embodiments of the present invention are in the field of improved performance of microalgae in the production of biological products such as but not limited to biofuels, biomass, pigments, starch, oils and the like through selection, mutagenesis or engineering to reduce expression or knockout the phototropin gene.

BACKGROUND

[0002] Phototropin is a blue light receptor, which mediates a variety of blue-light elicited physiological processes in plants and algae. In higher plants these processes include phototropism, chloroplast movement and stomatal opening. In the unicellular green alga *Chlamydomonas reinhardtii,* phototropin plays a vital role in the progression of the sexual life cycle and in the control of the eye spot size and light sensitivity. Phototropin is also involved in blue-light mediated changes in the synthesis of chlorophylls, carotenoids, and chlorophyll binding proteins. The UV-A/blue light sensing phototropins mediate a variety of light responses and are responsible in higher plants for optimization of photosynthetic yields (Chen, Chory et al. 2004).

[0003] Phototropins are composed of two domains, an amine terminal photosensory domain and a carboxy terminal serine/threonine protein kinase domain. The photosensory domain is a flavin mononucleotide binding domain, the LOV domain. Plants contain two of these domains in the phototropin regulatory sequence, LOV1 and LOV2 (Chen, Chory et al. 2004).

[0004] Phototropin knock-out mutants (PHOT K/O) have been made previously in plants (Suetsugu and Wada 2007, Moni, Lee et al. 2015) and algae (Huang and Beck 2003, Trippens, Greiner et al. 2012). However, all the PHOT K/O mutant prior art that has been located to date did not show improved productivity of the plant or alga.

[0005] In plants two phototropins have been reported, phot1 and phot2, these phototropins share sequence homology and have overlapping functions. These blue-light-sensitive receptors consist of two parts: a C-terminal serine-threonine kinase and two LOV domains that bind flavin mononucleotide as chromophores at the N-terminus. Recently, in the unicellular green alga, *Chlamydomonas reinhardtii*, a

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phototropin homolog was identified. It exhibits photochemical properties similar to those of higher plant phototropins and is also functional in *Arabidopsis*. Studies show that the basic mechanism of phototropin action is highly conserved, even though its apparent physiological functions are quite diverse.

Phototropin in higher plants:

[0006] Plants utilize several families of photoreceptors to better react to their environment, allowing them to fine tune pathways controlled by the photoreceptors - phototropin, phytochrome, and cryptochrome (Chen, Chory et al. 2004).

[0007] In higher plants phototropin mediates a variety of blue-light elicited physiological processes (Sullivan, Thomson et al. 2008). Phototropins are UV-A/blue light sensing photoreceptors that are known to optimize photosynthetic yields (Chen, Chory et al. 2004). The involvement of phototropin in photomovement in higher plants is well documented (Suetsugu and Wada 2007, Kagawa, Kimura et al. 2009). Studies involving *Arabidopsis* mutants lacking the phot1 and phot2 genes have revealed that in addition to regulating hypocotyl curvature of seedlings towards blue light, phototropins also regulate a diverse range of responses in flowering plants. These responses include chloroplast movements, nuclear positioning, stomatal opening, leaf expansion, leaf movements and leaf photomorphogenesis.

[0008] *Phototropin knock-out mutants (PHOT K/O)* have been made previously in plants (Suetsugu and Wada 2007, Moni, Lee et al. 2015). For instance in *Physcomitrella patens* (a moss) there are three PHOT genes and they have all been knocked out in different mutants (Suetsugu and Wada 2007). The focus of the *P. patens* study was the effect of PHOT K/O on phototropism (movement toward light) and the phenotypes they observed allowed them to determine which of the genes were necessary for phototropism (Suetsugu and Wada 2007).

[0009] PHOT expression was higher in darkness than in light, and PHOT1 *Arabidopsis* mutants was shown to increase the number of lateral roots produced (Moni, Lee et al. 2015). PHOT was also demonstrated to mediate phototropism, chloroplast relocation and leaf expansion (Matsuoka, Iwata et al. 2007). Using PHOT deficient *Arabidopsis* mutants, phototropin 2 was linked to palisade parenchyma cell development of leaves (Kozuka, Kong et al. 2011).

[0010] Another study looked at the role of phototropin under low photosynthetically active radiation (Takemiya, Inoue et al. 2005). They found that the wild-type and the PHOT1 mutant both showed increased but similar growth in low radiance blue light super imposed on red light. In white light there was no increase in biomass in both phot1 and phot2 mutants as well as in the double phot mutant.

[0011] A study by Folta and colleagues investigated the relationship between phot1 and phototropism and growth inhibition in *Arabidopsis* (Folta, Lieg et al. 2003). They found that the onset of

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phototropism and the phot1-mediated growth inhibition coincided and postulated that both were due to phot1 expression.

[0012] There is a substantial amount of patent literature around phototropin in higher plants. However, the focus has been on the commercial utility of the upstream, light regulated areas rather than on the phototropin gene itself. These light control domains that regulate PHOT expression – the light-oxygen-voltage-sensing (LOV) domains - have been carefully evaluated for potential commercial application in higher plants.

[0013] Shu & Tsien application (US20130330718) focused on using the LOV domain for control of proteins that generate singlet oxygen (SOGs). These fusion protein tags could be used for imaging under blue light for research purposes.

[0014] Other patents use light switchable regulatory sequences and contemplate the use of the phototropin LOV domain such as Yang and colleagues (EP2682469).

[0015] Hahn & Karginov (WO2011133493) focused on allosteric regulation of kinases using the light activated domains for control of expression in engineered fusion proteins (such as the LOV domains).

[0016] Hahn and colleagues (US8859232) demonstrated that the LOV domain of phototropin can be used as a light activated switch for the activation or inactivation of fusion proteins of interest. They contemplated using a LOV domain that could contain substantial portions of the phototropin molecule in addition to the LOV domain. They contemplated using the LOV domain isolated from algae and gave the specific example of *Vaucheria frigida*, a stramenopile or heterokont alga.

[0017] Kinoshita and colleagues (WO2014142334) demonstrated that overexpression of phototropin had no impact of stomatal opening in higher plants.

[0018] Bonger and colleagues (US20140249295) used the LOV domain as a fusion with another functional protein wherein the light switching ability of the LOV domain was used to control the stability and/or function of the fusion protein.

[0019] Folta and colleagues (WO2014085626) using mutants of phototropin 1 were able to show that the function of phot1 is mediation of the pathway in which green light reverses the effects of red and/or blue light on plant growth.

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[0020] Schmidt & Boyden (US20130116165) describe a new group of fusion proteins with light regulatory regions derived from *Avena sativa* phototropin 1. These regulatory domains are used for altering channel function in membranes.

[0021] To our knowledge the patent literature focused on the use of PHOT regulatory and promoter sequences, especially the LOV domains, rather than using PHOT K/O technology to improve plant productivity.

Phototropin in Algae (*Chlamydomonas*):

[0022] Phototropin has already been well studied in several different algae including *Chlamydomonas reinhardtii* (Briggs and Olney 2001). However, there are indications that phototropin presence is not universal or that the genes that function as phototropin are not very homologous to known phototropin genes. For instance it was reported that in *Thalassiosira pseudonana* (a diatom) and *Cyanidioschyzon merolae* (unicellular red alga) no genes were found encoding the phototropins (Grossman 2005). However putative genes with photosensory regions have been reported for both these organisms (Table 1).

[0023] In *Chlamydomonas reinhardtii,* phototropin plays a vital role in progression of the sexual life cycle (Huang and Beck 2003), control of the eye spot size and light sensitivity (Trippens, Greiner et al. 2012). Phototropin is also involved in blue-light mediated changes in the synthesis of chlorophylls, carotenoids, chlorophyll binding proteins. Phototropin has been localized to the flagella of *Chlamydomonas reinhardtii* (Huang, Kunkel et al. 2004). Phototropin is also known to be involved in expression of genes encoding chlorophyll and carotenoid biosynthesis and LHC apoproteins in *Chlamydomonas reinhardtii* (Im, Eberhard et al. 2006). The *Chlamydomonas reinhardtii* phototropin gene has been cloned and shown to function when expressed in *Arabidopsis* (Onodera, Kong et al. 2005).

[0024] Phototropin has been shown to control multiple steps in the sexual life cycle of *Chlamydomonas reinhardtii* (Huang and Beck 2003). PHOT knockdowns using RNAi were generated (Huang and Beck 2003). The entire focus of this study was on sexual mating and no mention of improved biomass, starch accumulation or photosynthesis rate was observed. It is also involved in the chemotaxis that is the initial phase of the sexual cycle of *Chlamydomonas reinhardtii* (Ermilova, Zalutskaya et al. 2004). However, no cell cycle implications of phototropin knockout have been published.

[0025] Detailed studies have carefully analyzed the function of the LOV domain in several algal species. An example is the *Chlamydomonas reinhardtii* mutant LOV2-C250S where careful studies of the light activation and regulation of this domain were carried out to better understand the mechanism of action (Sethi, Prasad et al. 2009).

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[0026] *Phototropin knock-out mutants (PHOT K/O)* have been made previously in algae (Huang and Beck 2003, Trippens, Greiner et al. 2012). PHOT minus strains had larger eyespots than the parental strain (Trippens, Greiner et al. 2012). This study focused on the impact of PHOT on eyespot structure function. These authors use RNAi knockdown of PHOT to reduce expression of phototropin (Trippens, Greiner et al. 2012).

[0027] *Novel phototropins* have been described in the green alga *Ostrecoccus tauri* and with a focus on their LOV domain structure/function (Veetil, Mittal et al. 2011).

[0028] Abad and colleagues (WO2013056212) provide the sequence for phototropin from a green alga, *Auxenochlorella protothecoides*, and indicate that the gene would be important for photosynthetic efficiency. However, they do not discuss the impact of deletion or inhibition of these gene on the alga.

DEFINITIONS

[0029] Unless otherwise defined, all technical and scientific terms have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the exemplary embodiments, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned are incorporated by reference in their entirety. In case of conflict, the present specification and definitions will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting for the practice of this invention.

[0030] Unless specifically referred to in the specification singular forms such as “a,” “an,” and “the,” include their plural forms. As an example, “an alga” includes its plural form “algae” and “a plant” includes the plural “plants.”

[0031] The term “algae” will refer to all organisms commonly referred to as algae including the prokaryotic cyanophyta (commonly called blue-green algae and cyanobacteria), prochlorophyta, glaucophyta, rhodophyta, heterokontophyta, haptophyte, cryptophyta, dinophyta, euglenophyta, chloroaracniophyta, chlorophyta, and those organisms of indeterminate nomenclature normally referred to as algae. A full description of these is found in the book “*Algae An Introduction to Phycology*” by Van Den Hoek, Mann & Jahns (1995) which is included by reference.

[0032] The term "expression" as used herein refers to transcription and/or translation of a nucleotide sequence within a host cell. The level of expression of a desired product in a host cell may

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be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the desired polypeptide encoded by the selected sequence.

[0033] The term "homologous" refers to the relationship between two proteins that possess a "common evolutionary origin", including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) in the same species, as well as homologous proteins from different species.

[0034] As used herein, "identity" means the percentage of identical nucleotide or amino acid residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions.

[0035] The term "sequence similarity" refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin (Reeck, de Haen et al. 1987). However, in common usage and in the current invention, the term "homologous", when modified with an adverb such as "highly", may refer to sequence similarity and may or may not relate to a common evolutionary origin.

[0036] In specific embodiments, two nucleic acid sequences are "substantially homologous" or "substantially similar" when at least about 85%, and more preferably at least about 90% or at least about 95% of the nucleotides match over a defined length of the nucleic acid sequences, as determined by a sequence comparison algorithm such as BLAST, FASTA, DNA Strider, CLUSTAL, and *etc.* An example of such a sequence is an allelic or species variant of the specific phototropin gene of the present invention. Sequences that are substantially homologous may also be identified by hybridization, *e.g.*, in a Southern hybridization experiment under stringency conditions as defined for that particular system.

[0037] Similarly, in particular embodiments of the invention, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 90% of the amino acid residues are identical. Two sequences are functionally identical when greater than about 95% of the amino acid residues are similar. Preferably the similar or homologous polypeptide sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Version 7, Madison, Wis.) pileup program, or using any of the programs and algorithms described above.

[0038] The term “knockout” or “gene knockout” refers herein to any organism and/or its corresponding genome where the gene of interest has been rendered unable to perform its function. This can be accomplished by both classical mutagenesis, natural mutation, specific or random inactivation, targeting in cis or trans, or any method wherein the normal expression of a protein is altered to reduce its effect. For example but not to limit the definition 1) one can use chemical mutagenesis to damage the

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gene and then select for organisms not expressing the gene, 2) one can target the gene and remove a portion or all of the gene by homologous recombination, 3) one can use RNAi methods to produce an inhibitor molecule for a particular protein and similar methods and 4) one can use genome editing tools to specifically modify the gene.

[0039] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature (Sambrook, Fritsch et al. 1989, Ausubel, Brent et al. 1997, Green and Sambrook 2012).

[0040] The term "transcriptome" refers to the set of RNA molecules present in a population of cells. It often reflects how an organism responds to particular situations and is looking at what genes are regulated under a particular condition. Examples of transcriptome analyses on algae are found in the following references (Hwang, Jung et al. 2008, Rismani-Yazdi, Haznedaroglu et al. 2011, Fu, Wang et al. 2014, Koid, Liu et al. 2014).

[0041] The term "biofuel" any fuel made through the application of biological processes not on a geological timescale. Examples include but are not limited to conversion of algal biomass to biocrude through hydrothermal liquifaction, anaerobic digestion of spent algal biomass for conversion to methane, extraction of lipid from algal biomass to convert to biodiesel, and conversion of water to biohydrogen through biological processes.

[0042] The term "bioproduct" is any product produced from biological processes either in whole or in part.

[0043] The term "sink molecules" refers to molecules used by an organism to store captured carbon. These can be but are not limited to sugars, starch, glycogen, lipids, fats, waxes, and similar biomolecules.

[0044] The publications discussed above are provided solely for their disclosure before the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosures by virtue of prior invention.

SUMMARY OF THE INVENTION

[0045] This and other unmet needs of the prior art are met by exemplary compositions and methods as described in more detail below.

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[0046] Exemplary embodiments of the compositions, systems, and methods disclosed herein wherein algae are treated so as to reduce or eliminate the expression of phototropin or a heterologous gene with the same function such that improved productivity is achieved.

[0047] In one aspect, embodiments of the present invention provide an organism and the method to use such organism where the phototropin gene is knocked out and the photosynthetic rate is improved and the biomass productivity improves.

[0048] In a further aspect, the mutant is produced from *Chlamydomonas reinhardtii* and the biomass productivity is doubled.

[0049] Another embodiment of the present invention provides an organism with reduced phot expression wherein the sexual cycle is arrested and the stability of the algal cell culture line is improved.

[0050] In a further embodiment the organism is derived from *Chlamydomonas reinhardtii* and has reduced promiscuity resulting in a more stable phenotype and genotype.

[0051] In one aspect, embodiments of the present invention provide an organism with reduced phototropin gene expression and the method to use such organism which as improved non-photochemical quenching providing the ability for better response to high light levels.

[0052] In a further embodiment the organism is derived from *Chlamydomonas reinhardtii*.

[0053] In one aspect, embodiments of the present invention provide an organism with reduced phototropin expression and the method to use such organism that results in higher levels of sink molecules, such as but not limited to lipid and starch.

[0054] In a further embodiment the organism has enhanced cell division compared to wild-type.

[0055] In a further embodiment the organism is derived from *Chlamydomonas reinhardtii*.

[0056] Another embodiment provides a method for increasing the productivity of an algal strain wherein the expression or function of the gene of the *Chlamydomnas reinhardtii* phototropin gene or genes that are substantially similar to the *Chlamydomnas reinhardtii* phototropin gene is reduced or eliminated.

[0057] In a further embodiment the method the gene has greater than 75% homology to the *Chlamydomnas reinhardtii* phototropin gene.

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[0058] In a further embodiment the method the gene has greater than 80% homology to the *Chlamydomnas reinhardtii* phototropin gene.

[0059] In yet another embodiment the biomass productivity of the algal strain is increased by greater than around 2-fold.

[0060] In a further embodiment the production of storage products in the algal strain is increased by greater than around 2-fold.

[0061] In yet a further embodiment the storage product is selected from starch, lipid, pigments and other sink compounds.

[0062] In a further embodiment the productivity of biomass is increased by greater than around 2-fold.

[0063] In a further embodiment the productivity is increased of bioproducts chosen from lipids, waxes, polysaccharides (*e.g.*, starch, glycogen, mannans, glycans, cellulose, hemicellulose), pigments (*e.g.*, xanthophyll).

[0064] In another embodiment of the method wherein the expression of the *Chlamydomnas reinhardtii* phototropin gene is reduced by chemical mutagenesis and selection.

[0065] In another embodiment of the method wherein the expression of the *Chlamydomnas reinhardtii* phototropin gene is reduced by genome editing.

[0066] In another embodiment of the method wherein the expression of the *Chlamydomnas reinhardtii* phototropin gene is reduced by trans acting elements (e.g., RNAi).

[0067] Another embodiment is an algal strain wherein relative to the wild-type parental line

- the expression of the phototropin gene or substantially similar gene is reduced,
- the photosynthetic pigments making up the antennae complex are reduced,
- and the content of sink compounds is increased.

[0068] In a further embodiment of the algal line the phototropin gene or substantially similar gene been rendered to be non-functional.

[0069] In a further embodiment of the algal line the phototropin gene or substantially similar gene been substantially deleted.

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[0070] In a further embodiment of the algal line the phototropin gene or substantially similar gene can be rendered to be non-functional on an inducible basis through a inducible promoter.

[0071] In another embodiment a gene downstream of phot can be used as an alternative target.

[0072] In a further embodiment the gene downstream has substantial homology to the *Arabidopsis* KIN10 or KIN11 genes.

[0073] In yet a further embodiment the KIN10 and KIN11 genes are chosen from genes substantially homologous to SEQUENCES 15 to 34.

BRIEF DESCRIPTION OF THE DRAWINGS

[0074] A better understanding of the exemplary embodiments of the invention will be had when reference is made to the accompanying drawings, and wherein:

[0075] FIG. 1 – Comparison of chlorophyll a/b ratios and chlorophyll content of PHOT K/O lines (PHOT K/O line G5 and parent cw15) and (PHOT K/O line A4 and parent UVM4): (A) chlorophyll a/b ratios in low light, (B) chlorophyll a/b ratios in low light and high light, (C) chlorophyll content in low light grown cells of cw15 parent and G5 mutant, and (D) chlorophyll content in low light grown cells of UV4 parent and A4 mutant.

[0076] FIG. 2 - Carotenoid pigment comparison of low light (LL) and high light (HL) grown cultures of *Chlamydomonas reinhardtii* PHOT K/O lines compared to wild-type. LL= Low light, HL = high light, CW15 = Parent for G5 PHOT K/O line, UV4 = parent for A4 PHOT K/O line, Neo = neoxanthin, Lutin = lutein, Viola = violaxanthin, Anthera = antheraxanthin, and Zea = zeaxanthin.

[0077] FIG. 3 – Xanthophyll cycle carotenoid de-epoxidation in *Chlamydomonas reinhardtii* PHOT K/O (lines G5 and A4) and their corresponding parental lines (CW15 and UVM4) grown at low and high light intensities.

[0078] FIG. 4 - Chlorophyll fluorescence induction kinetics of low-light grown *Chlamydomonas reinhardtii* PHOT K/O lines and respective wild-type parental strains. Cultures were either dark adapted or pre-illuminated with 715 nm light (photosystem I (PSI) actinic light) prior to measurement. For Chl fluorescence induction measurements, Chl fluorescence was measured under continuous, non-saturating illumination every microsecond.

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[0079] FIG. 5 - Photosynthetic rate comparison of *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines under increasing light intensity. CW15 and UV4 are parental wild-type lines while G5 and A4 are the PHOT K/O lines.

[0080] FIG. 6 – KEGG pathway graphical data on photosynthetic electron transport chain related gene expression *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines. Color intensity indicates fold change in transcript abundance relative to parent line. Red indicates up-regulated and green down regulated.

[0081] FIG. 7 – Growth and biomass comparison of *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines in environmental photobioreactors from Phenometric (ePBRs).

[0082] FIG. 8 – KEGG pathway graphical data on carbon fixation related gene expression *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines. Color intensity indicates fold change in transcript abundance relative to parent line. Red indicates up-regulated and green down regulated.

[0083] FIG. 9 – Cell cycle pathway diagram. *NIMA (Never in mitosis), NEK2, NEK6 (NIMA related kinases),* Cyclin and CDK (Cyclin- dependent kinases), *RB* (retinoblastoma)*/mat3* (mating type-linked) genes are up-regulated in cell cycle pathway.

[0084] FIG. 10 – Starch synthesis pathway.

[0085] FIG. 11 - Thylakoid membrane structure and starch accumulation comparison of PHOT K/O line with parent line. Inserts are a magnification of the thylakoid grana stacks.

[0086] FIG. 12 - KEGG pathway graphical data on terpenoid synthesis related gene expression *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines. Pink indicates up-regulated genes relative to parent line.

[0087] FIG. 13 - KEGG pathway graphical data on fatty acid elongation related gene expression *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines. Color intensity indicates fold change in transcript abundance relative to parent line. Green color indicates down regulated genes.

[0088] FIG. 14 – Lipid synthesis metabolic pathway. FAD = Fatty acid desaturase; ACP = Acyl carrier protein; FAB = Fatty acid biosynthesis. Black color indicates up-regulated genes.

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DETAILED DESCRIPTION

[0089] While there have been numerous studies on algal phototropin (Huang and Beck 2003, Ermilova, Zalutskaya et al. 2004, Huang, Kunkel et al. 2004, Im, Eberhard et al. 2006, Sethi, Prasad et al. 2009, Veetil, Mittal et al. 2011, Trippens, Greiner et al. 2012) to date there has been no correlation of the reduction or knock-out of phototropin to higher levels of biomass production and increased production of sink products such as starch and lipid.

[0090] The transcriptome of a *Chlamydomonas reinhardtii* phototropin knock out (PHOT K/O) mutant and the wild-type parent was compared to analyze differences in gene expression in high light grown cultures (500 µmol photons m^{-2} s^{-1}). An up-regulation of genes involved in photosynthetic electron transport chain, carbon fixation pathway, starch, lipid, and cell cycle control genes was observed in the PHOT K/O mutants. With respect to photosynthetic electron transport genes, genes encoding proteins of the cytochrome b_6f and ATP synthase complex were up regulated potentially facilitating rate limitations in proton-coupled electron transfer. In addition genes involved in the rate limiting steps in the Calvin cycle, including ribulose 1,5 bisphosphatase (RuBP), sidoheptulose 1,7 bisphosphatase (SBPase), glyceraldehyde-3-phosphate dehydrogenase (3PGDH) and that mediate cell-cycle control (CDK), were also up regulated in the PHOT K/O mutants as well as the starch synthase and fatty acid biosynthesis genes involved in starch and lipid synthesis. In addition, transmission electron micrographs show increased accumulation of starch granules in PHOT K/O mutant compared to wild-type, which is consistent with the higher expression of starch synthase genes. Collectively, the altered patterns of gene expression in the PHOT K/O mutants were associated with a two-fold increase in growth and biomass accumulation compared to wild-type when grown in environmental photobioreactors (PBR101 from Phenometrics, Inc., Lansing, MI) that simulate a pond environment. These surprising results suggest that phototropin may be a master gene regulator that suppresses rapid cell growth and promotes gametogenesis and sexual recombination in wild-type strains. Therefore, down regulating expression or eliminating the phototropin genes (*e.g.*, PHOTO K/O mutants) provides a valuable means to increase productivity of algae that has commercial applications.

[0091] Using a variety of methods exemplary embodiments of the invention are directed at improving the productivity of algal systems based on control of the phototropin gene and genes similar to phototropin in algal systems. This is particularly applicable to improving biomass productivity in algal mass culturing either for production of algal biofuels or bioproducts.

[0092] Productivity is a central issue in algae production and a doubling of the productivity could be very attractive to groups who hope to cross the threshold of commercial viability. However, one should note that widespread adoption of transgenic algae as a production system is not yet embraced. Several companies (for example Algenol, Ft. Meyers, Florida) are using transgenic algae (cyanobacteria) in

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closed tube reactors outdoors and, presumably, have a track to (national) regulatory approval. Use of transgenic algae has been approved in Florida and approvals have recently been granted by the US EPA for GMO field trials for Sapphire.

[0093] Production of bioproducts using this invention, owing to the observed doubling of productivity in biomass and sink compounds, could be pivotal in reaching commercial viability. The observed increase in starch production by this invention is especially important as it shows sink compounds are enhanced by the methods of this invention.

[0094] Alternative genome editing technologies such as CRIPR/Cas 9, Talen and Zinc finger nuclease approaches could also be used to inhibit expression of phototropin (Gaj, Gersbach et al. 2013, Sizova, Greiner et al. 2013).

[0095] It is possible to make PHOT knockouts using non-GMO approaches such as classical mutagenesis using chemical mutagens such as methylnitronitroso guanidine and ethyl methane sulfonate (Yan, Aruga et al. 2000).

[0096] To date, supporting data for this invention have been limited to the green alga, *Chlamydomonas reinhardtii*. Compared to wild-type *C. reinhardtii* PHOT K/O mutants of the invention show:

1. Reduction in chlorophyll and carotenoid pigments.
2. Reduced light harvesting antenna size
3. 2-fold increase in photosynthesis rate
4. Increased expression of genes that control rate limiting steps in photosynthetic electron transfer and Calvin Cycle activity
5. 2-fold increase in growth and biomass
6. Increased starch accumulation
7. Increased accumulation of xanthophyll cycle pigments
8. Higher accumulation of starch grains
9. Increased expression of lipid synthesis genes
10. Increased expression of the chloroplast localized MEP terpenoid synthesis pathway but not the cytoplasmic MVA terpenoid synthesis pathway.
11. Increased expression of cell cycle control genes potentially accelerating rates of cell division

[0097] Additionally, PHOT K/O mutants were unable to undergo sexual mating, which was attributed to an impact of the PHOT K/O on the cell cycle – effectively pausing the cell cycle and transferring energy into some of the functions above (*e.g.*, photosynthesis and storage product generation).

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PHOT Knockout (K/O) mutants of *Chlamydomonas reinhardtii*

[0098] Chlamydomonas *reinhardtii* PHOT knockout lines were generated in different parental backgrounds. PHOT K/O line G5 was made in cw15 parental background and A4 mutant line was made in UV4 background (Zorin, Lu et al. 2009).

Pigment analysis of phototropin knock out lines

[0099] Chlorophyll (Chl) and carotenoids are the central pigments of the photosynthetic apparatus. These pigments are associated with light-harvesting complexes and reaction-center complexes in photosynthetic organisms. The light environment plays a major role in governing the pigment composition of pigment–protein complexes of the photosynthetic apparatus. Blue light is especially important in modulating the synthesis of Chl and carotenoids, as well as the biogenesis of the photosynthetic apparatus in microalgae and vascular plants. Consistent with phototropin regulation of pigment biosynthetic pathways *C. reinhardtii* PHOT K/O lines showed:

1. *Chlorophyll content*: Higher chlorophyll a/b (Chl a/b) ratios compared to their respective wild-types when grown under low light intensities. As shown in Figure 1A, the G5 mutant line has Chl a/b ratios of 2.8 and 3.1 in low and high light, respectively while its parent CW15 has a Chl a/b ratio of 2.2 in low light with no significant increase in high light. Similarly the mutant A4 line has Chl a/b ratios of 2.9 and 3.4 in low light and high light respectively, and its parent has a Chl a/b ratio of 2 in low light with no significant change in high light. Chl a/b ratios are also higher in PHOT K/O lines under high light grown cultures, which is consistent with a reduction in chlorophyll antenna size at high light. Figure 1 C and 1 D shows a 50-60% reduced chlorophyll content per gram dry weight in the PHOT mutants compared to parent wild-type.
2. *Carotenoid content:* When grown under low light intensities PHOT K/O lines showed a 30-40% reduction in carotenoid content compared to parent wild (FIG. 2). The changes in xanthophyll cycle pigments were analyzed since the xanthophyll cycle pigments play an important role as antioxidants and for non-photochemical quenching of excess energy captured by the light harvesting complex. Both PHOT K/O lines show higher accumulation of photoprotective pigments in high light compared to their respective WT parents. G5 PHOT accumulates 2.5 fold more lutein and 4.1 fold more zeaxanthin compared to the parental line and A4 PHOT K/O accumulates 2.8 lutein and 3.8 fold zeaxanthin as well as 2.8 fold antheraxanthin compared to its respective parent. These results are consistent with the better photosynthetic performance of these lines when grown in high light intensities.
3. *De-epoxidation rates:* Consistent with the xanthophyll cycle pigment accumulation PHOT K/O lines show higher De-epoxidation compared to their respective wild-type under high light (FIG. 3). These data are consistent with the better performance of PHOT K/O lines in high light intensities as they have more robust photoprotection mechanisms.

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Photosynthetic state transition analysis in parent and PHOT K/O lines:

[0100] In *C. reinhardtii*, the peripheral PSII antenna is able to migrate laterally between PSII and PSI, in a process known as state transitions, to balance the excitation energy distribution between the two photosystems and to regulate the ratio of linear and cyclic electron flows. Linear electron transfer produces ATP and NADPH, while cyclic electron transfer driven by PSI produces only ATP. Increasing the antenna size of the PSI complex facilitates cyclic electron transfer and has been shown to enhance ATP production and support the optimal growth of *Chlamydomonas*. To assess the impact of reduced pigment content on the ability to carry out state transitions, chlorophyll (Chl) fluorescence induction kinetics were measured in low-light grown parent wild-type and PHOT K/O cells, that were either dark adapted or pre-illuminated with PSI (715 nm) actinic light (FIG. 4). PSI actinic light pre-illumination promotes light harvesting complex II (LHCII) migration from PSI to PSII. An increase in the PSII antenna size would accelerate Chl fluorescence rise kinetics and increase the maximal Chl fluorescence level at sub-saturating light intensities. Wild-type strains (FIG. 4 Left panels) and PHOT K/O lines (FIG. 4 Right panels) all had faster Chl fluorescence rise kinetics and achieved greater maximum Chl fluorescence levels following pre-illumination with PSI light as compared to dark adapted cells consistent with robust state transitions.

Photosynthetic rates in WILD-TYPE and PHOT K/O lines:

[0101] The photosynthetic rates of the PHOT lines were determined under increasing light conditions and PHOT K/O lines show 2 fold higher photosynthetic rates compared to their respective parent strains (FIG. 5). Rate limiting genes in photosynthetic electron transport genes were up-regulated in high light grown cultures (FIG. 6). Up-regulation of these genes may play a role in higher photosynthetic efficiency of PHOT K/O mutants.

Photosynthetic electron transport pathway genes:

[0102] The transcriptomic analysis of the PHOT K/O mutants compared to wild-type parental strains provided information on the different genes impacted by the elimination of phototropin expression (FIG. 6). These data are reported in the KEGG (Kyoto Encyclopedia of Genes and Genome) pathway format (Kanehisa and Goto 2000, Kanehisa, Goto et al. 2014). Rate limiting genes in photosynthetic electron transport pathway were up-regulated in high light grown cultures. Up-regulation of these genes may play a role in higher photosynthetic efficiency of PHOT K/O mutants.

1. *PetC:* Is a nuclear gene encoding the Rieske protein of the cytochrome b_6/f (cyt b_6/f) complex. The cytochrome b_6f complex catalyzes the rate-limiting step in photosynthetic electron transport. Increases in its expression levels or stoichiometry relative to the PSI and PSII reaction centers would be predicted to increase rates of electron and proton transfer. A 2-fold increase on *petC* expression was observed for the PHOT K/O mutants.

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2. *AtpD:* Encodes the delta subunit for ATPase. A 3-fold increase on *AtpD* expression was observed for the PHOT K/O mutants.
3. *F type ATPase genes*: The delta and gamma subunits of the F type ATPase gene were evaluated. Increases in expression of the ATPase complex would facilitate proton flux, increase ATP synthesis and reduce feedback inhibition on proton coupled electron transfer by accelerating dissipation of the delta pH gradient across the thylakoid membrane. A 3-fold increase was observed for the PHOT K/O mutants.

Growth and biomass analysis in parent and PHOT K/O lines:

[0103] Most importantly, phototropin knock out lines, had twice the cell density and accumulated twice the biomass of their respective parental wild-type strain when approaching the stationary phase of growth (after 12 days) (FIG. 7). These results are consistent with higher photosynthetic rates in phototropin knock out lines also impact biomass yield of cells grown under conditions mimicking the pond simulating conditions (ePBRs). These results are in concert with up-regulation of the genes involved in carbon fixation and cell cycle as determined by transcriptomic analysis.

Carbon fixation pathway genes upregulated:

[0104] Carbon fixation is the main pathway for storing energy and accumulating biomass in algae and plants. Many rate limiting genes were up-regulated in PHOT K/O lines (FIG. 8). SBPase and RuBP are limiting enzymes in the Calvin Cycle and their overexpression would increase carbon flux through the carbon reduction pathways.

1. Ribulose 1,5 bisphosphatase (RuBP): A 3-fold increase was observed for the PHOT K/O mutants.
2. Sidoheptulose 1,7 bisphosphatase (SBPase): A 3-fold increase was observed for the PHOT K/O mutants.
3. Glyceraldehyde-3-phosphate dehydrogenase (3PGDH): A 2-fold increase was observed for the PHOT K/O mutants.

Cell cycle pathway genes up regulated:

[0105] Cell cycle genes are up regulated in *Chlamydomonas reinhardtii* PHOT K/O mutants may enhance cell division in these lines contributing to the higher biomass in these lines (FIG. 9).

1. NIMA (Never in mitosis), NEK2, NEK6 (NIMA related kinases): Cell cycle progression (G2/M progression) 15, 5 and 5 fold increase, respectively, was observed for the PHOT K/O mutants.
2. RCC1 (Regulator of chromosome condensation): A16 fold increase was observed for the PHOT K/O mutants.
3. Cyclin and cyclin-dependent kinases (CDK): Cyclin-dependent kinases are involved in overall regulation of cell cycle progression and demonstrated a 2-fold increase for the PHOT K/O mutants..

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4. A 3-fold increase in MAT3 a homolog of retinoblastoma protein (MAT3/RB) was observed for the PHOT K/O mutants: These genes regulate the cell cycle at two key points: 1.) early/mid G1 control point, and 2) the size checkpoint for the dividing cell.

Thylakoid membrane structure and starch accumulation in parent and PHOT K/O lines:

[0106] We compared the chloroplast ultrastructure of the parental and PHOT K/O cells to determine whether there were changes in thylakoid membrane structure and starch accumulation. Starch represents the most widespread storage polysaccharide found in the plastids of both photosynthetic and non-photosynthetic cells of plants and algae. PHOT K/O lines exhibited higher accumulation of starch grains compared to their respective parent strains as well as up-regulation of starch synthesis genes (discussed below).

Starch biosynthesis pathway genes upregulated in PHOT K/O lines:

[0107] *Chlamydomonas reinhardtii* PHOT K/O mutants have higher starch accumulation due to up-regulation of the following genes involved in starch biosynthesis (FIG. 10). These results were consistent with the observed increase in starch content in PHOT K/O chloroplasts by EM.

1. *AGPase:* ADP glucose pyrophosphorylase catalyzes the rate-limiting step and first-dedicated step for starch biosynthesis. A 2-fold increase was observed for the PHOT K/O mutants.
2. *Starch synthase 2, 3 and 4*: A 5-fold increase was observed for the PHOT K/O mutants.
3. *Starch branching enzyme:* A 3-fold increase was observed for the PHOT K/O mutants.

[0108] A structural hallmark of thylakoid membranes in plants and microalgae is the stacking of the membranes associated with the localization of the PSII complex. The stromal membranes extending from the stacks are enriched in PSI and ATPase complexes. This arrangement of LHCII complexes provides functional flexibility, enabling their primary light harvesting function as well as ability to participate in multilevel regulatory mechanisms involving highly efficient energy dissipation through pigment interactions such as chlorophyll-xanthophyll interactions. These regulatory processes require a significant reorganization in the membrane, and a substantial degree of structural flexibility in thylakoid membranes to carry out short-term adaptations and long-term acclimations in response to change in light and environmental stimuli.

[0109] The thylakoid membrane structure in both parent strains and PHOT K/O lines can be observed in FIG. 11. Thylakoid membrane organization is drastically altered in PHOT K/O lines. These results are in concert with the phototropin involvement in regulation of LHC protein biosynthesis and pigment biosynthesis. When thylakoid membranes are tightly stacked, they are densely packed with proteins and inhibit efficient protein diffusion including diffusions of the electron transport carrier protein plastocyanin. This protein mobility is required for efficient photosynthetic electron transfer, as well as

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regulation and repair of photodamaged photosynthetic apparatus. In parent cells thylakoid membranes are very tightly stacked giving very little space for the movement of the molecules (FIG. 11, insert on left panel). In contrast, PHOT K/O lines have parallel grana stacks and wide luminal spacing enhancing protein diffusion and associated electron transport (FIG. 11; insert on right panel).

Other important genes upregulated in transcriptomic analysis:

Lipid biosynthesis pathway genes:

[0110] The following genes involved in lipid metabolism are up regulated in PHOT K/O mutants (FIG. 13 & FIG. 14):

1. *Acyl carrier protein (ACP)* is an important component in both FA and polyketide biosynthesis with the growing chain bound during synthesis as a thiol ester. A 3-fold increase was observed for the PHOT K/O mutants.
2. ω-3 fatty acid desaturase (FAD) A 4-fold increase was observed for the PHOT K/O mutants.
3. Fatty acid biosynthesis (FAB). A 3-fold increase was observed for the PHOT K/O mutants.

Terpenoid biosynthesis pathway genes:

[0111] The methyl erythritol 4-phosphate (MEP) pathway is the source of isoprenoid precursors for the chloroplast. The precursors lead to the formation of various isoprenoids having diverse roles in different biological processes. Some isoprenoids have important commercial uses. Isoprene, which is made in surprising abundance by some trees, plays a significant role in atmospheric chemistry. Multiple genes involved in MEP/DOXP pathway were up regulated in PHOT K/O mutants 9FIG. 12). In contrast, the mevalonate terpenoid pathway (cytoplasmic) genes were not up regulated in PHOT K/O mutants.

[0112] Note that all data so far were generated in cell wall free mutants of *Chlamydomonas reinhardtii*. Metabolomic analyses in *C. reinhardtii* clarified the pathways and gene up-regulation in high light in *C. reinhardtii* PHOT K/O mutants of this invention:

Heterologous Algal Phototropin Genes

[0113] The *Chlamydomonas reinhardtii* phototropin gene has already been sequenced and a provisional version is available publically (GenBank 5718965). Additional algal genes are available that have either been shown to be a phototropin, contain blue light receptors, have some homology to phototropin or are putative blue light receptors similar to phototropin (Table 1). Additional phototropin genes in two other production strains of microalgae.

[0114] *Chlorella* sp. Strain1412. Is a strain developed by the NAABB consortium and is housed at UTEX. The amino acid sequence is provided as SEQUENCE NO. 1 and the nucleotide sequence as SEQUENCE NO. 2. The phototropin B gene of Chlorella sp. Strain 1412 is provide as SEQUENCE NO. 3 and nucleotide as SEQUENCE NO. 4.

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[0115] *Chlorella* sp. Strain 1230. Is a UTEX strain. The amino acid sequence of phototropin A is provided as SEQUENCE NO. 5 and the nucleotide sequence as SEQUENCE NO. 6. The amino acid sequence of phototropin B is provided as SEQUENCE NO. 7 and the nucleotide sequence as SEQUENCE NO. 8.

[0116] *Chlorella* sp. Strain 1228. Is tentatively identified as *Chlorella sorokiniana*. The amino acid sequence of phototropin A is provided as SEQUENCE NO. 9and the nucleotide sequence as SEQUENCE NO. 10. The amino acid sequence of phototropin B is provided as SEQUENCE NO. 11 and the nucleotide sequence as SEQUENCE NO. 12.

[0117] *Picochlorum* sp. Strain DOE101. The amino acid sequence is provided as SEQUENCE NO. 13 and the nucleotide sequence as SEQUENCE NO. 14.

Table 1 - List of publically available sequences that may be phototropins or heterologous to phototropin genes.

GenBank #	Alga	Description	Aliases
9688782	*Micromonas pusila* CCMP1545	Phototropin, blue light receptor	MICPUCDRAFT_49739
9617508	*Volvox carteri f. nagariensis*	Phototropin	VOLCADRAFT_127319
23616146	*Auxenochlorella protothecoides*	Phototropin 2	F751_4755
23614975	*Auxenochlorella protothecoides*	Phototropin-1B	F751_3584
19011210	*Bathycoccus prasinos*	Phototropin	Bathy16g02310
9831018	*Ostrecoccus tauri*	Putative blue light receptor	Ot16g02900
8249220	*Micromonas sp, RCC299*	Blue light receptor	MICPUN_105003
16998047	*Cyanidioschyzon merolae* 10D	Serine/threonine kinase	MICPUT_105003
17089759	*Galdieria sulphuraria*	Serine/threonine kinase	Gasu_15820
17087623	*Galdieria sulphuraria*	Serine/threonine kinase	Gasu_38210
17041755	*Coccomyxa subellipsoidea* C-169	Putative blue light receptor	COCSUDRAFT_63287
17350696	*Chlorella variabilis*	Hypothetical protein	CHLNCDRAFT_141214
5005771	*Ostreococcus lucimarinus* CCE9901	Hypothetical protein	OSTLU_40751
17304390	*Guillarida theta* CCMP2712	Hypothetical protein	GUITHDRAFT_162563
7452793	*Thalassiosira pseudonana*	Hypothetical protein	THAPSDRAFT_33193

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	CCMP1355		
7442442	*Thalassiosira pseudonana* CCMP1355	Hypothetical protein, PAS domain	THAPSDRAFT_261631
7200921	*Phaeodactylum tricornutum* CCAP 1055/1	Hypothetical protein; one PAS domain	PHATRDRAFT_51933

Alternative Targets

[0118] Additional PHOT downstream signal transduction targets can be use as alternatives to the knockout or reduction in phot expression to generate the desirable phenotypes of this invention, including but not limited to improved photosynthetic efficiency, higher biomass productivity, increase yield of sink compounds, and improved genetic stability. An example of this could be the algal gene homologous to the *Arabidopsis* KIN10 and KIN11 kinases (Baena-Gonzalez, Rolland et al. 2007). Genes substantially homologous to the *Chlorella* genes in SEQUENCES 15 to 27 and the *Chlamydomonas* genes in SEQUENCES 28-34 would be applicable to this current invention.

EXAMPLES

[0119] Certain embodiments of the invention will be described in more detail through the following examples. The examples are intended solely to aid in more fully describing selected embodiments of the invention, and should not be considered to limit the scope of the invention in any way.

Example 1 – Growth of *Chlamydomonas reinhardtii*

[0120] *Chlamydomonas reinhardtii* parental strains (cw15 and UV4) and the phototropin knockout (PHOT K/O) mutants (CW15 and A4) were grown at 25°C in 250 mL Erlenmeyer flasks containing 100 mL of High-Salt (HS) or Tris-Acetate-Phosphate (TAP) media and shaken at 150 rpm (http://www.chlamy.org/media.html). Cultures were typically inoculated from a log phase culture using 1 mL of cells. Flasks were illuminated using fluorescent light at the light intensities as indicated for each experiment.

Example 2 – Measurement of photoautotrophic growth and biomass estimation

[0121] Photoautotrophic growth of the parent strains CW15 and UV4) and the phototropin knock out mutants (G5 and A4) was measured in environmental photobioreactors (ePBRs; Phenometrics) in 500 mL of liquid HS media. All experiments were done in triplicates for each time point and each treatment. Light intensity was programmed for a 12 h sinusoidal light period with a peak mid-day intensity of 2,000 µmol photons $m^{-2} s^{-1}$. Temperature was a constant 25°C, and the ePBRs were stirred with a magnetic stir bar at 200 rpm. Filtered air was bubbled constantly through the growing cultures. The optical

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density of the cultures was monitored on a daily basis at 750 nm using a Cary 300 Bio UV–Vis spectrophotometer (Agilent). After completion of growth measurements, the total contents of individual ePBRs were harvested by centrifugation at 11,000 rpm for 15 min. Cell pellets were frozen immediately in liquid N_2 and later freeze-dried using a Microprocessor Controlled Lyophilizer (Flexi-Dry). After drying, pellets were weighed for total biomass.

Example 3 – Measurement of chlorophyll fluorescence

[0122] For Chl fluorescence induction analysis, cell suspensions of the parental wild-type and transgenic *Chlamydomonas* strains were adjusted to a Chl concentration of ~ 2.5 µg/mL. Quenching of Chl fluorescence was measured using the FL-3500 fluorometer (Photon System Instruments) (Kaftan, Meszaros et al. 1999). The cells were dark adapted for 10 min prior to the measurement. Chl fluorescence was induced using non-saturating continuous illumination and Chl fluorescence levels were measured every 1 µs using a weak pulse-modulated measuring flash. For the state transition experiments, low light grown cultures were dark adapted or pre-illuminated with 715 nm light for 10 min prior to the induction of Chl fluorescence. The actinic flash duration for this experiment was set to 50 µs and Chl fluorescence was measured every 1 µs.

Example 4 - Measurement of photosynthetic oxygen evolution –

[0123] CO_2-supported rates of oxygen evolution were determined for low light (50 µmol photons $m^{-2} s^{-1}$) HS grown log-phase cultures (0.4–0.6 $OD_{750\ nm}$) using a Clark-type oxygen electrode (Hansatech Instruments). Cells were re-suspended in 20 mM HEPES buffer (pH 7.4) and air-saturated rates of oxygen evolution were measured as a function of light intensity (650 nm) at 50, 150, 300, 450, 600, 750 and 850 µmol photons $m^{-2} s^{-1}$. The same experiment was repeated in the presence of 10 mM $NaHCO_3$. Light saturation curves were normalized on the basis of Chl as well as cell density ($A_{750\ nm}$). Chl was determined by method described by Arnon (Arnon 1949).

Example 5 – Measurement of pigment content by HPLC

[0124] *Chlamydomonas* cultures were grown at low (50 µmol photons $m^{-2} s^{-1}$) and high (saturating) light (500 µmol photons $m^{-2} s^{-1}$) intensities for 5 days in HS media in shaker flasks. Cells were centrifuged at 3,000 rpm for 3 min and immediately frozen in liquid nitrogen and lyophilized. Carotenoids and chlorophylls were extracted with 100% acetone in the dark for 20 min. After incubation samples were centrifuged at 14,000 rpm for 2 min in a microfuge and the supernatant was transferred to a glass tube and dried under vacuum. The dried samples were re-suspended in 1 mL of acetonitrile:water:triethylamine (900:99:1, v/v/v) for HPLC analysis. Pigment separation and chromatographic analysis were performed on a Beckman HPLC equipped with a UV–Vis detector, using a C18 reverse phase column at a flow rate of 1.5 ml/min. Mobile phases were (A) acetonitrile/H_2O/triethylamine (900:99:1, v/v/v) and (B) ethyl acetate. Pigment detection was carried out at

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445 nm with reference at 550 nm (Tian and DellaPenna 2001). Individual algal pigments were identified on the basis of their retention times and optical absorbance properties and quantified on the basis of their integrated absorbance peaks relative to known carotenoid standards. Carotenoid standards were purchased from DHI, Denmark. Pigments were standardized on the basis of dry weight of three replicates.

[0125] Example 7 – Transmission electron microscopy

[0126] Cells were prepared for electron microscopy by immobilizing cells in 3% sodium alginate (w/v) and the alginate beads were then solidified by incubation in cold 30mM CaCl2 for 30min. We used alginate encapsulated algal cells to keep cells intact as well as to protect from direct and harmful effect of chemicals during fixation processes. These cells were fixed using 2% glutaraldehyde for 1.5-2 hours and after fixation, these cells were post fixed in buffered 2% osmium tetroxide for 1.5 hours. After dehydration these cells were embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate. LEO 912 transmission electron microscope was used to view and collect images at 120kv and a Proscan digital camera.

Example 8 – Transcriptome analysis

[0127] Total RNA was extracted from 100 mg of cells/sample, flash frozen in liquid nitrogen, grown at high light (500 µmol photons m^{-2} s^{-1}) intensities for 5 days in HS media in shaker flasks) using the Direct-zol RNA-miniprep kit (ZYMO, P/N 2051) according to the manufacturer's instructions. Each total RNA sample was enriched for mRNA by hybridizing the poly(A) tail to oligo d(T)25 probes covalently coupled to magnetic beads, followed by elution (NEB, P/N S1419S). The enriched mRNA fractions were prepared for Illumina sequencing using the ScriptSeq V.2 RNA-seq Library Preparation Kit (Epicentre, P/N SSV21106) and sequenced on a Hi-Seq 2000 (2 X 150bp), multiplexed at 6 samples per lane. The resultant sequence reads were trimmed for quality and mapped to the coding sequences present in version 9 of the *Chlamydomonas reinhardtii* genome annotation (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Creinhardtii) using bowtie2. The relative transcript abundance of each gene (mean of 3 biological samples) was determined using RSEM and differential expression values (UV4 vs A4) were calculated using EdgeR. All genes identified as differentially expressed were mapped to KEGG biochemical maps using the v.9 annotation assignments.

Example 9- Identification of *Chlorella* spp. Phototropin coding sequence

[0128] Phototropin genes were identified in three *Chlorella* species (herein designated as strain 1412, strain 1228 and *Chlorella sorokiniana* UTEX1230) and a *Picochlorum* sp. DOE101 by conducting homologous BLASTp searches against the annotations of *Chlorella* species using *Chlamydomonas reinhardtii* phototropin genes/proteins (NP_851210) and *Arabidopsis thaliana* protein sequences (Accession # AED97002.1 and AEE78073) as query proteins. The *Chlorella* spp. and *Picochlorum*

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phototropin homologs were aligned to other phototropin amino acid sequences using CLUSTALW, then truncated based on conserved sequence alignments and phylogenetically analyzed using a Maximum-Likelihood algorithm. Each *Chlorella* strain contains two paralogous copies of photropin and *Picochlorum sp.* DOE101 was found to contain 1 homolog of phototropin. These sequences are provided as SEQUENCE Nos. 1-14.

Example 10 – Inducible control of phototropin expression in *Chlamydomonas reinhardtii*

[0129] One method to reduce expression of algal PHOT gene(s) is to use RNAi technology driving the expression of double stranded, fold-back RNA elements to reduce the PHOT expression. A strong gene promoter such as *psa*D or other strong constitutive gene promoters could be used to drive expression of the RNAi construct similar to methods used previously in *Chlamydmonas* for modulation of light harvesting antennae complex (Perrine, Negi et al. 2012).

Example 11 – Production of a *Chlorella* phototropin minus mutant

[0130] PHOT gene knockouts could be potentially generated by tradition mutagenesis approaches including chemical, UV, random insertional mutagenesis screened by TILLING (Comai, Young et al. 2004, Nieto, Piron et al. 2007), and by targeted knock outs using CRISPR/cas9 (Wang, Yang et al. 2013, Xiao, Wang et al. 2013, Dubrow 2014). Pooled PHOT-based PCR screening coupled with sequencing of PHOT PCR products could be used to screen for PHOT mutants.

Example 12 – Chemical mutagenesis for production of a phototropin K/O mutant in *Chlorella protothecoides*

[0131] Classical chemical mutagenesis is carried out using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). This mutagen makes nucleotide changes in the DNA and these changes, depending on their position, can have effects that are either positive or negative in the use of the strain being treated. By careful observation of phenotypes produced, as well as implementation of selective pressure, one selects mutants with improved traits for the desired purpose. This method has been applied to algae previously (Yan, Aruga et al. 2000).

[0132] Identifying strains of algae that grow rapidly and produce high starch is used as a selection marker for PHOT K/O mutants. Because this approach does not involve adding foreign DNA (in fact is focused only on existing genetic potential of the strain being mutagenized), strains generated by chemical mutagenesis are not considered to be "genetically modified", allowing deployment in the field without additional government regulation.

[0133] N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was chosen based on its proven use for modifying blue-green algae, as well as its ability to eliminate toxicity by degradation in dilute acid. First, the conditions required to result in approximately 99% lethality for *Chlorella protothecoides* are

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determined; this degree of lethality generated optimal mutation frequency in blue-green algae (Chapman and Meeks 1987). Two treatments, exposure to 0.25 mg/mL MNNG for 30 minutes and 0.025 mg/mL MNNG for 60 minutes, result in approximately 99% lethality for this strain (unpublished data). Both treatments are used to generate mutagenized populations of *Chlorella* using enrichment strategies.

[0134] Approximately 10^8 cells are mutagenized with four concentrations of MNNG and incubated for three different durations. After rinsing out the mutagen, approximately 10^4 cells are spread plated on nutrient plates, and the number of colonies scored after 12 days. Treatments with approximately 100 surviving colonies, representing 99% lethality, are chosen as optimal for generating mutations.

[0135] PHOT K/O mutants are expected to be more rapidly growing and to produce excess sink material. In *C. protothecoides* the sink is lipid which could be used as a screen for selection of cells representing high lipid cells. Numerous methods are in the literature for such selection such as Nile red (Pick and Rachutin-Zalogin 2012) and BODIPY 493/503 (Ohsaki, Shinohara et al. 2010). High lipid cells are selected by flow cytometry and then placed in flask for cell culture. Rapid growing high lipid cells will dominate the culture and should be PHOT K/O as determined in this invention.

Example 13 – Genome editing using CRISPR/cas9 to reduce expression of phototropin in *Chlamydomonas reinhardtii.*

[0136] Recently, it has been demonstrated that CRISPR/cas9 genome editing techniques can be used to knock out genes of interest in Chlamydomonas when the Cas9 gene is expressed constitutively. By incorporating multiple guide RNA elements to specifically recognize the PHOT gene high high efficiencies of gene mutagenesis can occur during miss-repair of the double stranded break in the target gene catalyzed by Cas/9 by the endogenous repair enzymes. By targeting repair of a recognized restriction endonuclease site, inhibition of the digestion of the PHOT-specific PCR product by the diagnostic restriction endonuclease can be used as an effective screen for PHOT mutants. Similarly, DNA repair mistakes that occur following double stranded DNA breaks in the PHOT gene generated by TALEN complexes can be used to generate PHOT-specific mutants.

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IMPROVED PRODUCTIVITY AND BIOPRODUCT FORMATION IN PHOTOTROPIN KNOCK/OUT MUTANTS IN MICROALGAE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and benefit of the filing of U.S. Provisional Patent Application No. 62/171,176 entitled "Improved Productivity and Bioproduct Formation in Phototropin Knock/out Mutants in Microalgae" filed on June 4. 2015, and the specification and claims thereof are incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under grants Nos. Prime Contract No. DE-AC52-06NA25396 and NMC subcontract No. 277529. The U.S. government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on June 6, 2016, is named PHOT_PCT_Sequences_060316_ST25.txt and is 317 Kbytes in size.

TECHNICAL FIELD

[0004] Disclosed embodiments of the present invention are in the field of improved performance of microalgae in the production of biological products such as but not limited to biofuels, biomass, pigments, starch, oils and the like through selection, mutagenesis or engineering to reduce expression or knockout the phototropin gene for example.

BACKGROUND

[0005] Phototropin is a blue light receptor, which mediates a variety of blue-light elicited physiological processes in plants and algae. In higher plants these processes include phototropism, chloroplast movement and stomatal opening. In the unicellular green alga *Chlamydomonas reinhardtii,* phototropin (PHOT) plays a vital role in the progression of the sexual life cycle and in the control of the eye spot size and light sensitivity. Phototropin is also involved in blue-light mediated changes in the

synthesis of chlorophylls, carotenoids, and chlorophyll binding proteins. The UV-A/blue light sensing phototropins mediate a variety of light responses and are responsible in higher plants for optimization of photosynthetic yields (Chen, Chory et al. 2004).

[0006] Phototropins are commonly composed of two domains, an amine terminal photosensory domain and a carboxy terminal serine/threonine protein kinase domain. The photosensory domain is a flavin mononucleotide binding domain, the LOV domain. Plants and green algae contain two of these domains in the phototropin regulatory sequence, LOV1 and LOV2 (Chen, Chory et al. 2004).

[0007] Phototropin knock-out mutants (PHOT K/O) have been made previously in plants (Suetsugu and Wada 2007, Moni, Lee et al. 2015) and algae (Zorin, Lu et al. 2009; Trippens, Greiner et al. 2012). However, all the PHOT K/O mutant prior art that has been located to date did not show improved productivity of the plant or alga.

[0008] In plants two phototropins have been reported, phot1 and phot2, these phototropins share sequence homology and have overlapping functions. These blue-light-sensitive receptors consist of two parts: a C-terminal serine-threonine kinase and two LOV domains that bind flavin mononucleotide as chromophores at the N-terminus. Recently, in the unicellular green alga, *Chlamydomonas reinhardtii*, a phototropin homolog was identified. It exhibits photochemical properties similar to those of higher plant phototropins and is also functional in *Arabidopsis*. Studies show that the basic mechanism of phototropin action is highly conserved, even though its apparent physiological functions are quite diverse.

Phototropin in higher plants:

[0009] Plants utilize several families of photoreceptors to better react to their environment, allowing them to fine tune pathways controlled by the photoreceptors - phototropin, phytochrome, and cryptochrome (Chen, Chory et al. 2004).

[0010] In higher plants phototropin mediates a variety of blue-light elicited physiological processes (Sullivan, Thomson et al. 2008). Phototropins are UV-A/blue light sensing photoreceptors that are known to optimize photosynthetic yields (Chen, Chory et al. 2004). The involvement of phototropin in photomovement in higher plants is well documented (Suetsugu and Wada 2007, Kagawa, Kimura et al. 2009). Studies involving *Arabidopsis* mutants lacking the phot1 and phot2 genes have revealed that in addition to regulating hypocotyl curvature of seedlings towards blue light, phototropins also regulate a diverse range of responses in flowering plants. These responses include chloroplast movements, nuclear positioning, stomatal opening, leaf expansion, leaf movements and leaf photomorphogenesis.

[0011] *Phototropin knock-out mutants (PHOT K/O)* have been made previously in plants (Suetsugu and Wada 2007, Moni, Lee et al. 2015). For instance in *Physcomitrella patens* (a moss) there are three PHOT genes and they have all been knocked out in different mutants (Suetsugu and Wada 2007). The focus of the *P. patens* study was the effect of PHOT K/O on phototropism (movement toward light) and the phenotypes they observed allowed them to determine which of the genes were necessary for phototropism (Suetsugu and Wada 2007).

[0012] PHOT expression was higher in darkness than in light, and phot1 *Arabidopsis* mutants was shown to increase the number of lateral roots produced (Moni, Lee et al. 2015). phot was also demonstrated to mediate phototropism, chloroplast relocation and leaf expansion (Matsuoka, Iwata et al. 2007). Using phot deficient *Arabidopsis* mutants, phototropin 2 was linked to palisade parenchyma cell development of leaves (Kozuka, Kong et al. 2011).

[0013] Another study looked at the role of phototropin under low photosynthetically active radiation (Takemiya, Inoue et al. 2005). They found that the wild-type and the PHOT1 mutant both showed increased but similar growth in low radiance blue light super imposed on red light. In white light there was no increase in biomass in both phot1 and phot2 mutants as well as in the double phot mutant.

[0014] A study by Folta and colleagues investigated the relationship between phot1 and phototropism and growth inhibition in *Arabidopsis* (Folta, Lieg et al. 2003). They found that the onset of phototropism and the phot1-mediated growth inhibition coincided and postulated that both were due to phot1 expression.

[0015] There is a substantial amount of patent literature around phototropin in higher plants. However, the focus has been on the commercial utility of the upstream, light regulated areas rather than on the phototropin gene itself. These light control domains that regulate PHOT expression -- the light-oxygen-voltage-sensing (LOV) domains - have been carefully evaluated for potential commercial application in higher plants.

[0016] Shu & Tsien application (US20130330718) focused on using the LOV domain for control of proteins that generate singlet oxygen (SOGs). These fusion protein tags could be used for imaging under blue light for research purposes.

[0017] Other patents use light switchable regulatory sequences and contemplate the use of the phototropin LOV domain such as Yang and colleagues (EP2682469).

[0018] Hahn & Karginov (WO2011133493) focused on allosteric regulation of kinases using the light activated domains for control of expression in engineered fusion proteins (such as the LOV domains).

[0019] Hahn and colleagues (US8859232) demonstrated that the LOV domain of phototropin can be used as a light activated switch for the activation or inactivation of fusion proteins of interest. They contemplated using a LOV domain that could contain substantial portions of the phototropin molecule in addition to the LOV domain. They contemplated using the LOV domain isolated from algae and gave the specific example of *Vaucheria frigida*, a stramenopile or heterokont alga.

[0020] Kinoshita and colleagues (WO2014142334) demonstrated that overexpression of phototropin had no impact of stomatal opening in higher plants.

[0021] Bonger and colleagues (US20140249295) used the LOV domain as a fusion with another functional protein wherein the light switching ability of the LOV domain was used to control the stability and/or function of the fusion protein.

[0022] Folta and colleagues (WO2014085626) using mutants of phototropin 1 were able to show that the function of phot1 is mediation of the pathway in which green light reverses the effects of red and/or blue light on plant growth.

[0023] Schmidt & Boyden (US20130116165) describe a new group of fusion proteins with light regulatory regions derived from *Avena sativa* phototropin 1. These regulatory domains are used for altering channel function in membranes.

[0024] To date there is no disclosure of the use of PHOT knockout or knockdown (suppression) technology to improve or algae plant productivity.

Phototropin in Algae:

[0025] Phototropin has already been well studied in several different algae including *Chlamydomonas reinhardtii* (Briggs and Olney 2001). However, there are indications that phototropins have diverged significantly or that the genes that function as phototropin are not very homologous to plant phototropin genes. For instance it was reported that in *Thalassiosira pseudonana* (a diatom) and *Cyanidioschyzon merolae* (unicellular red alga) no genes were found encoding the phototropins (Grossman 2005). However putative genes with photosensory LOV domains, aurechromes, have been reported for these and other photosynthetic stramenopiles (Table 1). Most aureochromes contain a

single LOV domain and function as transcription factors that regulate cell division, chloroplast movement, pigment production, and phototropism. (Takahashi. J Plant Res (2016) 129:189–197)

[0026] In *Chlamydomonas reinhardtii,* phototropin plays a vital role in progression of the sexual life cycle (Huang and Beck 2003), control of the eye spot size and light sensitivity (Trippens, Greiner et al. 2012). Phototropin is also involved in blue-light mediated changes in the synthesis of chlorophylls, carotenoids, chlorophyll binding proteins. Phototropin has been localized to the flagella of *Chlamydomonas reinhardtii* (Huang, Kunkel et al. 2004). Phototropin is also known to be involved in expression of genes encoding chlorophyll and carotenoid biosynthesis and LHC apoproteins in *Chlamydomonas reinhardtii* (Im, Eberhard et al. 2006). The *Chlamydomonas reinhardtii* phototropin gene has been cloned and shown to function when expressed in *Arabidopsis* (Onodera, Kong et al. 2005).

[0027] Phototropin has been shown to control multiple steps in the sexual life cycle of *Chlamydomonas reinhardtii* (Huang and Beck 2003). PHOT knockdowns using RNAi were generated (Huang and Beck 2003). The entire focus of this study was on sexual mating and no mention of improved biomass, starch accumulation or photosynthesis rate was observed. It is also involved in the chemotaxis that is the initial phase of the sexual cycle of *Chlamydomonas reinhardtii* (Ermilova, Zalutskaya et al. 2004). However, no cell cycle implications of phototropin knockout or knockdowns have been published.

[0028] Detailed studies have carefully analyzed the function of the LOV domain in several algal species. An example is the *Chlamydomonas reinhardtii* mutant LOV2-C250S where careful studies of the light activation and regulation of this domain were carried out to better understand the mechanism of action (Sethi, Prasad et al. 2009).

[0029] *Phototropin knock-out mutants (PHOT K/O)* have been made previously in algae (Zorin, Lu et al. 2009 Trippens, Greiner et al. 2012). PHOT minus strains had larger eyespots than the parental strain (Trippens, Greiner et al. 2012). This study focused on the impact of PHOT on eyespot structure function. These authors used a knock-out mutant of PHOT to reduce expression of phototropin (Trippens, Greiner et al. 2012).

[0030] *Novel phototropins* have been described in the green alga *Ostreococcus tauri* and with a focus on their LOV domain structure/function (Veetil, Mittal et al. 2011).

[0031] Abad and colleagues (WO2013056212) provide the sequence for phototropin from a green alga, *Auxenochlorella protothecoides*, and indicate that the gene would be important for photosynthetic efficiency. However, they do not discuss the impact of deletion or inhibition of this gene on the alga.

DEFINITIONS

[0032] Unless otherwise defined, all technical and scientific terms have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the exemplary embodiments, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned are incorporated by reference in their entirety. In case of conflict, the present specification and definitions will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting for the practice of this invention.

[0033] Unless specifically referred to in the specification singular forms such as "a," "an," and "the," include their plural forms. As an example, "an alga" includes its plural form "algae" and "a plant" includes the plural "plants."

[0034] The term "algae" will refer to all organisms commonly referred to as algae including the prokaryotic cyanophyta (commonly called blue-green algae and cyanobacteria), prochlorophyta, glaucophyta, rhodophyta, heterokontophyta, haptophyte, cryptophyta, dinophyta, euglenophyta, chloroaracniophyta, chlorophyta, and those organisms of indeterminate nomenclature normally referred to as algae. A full description of these is found in the book "*Algae An Introduction to Phycology*" by Van Den Hoek, Mann & Jahns (1995), which is included by reference.

[0035] The term "expression" as used herein refers to transcription and/or translation of a nucleotide sequence within a host cell. The level of expression of a desired product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the desired polypeptide encoded by the selected sequence.

[0036] The term "overexpression" as used herein refers to excessive expression of a gene product (RNA or protein) in greater-than-normal amounts.

[0037] The term "homologous" refers to the relationship between two proteins that possess a "common evolutionary origin", including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) in the same species, as well as homologous proteins from different species.

[0038] As used herein, "identity" means the percentage of identical nucleotide or amino acid residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions.

[0039] The term "sequence similarity" refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin (Reeck, de Haen et al. 1987). However, in common usage and in the current invention, the term "homologous", when modified with an adverb such as "highly", may refer to sequence similarity and may or may not relate to a common evolutionary origin.

[0040] In specific embodiments, two nucleic acid sequences are "substantially homologous" or "substantially similar" when at least about 75%, and more preferably at least 80%, and more preferably at least 85%, and more preferably at least about 90% or at least about 95% of the nucleotides (or any integer value in between) match over a defined length of the nucleic acid sequences, as determined by a sequence comparison algorithm such as BLAST, CLUSTAL, MUSCLE, *etc.* An example of such a sequence is an allelic or species variant of the specific phototropin gene of the present invention. Sequences that are substantially homologous may also be identified by hybridization, *e.g.*, in a Southern hybridization experiment under stringency conditions as defined for that particular system. The homology may be as high as about 93-95%, 98%, or 99% (or any integer value in between). For example, the sequence to which homology is matched is a wild-type parental line and the length of the sequence is the full length of the sequence from wild-type parental line.

[0041] Similarly, in particular embodiments of the invention, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 75% of the amino acid residues are identical wherein identical contemplates a conservative substitution at a nucleic acid position. In a preferred embodiment there is at least 80%, and more preferably at least 85%, and more preferably at least about 90% and more preferably at least about 90-95% of the amino acid residues are identical (or any integer value in between). Two sequences are functionally identical when greater than about 95% of the amino acid residues are similar. Preferably the similar or homologous polypeptide sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Version 7, Madison, Wis.) pileup program, or using any of the programs and algorithms described above. Conservative amino acid substitutions are among: acidic (negatively charged) amino acids such as aspartic acid and glutamic acid; basic (positively charged) amino acids such as arginine, histidine, and lysine; neutral polar amino acids such as glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; neutral nonpolar (hydrophobic) amino acids such as alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; amino acids having aliphatic side chains such as glycine, alanine, valine, leucine, and isoleucine; amino acids having aliphatic-hydroxyl side chains such as serine and threonine; amino acids having amide-containing side chains such as asparagine and glutamine; amino acids having aromatic side chains such as phenylalanine, tyrosine, and tryptophan; amino acids having basic side chains such as lysine, arginine, and histidine; amino acids having sulfur-containing side chains such as cysteine and methionine; naturally conservative amino acids

such as valine-leucine, valine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, aspartic acid-glutamic acid, and asparagine-glutamine. A further aspect of the homologs encoded by DNA useful in the transgenic plants or algae of the invention are those proteins that differ from a disclosed protein as the result of deletion or insertion of one or more amino acids in a native sequence.

[0042] The term "knockout" or "gene knockout" refers herein to any organism and/or its corresponding genome where the gene of interest has been rendered unable to perform its function. This can be accomplished by both classical mutagenesis, natural mutation, specific or random inactivation, targeting in cis or trans, or any method wherein the normal expression of a protein is altered to reduce its effect. For example but not to limit the definition 1) one can use chemical mutagenesis to damage the gene and then select for organisms not expressing the gene, 2) one can target the gene and remove a portion or all of the gene by homologous recombination, 3) one can use RNAi methods to produce an inhibitor molecule for a particular protein and similar methods and 4) one can use genome editing tools (i.e. CRISPR-Cas) to specifically modify the gene.

[0043] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature (Sambrook, Fritsch et al. 1989, Ausubel, Brent et al. 1997, Green and Sambrook 2012).

[0044] The term "transcriptome" refers to the set of RNA molecules present in a population of cells. It often reflects how an organism responds to particular situations and is looking at what genes are regulated under a particular condition. Examples of transcriptome analyses on algae are found in the following references (Hwang, Jung et al. 2008, Rismani-Yazdi, Haznedaroglu et al. 2011, Fu, Wang et al. 2014, Koid, Liu et al. 2014).

[0045] The term "biofuel" refers to any fuel made through the application of biological processes not on a geological timescale. Examples include but are not limited to conversion of algal biomass to biocrude through hydrothermal liquefaction, anaerobic digestion of spent algal biomass for conversion to methane, extraction of lipid from algal biomass to convert to biodiesel, and conversion of water to biohydrogen through biological processes.

[0046] The term "bioproduct" is any product produced from biological processes either in whole or in part.

[0047] The term biomass productivity or production as used herein refers to to the rate of generation of **biomass** in an ecosystem. It is usually expressed in units of mass per unit surface (or volume) per unit time, for instance grams per square metre per day (g m^{-2} d^{-1}). The mass unit may relate to biologically produced dry matter generated.

[0048] The term "sink molecules", "sink compounds", sink materials" refers to molecules used by an organism to store captured carbon. These can be but are not limited to sugars, starch, glycogen, lipids, fats, waxes, and similar biomolecules.

[0049] The publications discussed above are provided solely for their disclosure before the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosures by virtue of prior invention.

SUMMARY OF THE INVENTION

[0050] This and other unmet needs of the prior art are met by exemplary compositions and methods as described in more detail below.

[0051] One embodiment of the present invention provides for a method for increasing a biomass productivity of an algal strain wherein the expression or function of a *Chlamydomonas reinhardtii* phototropin gene, a gene substantially similar to the *Chlamydomonas reinhardtii* phototropin gene or a sequence substantially similar to SEQ ID NO 1-14 and 51-66 is reduced or eliminated. In a preferred embodiment the gene substantially similar has greater than 75% homology, more preferably greater than 80% homology to the *Chlamydomonas reinhardtii* phototropin gene or the sequence identified in SEQ ID NO 1-14 and 51-66.

[0052] For example, the biomass productivity of the algal strain is increased by greater than around 2-fold. The biomass production of storage product(s) in the algal strain is increased by greater than around 2-fold, for example the storage product(s) is selected from starch, lipid, pigments and other sink molecules and for example the productivity of biomass is increased by greater than around 2-fold. Further, the biomass productivity may be increased for bioproducts chosen from lipids, waxes, polysaccharides (*e.g.*, starch, glycogen, mannans, glycans, cellulose, hemicellulose), pigments (*e.g.*, xanthophyll). In a preferred embodiment the expression of the *Chlamydomonas reinhardtii* phototropin gene, the gene substantially similar to the *Chlamydomonas reinhardtii* phototropin gene or the sequence substantially similar to SEQ ID NO 1-14 and 51-66 is reduced by example chemical mutagenesis and selection, genome editing, trans acting elements (e.g., RNAi), and/or an inducible basis through an inducible promoter.

[0053] Another embodiment of the present invention provides for an algal strain wherein relative to the wild-type parental line the expression of the phototropin gene or a substantially similar gene is reduced, the photosynthetic pigments making up the antenna complex are reduced, and/or the content of sink molecules is increased. In a preferred embodiment the phototropin gene or a substantially similar gene been rendered to be non-functional. In a preferred embodiment the non-functional gene has been substantially deleted or is rendered to be non-functional on an inducible basis through an inducible promoter. In a preferred embodiment the algal line having the phototropin gene deletion would generate sterile and stable diploid population of polyploid algae to avoid recombination of genetic material during sexual reproduction or in another embodiment would be used to generate stable transgene-stacking traits in polyploid algal strains. In a preferred embodiment the photogropin gene or a substantially similar gene is selected from SEQ ID NO 1-14 and 51-66. In another preferred embodiment the gene the gene substantially similar has greater than 75% homology, or more preferably greater than 80% homology to the *Chlamydomonas reinhardtii* phototropin gene or the sequence identified in SEQ ID NO 1-14 and 51-66.

[0054] In another embodiment a method for increasing a biomass productivity of an algal strain wherein the expression or function of a *Chlamydomonas reinhardtii* NTR2 or NTRC gene, a gene substantially similar to a *Arabidopsis* NTR2 or NTRC gene or a sequence substantially similar to SEQ ID NO 35-50 and 67-68 is over expressed in the algal strain is provided. In a preferred embodiment the gene substantially similar has greater than 75% homology, or preferrably greater than 80% homology to the *Arabidopsis* NTR2 or NTRC gene or the sequence identified in SEQ ID NO 35-50 and 67-68.

[0055] For example, the biomass productivity of the algal strain is increased by greater than around 2-fold. The biomass production of storage product(s) in the algal strain is increased by greater than around 2-fold, for example the storage product(s) is selected from starch, lipid, pigments and other sink molecules and for example the productivity of biomass is increased by greater than around 2-fold. Further, the biomass productivity may be increased for bioproducts chosen from lipids, waxes, polysaccharides (*e.g.*, starch, glycogen, mannans, glycans, cellulose, hemicellulose), pigments (*e.g.*, xanthophyll).

[0056] In yet another embodiment a method for increasing a productivity of an algal strain wherein the expression or function of a *Chlamydomonas reinhardtii* KIN10 or KIN11 gene, a gene substantially similar to a *Arabidopsis* KIN10 or KIN11 gene or a sequence substantially similar to SEQ ID NO 15-34 is over expressed in the algal strain is provided. In a preferred embodiment the gene substantially similar has greater than 75% homology, or preferrably greater than 80% homology to the *Arabidopsis* KIN10 or KIN11 gene or the sequence identified in SEQ ID NO 15-34.For example, the biomass productivity of the algal strain is increased by greater than around 2-fold. The biomass

production of storage product(s) in the algal strain is increased by greater than around 2-fold, for example the storage product(s) is selected from starch, lipid, pigments and other sink molecules and for example the productivity of biomass is increased by greater than around 2-fold. Further, the biomass productivity may be increased for bioproducts chosen from lipids, waxes, polysaccharides (*e.g.*, starch, glycogen, mannans, glycans, cellulose, hemicellulose), pigments (*e.g.*, xanthophyll).

[0057] Exemplary embodiments of the compositions, systems, and methods disclosed herein wherein algae are treated so as to reduce or eliminate the expression of phototropin or a heterologous gene with the same function such that improved productivity is achieved.

[0058] In one aspect, embodiments of the present invention provide an organism and the method to use such organism where the phototropin gene is knocked out and the photosynthetic rate is improved and the biomass productivity improves.

[0059] In a further aspect, the mutant is produced from *Chlamydomonas reinhardtii* and the biomass productivity is doubled.

[0060] Another embodiment of the present invention provides an organism with reduced PHOT expression wherein the sexual cycle is arrested and the genetic stability of the algal cell culture line is improved.

[0061] In a further embodiment the organism is derived from *Chlamydomonas reinhardtii* and has reduced promiscuity resulting in a more stable genotype and phenotype.

[0062] In one aspect, embodiments of the present invention provide an organism with reduced phototropin gene expression and the method to use such organism which as improved non-photochemical quenching providing the ability for better response to high light levels.

[0063] In one aspect, embodiments of the present invention provide an organism with reduced phototropin expression and the method to use such organism that results in higher levels of sink molecules, such as but not limited to lipid and starch.

[0064] In a further embodiment the organism has enhanced cell division compared to wild-type.

[0065] In a further embodiment the organism is derived from *Chlamydomonas reinhardtii*.

[0066] In another embodiment of the method wherein the expression of the *Chlamydomonas reinhardtii* phototropin gene is reduced by genome editing (i.e. CRISPR/Cas).

[0067] In another embodiment of the method wherein the expression of the *Chlamydomonas reinhardtii* phototropin gene is reduced by trans acting elements (e.g., RNAi).

[0068] In a further embodiment the gene downstream of PHOT has substantial homology to the *Arabidopsis* KIN10 or KIN11 genes or a portion thereof (Snf1 related kinases, SNRK) and can be overexpressed to increase the productivity of an algal strain.

[0069] In yet a further embodiment the KIN10 and KIN11 genes or a portion thereof are chosen from genes substantially homologous to a nucleic acid sequence identified in SEQ ID NO 15 to 34 or a nucleic acid sequence encoding for an amino acid sequence identified in SEQ ID NO15 to 34.

[0070] In a further embodiment the gene downstream of phot has substantial homology to the *Arabidopsis* NTRC and NTR2 gene(s) or a portion thereof and can be overexpressed to increase the productivity of an algal strain.

[0071] In yet a further embodiment the NTRC and NTR2 genes or a portion thereof are chosen from genes substantially homologous to a nucleic acid sequence identified in SEQ ID NO 35 to 50 or a nucleic acid sequence encoding for an amino acid sequence selected in SEQ ID NO 35 to 50.

BRIEF DESCRIPTION OF THE DRAWINGS

[0072] A better understanding of the exemplary embodiments of the invention will be had when reference is made to the accompanying drawings, and wherein:

[0073] FIG. A-D Comparison of chlorophyll a/b ratios and chlorophyll content of PHOT K/O lines (PHOT K/O line G5 and parent cw15) and (PHOT K/O line A4 and parent UVM4): (A) chlorophyll a/b ratios in low light, (B) chlorophyll a/b ratios in low light and high light, (C) chlorophyll content in low light grown cells of cw15 parent and G5 mutant, and (D) chlorophyll content in low light grown cells of UV4 parent and A4 mutant.

[0074] FIG. 2 A-D - Carotenoid pigment comparison of low light (LL) and high light (HL) grown cultures of *Chlamydomonas reinhardtii* PHOT K/O lines compared to wild-type. LL= Low light, HL = high light, CW15 = Parent for G5 PHOT K/O line, UV4 = parent for A4 PHOT K/O line, Neo = neoxanthin, Lutein = lutein, Viola = violaxanthin, Anthera = antheraxanthin, and Zea = zeaxanthin.

[0075] FIG. 3 A-B– Xanthophyll cycle carotenoid de-epoxidation in *Chlamydomonas reinhardtii* PHOT K/O (lines G5 and A4) and their corresponding parental lines (CW15 and UVM4) grown at low and high light intensities.

[0076] FIG. 4 A-D- Chlorophyll fluorescence induction kinetics of low-light grown *Chlamydomonas reinhardtii* PHOT K/O lines and respective wild-type parental strains. Cultures were either dark adapted or pre-illuminated with 715 nm light (photosystem I (PSI) actinic light) prior to measurement. For Chl fluorescence induction measurements, Chl fluorescence was measured under continuous, non-saturating illumination every microsecond.

[0077] FIG. 5 A-B- Photosynthetic rate comparison of *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines under increasing light intensity. CW15 and UV4 are parental wild-type lines while G5 and A4 are the PHOT K/O lines.

[0078] FIG. 6 – KEGG pathway graphical data on photosynthetic electron transport chain related gene expression *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines. Star indicates fold change in transcript abundance relative to parent line..

[0079] FIG. 7 A-D– Growth and biomass comparison of *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines in environmental photobioreactors from Phenometric (ePBRs).

[0080] FIG. 8 – KEGG pathway graphical data on carbon fixation related gene expression *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines. Hatched lineand/or star indicates fold change in transcript abundance relative to parent line.

[0081] FIG. 9 – Cell cycle pathway diagram. *NIMA (Never in mitosis), NEK2, NEK6 (NIMA related kinases),* Cyclin and CDK (Cyclin- dependent kinases), *RB* (retinoblastoma)*/mat3* (mating type-linked) genes are up-regulated in cell cycle pathway.

[0082] FIG. 10 – Starch synthesis pathway.

[0083] FIG. 11 - Thylakoid membrane structure and starch accumulation comparison of PHOT K/O line with parent line. Inserts are a magnification of the thylakoid grana stacks.

[0084] FIG. 12 - KEGG pathway graphical data on terpenoid synthesis related gene expression *Chlamydomonas reinhardtii* PHOT K/O lines and parent lines. Star indicates up-regulated genes relative to parent line.

DETAILED DESCRIPTION

[0085] While there have been numerous studies on algal phototropin (Huang and Beck 2003, Ermilova, Zalutskaya et al. 2004, Huang, Kunkel et al. 2004, Im, Eberhard et al. 2006, Sethi, Prasad et al. 2009, Veetil, Mittal et al. 2011, Trippens, Greiner et al. 2012) to date there has been no correlation of the reduction or knock-out of phototropin to higher levels of biomass production and increased production of sink molecules/products such as starch and lipid.

[0086] The transcriptome of a *Chlamydomonas reinhardtii* phototropin knock out (PHOT K/O) mutant and the wild-type parent were compared to analyze differences in gene expression in high light grown cultures (500 µmol photons m^{-2} s^{-1}). An up-regulation of genes involved in photosynthetic electron transport chain, carbon fixation pathway, starch, lipid, and cell cycle control genes was observed in the PHOT K/O mutants. Referring now to FIG. 6, with respect to photosynthetic electron transport genes, genes encoding proteins of the cytochrome b_6f and ATP synthase complex were up regulated potentially facilitating rate limitations in proton-coupled electron transfer. In addition genes involved in the rate limiting steps in the Calvin cycle, including Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), sidoheptulose 1,7 bisphosphatase (SBPase), glyceraldehyde-3-phosphate dehydrogenase (3PGDH) and that mediate cell-cycle control (CDK), were also up regulated in the PHOT K/O mutants as well as the starch synthase and fatty acid biosynthesis genes involved in starch and lipid synthesis. In addition, transmission electron micrographs show increased accumulation of starch granules in PHOT K/O mutant compared to wild-type, which is consistent with the higher expression of starch synthase genes. Collectively, the altered patterns of gene expression in the PHOT K/O mutants were associated with a two-fold increase in growth and biomass accumulation compared to wild-type when grown in environmental photobioreactors (PBR101 from Phenometrics, Inc., Lansing, MI) that simulate a pond environment as evidence of increase productivity of algae. These surprising results suggest that phototropin may be a master gene regulator that suppresses rapid cell growth and promotes gametogenesis and sexual recombination in wild-type strains. Therefore, down regulating expression or eliminating the phototropin genes (*e.g.*, PHOTO K/O mutants) provides a valuable means to increase productivity of algae that has commercial applications.

[0087] Using a variety of methods exemplary embodiments of the invention are directed at improving the productivity of algal systems based on control of the phototropin gene and genes similar to phototropin in algal systems. This is particularly applicable to improving biomass productivity in algal mass culturing either for production of algal biofuels or bioproducts.

[0088] Productivity is a central issue in algae production and a doubling of the productivity could be very attractive to groups who hope to cross the threshold of commercial viability. However, one should

note that widespread adoption of transgenic algae as a production system is not yet embraced. Several companies (for example Algenol, Ft. Meyers, Florida) are using transgenic algae (cyanobacteria) in closed tube reactors outdoors and, presumably, have a track to (national) regulatory approval. Use of transgenic algae has been approved in Florida and approvals have recently been granted by the US EPA for GMO field trials for Sapphire Energy Company.

[0089] Production of bioproducts using this invention, owing to the observed doubling of productivity in biomass and sink molecules/compounds, could be pivotal in reaching commercial viability. The observed increase in starch production by this invention is especially important as it shows sink molecules/compounds are enhanced by the methods of this invention.

[0090] Alternative genome editing technologies such as CRISPR/Cas 9, Talen and Zinc finger nuclease approaches could also be used to inhibit expression of phototropin (Gaj, Gersbach et al. 2013, Sizova, Greiner et al. 2013).

[0091] It is possible to make PHOT knockouts using non-GMO approaches such as classical mutagenesis using chemical mutagens such as methylnitronitroso guanidine and ethyl methane sulfonate (Yan, Aruga et al. 2000).

[0092] To date, supporting data for this invention have been limited to the green alga, *Chlamydomonas reinhardtii*. Compared to wild-type *C. reinhardtii,* PHOT K/O mutants of the invention show:

1. Reduction in chlorophyll and carotenoid pigments (see FIG 1).
2. Reduced light harvesting antenna size (see FIG 1).
3. 2-fold increase in photosynthesis rate (see in FIG 5).
4. Increased expression of genes that control rate limiting steps in photosynthetic electron transfer and Calvin Cycle activity (see FIG 6 and FIG 8).
5. 2-fold increase in growth and biomass (see in FIG 7.)
6. Increased expression of starch synthesis genes (see in FIG 10.)
7. Increased accumulation of xanthophyll cycle pigments (see in FIG 12).
8. Higher accumulation of starch grains (see in FIG 11).
9. Increased expression of the chloroplast localized MEP terpenoid synthesis pathway but not the cytoplasmic MVA terpenoid synthesis pathway (see in FIG 12)
10. Increased expression of cell cycle control genes potentially accelerating rates of cell division (see in FIG 9).
11. Increased expression of glycolysis pathway genes.
12. Increased expression of Kin10/Kin11 (SNRK) genes.

13. Increased expression of NTR2 and NTRC genes.

[0093] Additionally, PHOT K/O mutants were unable to undergo sexual mating, which was attributed to an impact of the PHOT K/O on the cell cycle – effectively blocking meiosis while accelerating photosynthetic and cell division rates.

PHOT Knockout (K/O) mutants of *Chlamydomonas reinhardtii*

[0094] Chlamydomonas *reinhardtii* PHOT knockout lines were generated in different parental backgrounds. PHOT K/O line G5 was made in cw15 parental background and A4 mutant line was made in UV4 background (Zorin, Lu et al. 2009).

Pigment analysis of phototropin knock out lines

[0095] Chlorophyll (Chl) and carotenoids are the central pigments of the photosynthetic apparatus. These pigments are associated with light-harvesting complexes and reaction-center complexes in photosynthetic organisms. The light environment plays a major role in governing the pigment composition of pigment–protein complexes of the photosynthetic apparatus. Blue light is especially important in modulating the synthesis of Chl and carotenoids, as well as the biogenesis of the photosynthetic apparatus in microalgae and vascular plants. Consistent with phototropin regulation of pigment biosynthetic pathways *C. reinhardtii* PHOT K/O lines showed:
Chlorophyll content: Higher chlorophyll a/b (Chl a/b) ratios compared to their respective wild-types when grown under low light intensities. As shown in Figure 1A and 1B , the G5 mutant line has Chl a/b ratios of 2.8 and 3.1 in low and high light, respectively while its parent CW15 has a Chl a/b ratio of 2.2 in low light with no significant increase in high light. Similarly the mutant A4 line has Chl a/b ratios of 2.9 and 3.4 in low light and high light respectively, and its parent has a Chl a/b ratio of 2 in low light with no significant change in high light. Chl a/b ratios are also higher in PHOT K/O lines under high light grown cultures, which is consistent with a reduction in chlorophyll antenna size at high light. Figure 1 C and 1 D shows a 50-60% reduced chlorophyll content per gram dry weight in the PHOT mutants compared to parent wild-type.

[0096] *Carotenoid content:* When grown under low light intensities PHOT K/O lines showed a 30-40% reduction in carotenoid content compared to parent wild . The changes in xanthophyll cycle pigments were analyzed since the xanthophyll cycle pigments play an important role as antioxidants and for non-photochemical quenching of excess energy captured by the light harvesting complex. Both PHOT K/O lines show higher accumulation of photoprotective pigments in high light compared to their respective WT parents. Referring now to FIG 2B, G5 PHOT accumulates 2.5 fold more lutein and 4.1 fold more zeaxanthin compared to the parental line as shown in FIG 2A. Referring now to FIG 2D,A4 PHOT K/O accumulates 2.8 lutein and 3.8 fold zeaxanthin as well as 2.8 fold antheraxanthin compared to its

respective parent as shown in FIG 2C. These results are consistent with the better photosynthetic performance of these lines when grown in high light intensities.

[0097] *De-epoxidation rates:* Consistent with the xanthophyll cycle pigment accumulation PHOT K/O lines show higher De-epoxidation in high light conditions as compared to their respective wild-type under high light (FIG. 3 A-B). These data are consistent with the better performance of PHOT K/O lines in high light intensities as they have more robust photoprotection mechanisms.

Photosynthetic state transition analysis in parent and PHOT K/O lines:

[0098] In *C. reinhardtii*, the peripheral PSII antenna is able to migrate laterally between PSII and PSI, in a process known as state transitions, to balance the excitation energy distribution between the two photosystems and to regulate the ratio of linear and cyclic electron flows. Linear electron transfer produces ATP and NADPH, while cyclic electron transfer driven by PSI produces only ATP. Increasing the antenna size of the PSI complex facilitates cyclic electron transfer and has been shown to enhance ATP production and support the optimal growth of *Chlamydomonas*. To assess the impact of reduced pigment content on the ability to carry out state transitions, chlorophyll (Chl) fluorescence induction kinetics were measured in low-light grown parent wild-type (FIG 4 A and C) and PHOT K/O cells (FIG B and D), that were either dark adapted (sold line) or pre-illuminated with PSI (715 nm) actinic light (broken line). PSI actinic light pre-illumination promotes light harvesting complex II (LHCII) migration from PSI to PSII. An increase in the PSII antenna size would accelerate Chl fluorescence rise kinetics and increase the maximal Chl fluorescence level at sub-saturating light intensities. Wild-type strains (FIG. 4 A and C) and PHOT K/O lines (FIG. 4 B and D) all had faster Chl fluorescence rise kinetics and achieved greater maximum Chl fluorescence levels following pre-illumination with PSI light as compared to dark adapted cells consistent with robust state transitions.

Photosynthetic rates in WILD-TYPE and PHOT K/O lines:

[0099] Referring now to FIG. 5 A_and FIG. 5 B, the photosynthetic rates of the PHOT lines were determined under increasing light conditions and PHOT K/O lines (open boxes) show 2 fold higher photosynthetic rates compared to their respective parent strains (filled circles). Rate limiting genes in photosynthetic electron transport genes were up-regulated in high light grown cultures (FIG. 6). Up-regulation of these genes may play a role in higher photosynthetic efficiency of PHOT K/O mutants.

Photosynthetic electron transport pathway genes:

[0100] The transcriptomic analysis of the PHOT K/O mutants compared to wild-type parental strains provided information on the different genes impacted by the elimination of phototropin expression (FIG. 6). These data are reported in the KEGG (Kyoto Encyclopedia of Genes and Genome) pathway format (Kanehisa and Goto 2000, Kanehisa, Goto et al. 2014) found on the world wide web at

genome.jp/kegg/mapper.html last visited May 25, 2016. Rate limiting genes in photosynthetic electron transport pathway were up-regulated in high light grown cultures. Up-regulation of these genes may play a role in higher photosynthetic efficiency of PHOT K/O mutants.

1. *PetC:* Is a nuclear gene encoding the Rieske protein of the cytochrome b_6/f (cyt b_6/f) complex. The cytochrome b_6f complex catalyzes the rate-limiting step in photosynthetic electron transport. Increases in its expression levels or stoichiometry relative to the PSI and PSII reaction centers would be predicted to increase rates of electron and proton transfer. A 2-fold increase on *petC* expression was observed for the PHOT K/O mutants (see FIG 6).

AtpD: Encodes the delta subunit for ATPase. A 3-fold increase on *AtpD* expression was observed for the PHOT K/O mutants (see FIG 6).

F type ATPase genes: The delta and gamma subunits of the F type ATPase gene were evaluated. Increases in expression of the ATPase complex would facilitate proton flux, increase ATP synthesis and reduce feedback inhibition on proton coupled electron transfer by accelerating dissipation of the delta pH gradient across the thylakoid membrane. A 3-fold increase was observed for the PHOT K/O mutants (see FIG 6).

PGRL1: Is an important gene for efficient cyclic electron flow. A 2.2 fold increase was observed for PHOT K/O mutants

PGR7: Is a gene necessary for efficient photosynthetic electron transport. A 6.4 fold increase was observed for PHOT K/O mutants.

Growth and biomass analysis in parent and PHOT K/O lines:

[0101] Most importantly, phototropin knock out lines (open boxes), had twice the cell density (FIG. 7A and 7C) and accumulated twice the biomass (FIG. 7B and 7D) of their respective parental wild-type strain (solid boxes) when approaching the stationary phase of growth (after 12 days) (FIG. 7). These results are consistent with higher photosynthetic rates in phototropin knock out lines also impact biomass yield of cells grown under conditions mimicking the pond simulating conditions (ePBRs). These results are in concert with up-regulation of the genes involved in carbon fixation and cell cycle as determined by transcriptomic analysis.

Carbon fixation pathway genes upregulated:

[0102] Carbon fixation is the main pathway for storing energy and accumulating biomass in algae and plants. Many rate limiting genes were up-regulated in PHOT K/O lines (FIG. 8). SBPase and RuBisCO are limiting enzymes in the Calvin Cycle and their overexpression would increase carbon flux through the carbon reduction pathways. Carbonic anhydrase (CA), an enzyme active in the interconversion of bicarbonate and CO_2 facilitating CO_2 fixation.

1. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) A 3-fold increase was observed for the PHOT K/O mutants (see FIG 8).

2. Sidoheptulose 1,7 bisphosphatase (SBPase): A 3-fold increase was observed for the PHOT K/O mutants (see FIG 8).
3. Glyceraldehyde-3-phosphate dehydrogenase (3PGDH): A 2-fold increase was observed for the PHOT K/O mutants (see FIG 8).
4. α carbonic anhydrases: A 2.6 to 5 fold increase was observed for the PHOT K/O mutants.
5. β carbonic anhydrases: A 8 fold to 6 fold increase was observed for the PHOT K/O mutants.

Thioredoxin reductase genes are up-regulated in PHOT K/O lines:

[0103] Thioredoxins are small ubiquitous redox proteins, which are crucial components of the regulatory redox networks in all living cells. Thioredoxins are reduced by different reductases , depending on their subcellular localization. Among these reductases, NADPH-dependent thioredoxin reductases (NTR) genes are known to regulate multiple gene targets involved in photosynthesis, non-photochemical quenching (NPQ), Calvin-Benson cycle, starch biosynthesis, cold stress tolerance and thermotolerance.

1. NADPH-dependent thioredoxin reductase C (NTRC): A 2.4 fold increase was observed for the PHOT K/O mutants
2. NADPH-dependent thioredoxin reductase 2 (NTR2): A 4 fold increase was observed for the PHOT K/O mutants

Key growth regulatory genes are up-regulated in PHOT K/O lines:

[0104] KIN10 or KIN11 ((Snf1 related kinases, SNRK) are one of the very well-studied central regulators of energy and stress metabolism in plants. SNRK1 proteins play central roles in coordinating energy balance and nutrient metabolism in plants. A 10-fold increase was observed for the PHOT K/O mutants.

Cell cycle pathway genes up regulated:

[0105] Cell cycle genes are up regulated in *Chlamydomonas reinhardtii* PHOT K/O mutants may enhance cell division in these lines contributing to the higher biomass in these lines (FIG. 9).

1. NIMA (Never in mitosis), NEK2, NEK6 (NIMA related kinases): Cell cycle progression (G2/M progression) 15, 5 and 5 fold increase, respectively, was observed for the PHOT K/O mutants.

RCC1 (Regulator of chromosome condensation): A16 fold increase was observed for the PHOT K/O mutants.Cyclin and cyclin-dependent kinases (CDK): Cyclin-dependent kinases are involved in overall regulation of cell cycle progression and demonstrated a 2-fold increase for the PHOT K/O mutants.
A 3-fold increase in MAT3 a homolog of retinoblastoma protein (MAT3/RB) was observed for the PHOT K/O mutants: These genes regulate the cell cycle at two key points: 1.) early/mid G1 control point, and 2) the size checkpoint for the dividing cell.

Glycolysis pathway genes are up-regulated in PHOT K/O lines:

[0106] Glycolysis is the first step in the breakdown of glucose to extract energy for cellular metabolism, which converts glucose to pyruvate and generates ATP (energy) and NADH (reducing power). Many important genes of this pathway show higher expression in PHOT K/O mutants.

1. Hexokinase: A 3.4 fold increase was observed for the PHOT K/O mutants.
2. Glyceraldehyde phosphate dehydrogenase: A 6 fold increase was observed for the PHOT K/O mutants
3. Fructose – bisphosphate Aldolase: A 4 fold increase was observed for the PHOT K/O mutants.
4. Pyruvate Kinase: A 16 fold increase was observed for the PHOT K/O mutants.

Thylakoid membrane structure and starch accumulation in parent and PHOT K/O lines:

[0107] We compared the chloroplast ultrastructure of the parental and PHOT K/O cells to determine whether there were changes in thylakoid membrane structure and starch accumulation. Starch represents the most widespread storage polysaccharide found in the plastids of both photosynthetic and non-photosynthetic cells of plants and algae. PHOT K/O lines exhibited higher accumulation of starch grains compared to their respective parent strains as well as up-regulation of starch synthesis genes (FIG. 10 and 11) (discussed below).

Starch biosynthesis pathway genes upregulated in PHOT K/O lines:

[0108] *Chlamydomonas reinhardtii* PHOT K/O mutants have higher starch accumulation due to up-regulation of the following genes involved in starch biosynthesis is FIG. 10. These results were consistent with the observed increase in starch content in PHOT K/O chloroplasts by EM.

1. *AGPase:* ADP glucose pyrophosphorylase catalyzes the rate-limiting step and first-dedicated step for starch biosynthesis. A 2-fold increase was observed for the PHOT K/O mutants.
2. *Starch synthase 2, 3 and 4*: A 5-fold increase was observed for the PHOT K/O mutants.
3. *Starch branching enzyme:* A 3-fold increase was observed for the PHOT K/O mutants.

[0109] A structural hallmark of thylakoid membranes in plants and microalgae is the stacking of the membranes associated with the localization of the PSII complex. The stromal membranes extending from the stacks are enriched in PSI and ATPase complexes. This arrangement of LHCII complexes provides functional flexibility, enabling their primary light harvesting function as well as ability to participate in multilevel regulatory mechanisms involving highly efficient energy dissipation through pigment interactions such as chlorophyll-xanthophyll interactions. These regulatory processes require a significant reorganization in the membrane, and a substantial degree of structural flexibility in thylakoid membranes to carry out short-term adaptations and long-term acclimations in response to change in light and environmental stimuli.

[0110] An electron micrograph illustration showing the thylakoid membrane structure in both parent strain and PHOT K/O line is drastically altered in PHOT K/O lines. These results are in concert with the phototropin involvement in regulation of LHC protein biosynthesis and pigment biosynthesis. When thylakoid membranes are tightly stacked, they are densely packed with proteins and inhibit efficient protein diffusion including diffusions of the electron transport carrier protein plastocyanin. This protein mobility is required for efficient photosynthetic electron transfer, as well as regulation and repair of photodamaged photosynthetic apparatus. In parent cells thylakoid membranes are very tightly stacked giving very little space for the movement of the molecules). In contrast, PHOT K/O lines have parallel grana stacks and wide luminal spacing

Other important genes upregulated in transcriptomic analysis:

Lipid biosynthesis pathway genes:

[0111] The following genes involved in lipid metabolism are up regulated in PHOT K/O mutants:

1. *Acyl carrier protein (ACP)* is an important component in both FA and polyketide biosynthesis with the growing chain bound during synthesis as a thiol ester. A 3-fold increase was observed for the PHOT K/O mutants.

ω-3 fatty acid desaturase (FAD) A 4-fold increase was observed for the PHOT K/O mutants.
Fatty acid biosynthesis (FAB). A 3-fold increase was observed for the PHOT K/O mutants.

Terpenoid biosynthesis pathway genes:

[0112] The methyl erythritol 4-phosphate (MEP) pathway is the source of isoprenoid precursors for the chloroplast. The precursors lead to the formation of various isoprenoids having diverse roles in different biological processes. Some isoprenoids have important commercial uses. Isoprene, which is made in surprising abundance by some trees, plays a significant role in atmospheric chemistry. Multiple genes involved in MEP/DOXP pathway were up regulated in PHOT K/O mutants (FIG. 12). In contrast, the mevalonate terpenoid pathway (cytoplasmic) genes were not up regulated in PHOT K/O mutants.

[0113] Note that all data so far were generated in cell wall free mutants of *Chlamydomonas reinhardtii*. Metabolomic analyses in *C. reinhardtii* clarified the pathways and gene up-regulation in high light in *C. reinhardtii* PHOT K/O mutants of this invention:

Heterologous Algal Phototropin Genes

[0114] The *Chlamydomonas reinhardtii* phototropin gene has already been sequenced and a provisional version is available publically (GenBank 5718965). Additional algal genes are available that have either been shown to be a phototropin, contain blue light receptors, have some homology to phototropin or are putative blue light receptors similar to phototropin (Table 1). Additional phototropin genes in two other production strains of microalgae are known.

[0115] *Chlorella* sp. Strain1412. Is a strain developed by the National Alliance of Biofuels and Bio-products (NAABB) consortium and is housed at UTEX Culture Collection Of Algae at the University of Texas at Austin (UTEX). The amino acid sequence is provided as SEQ ID NO. 1 and the nucleotide sequence as SEQ ID NO. 2. The phototropin B gene of *Chlorella* sorokiniana. Strain 1412 is provide as SEQ ID NO. 3 and nucleotide as SEQ ID NO. 4.

[0116] *Chlorella* sp. *sorokiniana* strain 1230. Is a UTEX strain. The amino acid sequence of phototropin A is provided as SEQ ID NO. 5 and the nucleotide sequence as SEQ ID NO. 6. The amino acid sequence of phototropin B is provided as SEQ ID NO. 7 and the nucleotide sequence as SEQ ID NO. 8.

[0117] *Chlorella* sp. *sorokiniana* strain 1228. The amino acid sequence of phototropin A is provided as SEQ ID NO. 9 and the nucleotide sequence as SEQ ID NO. 10. The amino acid sequence of phototropin B is provided as SEQ ID NO. 11 and the nucleotide sequence as SEQ ID NO. 12.

[0118] *Picochlorum soloecismus* (DOE101). The amino acid sequence is provided as SEQ. ID NO. 13 and the nucleotide sequence as SEQ. ID NO. 14.

Table 1 - List of publically available sequences that may be phototropins or heterologous to phototropin genes based upon homology or function.

GenBank #	Alga	Description	Aliases
9688782	*Micromonas pusila* CCMP1545	Phototropin, blue light receptor	MICPUCDRAFT_49739
9617508	*Volvox carteri f. nagariensis*	Phototropin	VOLCADRAFT_127319
23616146	*Auxenochlorella protothecoides*	Phototropin 2	F751_4755
23614975	*Auxenochlorella protothecoides*	Phototropin-1B	F751_3584
19011210	*Bathycoccus prasinos*	Phototropin	Bathy16g02310
9831018	*Ostrecoccus tauri*	Putative blue light receptor	Ot16g02900
8249220	*Micromonas sp, RCC299*	Blue light receptor	MICPUN_105003
16998047	*Cyanidioschyzon merolae* 10D	Serine/threonine kinase	MICPUT_105003
17089759	*Galdieria sulphuraria*	Serine/threonine kinase	Gasu_15820
17087623	*Galdieria sulphuraria*	Serine/threonine kinase	Gasu_38210
17041755	*Coccomyxa subellipsoidea* C-169	Putative blue light	COCSUDRAFT_63287

		receptor	
17350696	*Chlorella variabilis*	Hypothetical protein	CHLNCDRAFT_141214
5005771	*Ostreococcus lucimarinus* CCE9901	Hypothetical protein	OSTLU_40751
17304390	*Guillarida theta* CCMP2712	Hypothetical protein	GUITHDRAFT_162563
7452793	*Thalassiosira pseudonana* CCMP1355	Hypothetical protein	THAPSDRAFT_33193
7442442	*Thalassiosira pseudonana* CCMP1355	Hypothetical protein, PAS domain	THAPSDRAFT_261631
7200921	*Phaeodactylum tricornutum* CCAP 1055/1	Hypothetical protein; one PAS domain	PHATRDRAFT_51933
CBJ25875	*Ectocarpus siliculosus* CCAP:1310/4	aureochrome 1	AUR1; Esi_0017_0027
XP_005854445	*Nannochloropsis gaditana* CCMP526	PAS and BZIP domain containing protein, putative aureochrome	GA_0015702
BAF91488	*Vaucheria frigida*	aureochrome1	AUREO1

Alternative Targets

[0119] Additional PHOT downstream signal transduction targets can be use as alternatives to the knockout or reduction in phot expression to generate the desirable phenotypes of this invention, including but not limited to improved photosynthetic efficiency, higher biomass productivity, increase yield of sink molecules/compounds, and improved genetic stability. An example of this could be the algal gene homologous to the *Arabidopsis* KIN10 and KIN11 kinases (Baena-Gonzalez, Rolland et al. 2007). Genes substantially homologous to the *Chlorella* genes in SEQ ID 15 to 27 and the *Chlamydomonas* genes in SEQ ID 28-34 would be applicable to this current invention.

[0120] Additional gene targets can be used as alternatives to the knockout or reduction in phot expression to generate the desirable phenotypes of this invention with desirable phenotypes having but not limited to improved photosynthetic efficiency, higher biomass productivity, increase yield of sink molecules. These genes could include the algal genes homologous to the *Arabidopsis NADPH thioredoxin reductase C* (NTRC) and *NADPH thioredoxin reductase 2* genes (Toivola et al. 2013) Genes substantially homologous to the *Chlorella* genes in SEQ ID NO 35- 40, 43-44 and 47 to 50 and the *Chlamydomonas* genes in SEQ ID 67-68 would be applicable to this current invention

EXAMPLES

[0121] Certain embodiments of the invention will be described in more detail through the following examples. The examples are intended solely to aid in more fully describing selected embodiments of the invention, and should not be considered to limit the scope of the invention in any way.

Example 1 – Growth of *Chlamydomonas reinhardtii*

[0122] *Chlamydomonas reinhardtii* parental strains (cw15 and UV4) and the phototropin knockout (PHOT K/O) mutants (CW15 and A4) were grown at 25°C in 250 mL Erlenmeyer flasks containing 100 mL of High-Salt (HS) or Tris-Acetate-Phosphate (TAP) media and shaken at 150 rpm (http://www.chlamy.org/media.html). Cultures were typically inoculated from a log phase culture using 1 mL of cells. Flasks were illuminated using fluorescent light at the light intensities as indicated for each experiment.

Example 2 – Measurement of photoautotrophic growth and biomass estimation

[0123] Photoautotrophic growth of the parent strains CW15 and UV4) and the phototropin knock out mutants (G5 and A4) was measured in environmental photobioreactors ("ePBRs") (obtained from Phenometrics, Inc.) in 500 mL of liquid HS media. All experiments were done in triplicates for each time point and each treatment. Light intensity was programmed for a 12 h sinusoidal light period with a peak mid-day intensity of 2,000 µmol photons m^{-2} s^{-1}. Temperature was a constant 25°C, and the ePBRs were stirred with a magnetic stir bar at 200 rpm. Filtered air was bubbled constantly through the growing cultures. The optical density of the cultures was monitored on a daily basis at 750 nm using a Cary 300 Bio UV–Vis spectrophotometer (Agilent). After completion of growth measurements, the total contents of individual ePBRs were harvested by centrifugation at 11,000 rpm for 15 min. Cell pellets were frozen immediately in liquid N_2 and later freeze-dried using a Microprocessor Controlled Lyophilizer (Flexi-Dry). After drying, pellets were weighed for total biomass.

Example 3 – Measurement of chlorophyll fluorescence

[0124] For Chl fluorescence induction analysis, cell suspensions of the parental wild-type and transgenic *Chlamydomonas* strains were adjusted to a Chl concentration of ~ 2.5 µg/mL. Quenching of Chl fluorescence was measured using the FL-3500 fluorometer (Photon System Instruments) (Kaftan, Meszaros et al. 1999). The cells were dark adapted for 10 min prior to the measurement. Chl fluorescence was induced using non-saturating continuous illumination and Chl fluorescence levels were measured every 1 µs using a weak pulse-modulated measuring flash. For the state transition experiments, low light grown cultures were dark adapted or pre-illuminated with 715 nm light for 10 min

prior to the induction of Chl fluorescence. The actinic flash duration for this experiment was set to 50 µs and Chl fluorescence was measured every 1 µs.

Example 4 - Measurement of photosynthetic oxygen evolution –

[0125] CO_2-supported rates of oxygen evolution were determined for low light (50 µmol photons m^{-2} s^{-1}) HS grown log-phase cultures (0.4–0.6 $OD_{750\ nm}$) using a Clark-type oxygen electrode (Hansatech Instruments). Cells were re-suspended in 20 mM HEPES buffer (pH 7.4) and air-saturated rates of oxygen evolution were measured as a function of light intensity (650 nm) at 50, 150, 300, 450, 600, 750 and 850 µmol photons m^{-2} s^{-1}. The same experiment was repeated in the presence of 10 mM $NaHCO_3$. Light saturation curves were normalized on the basis of Chl as well as cell density ($A_{750\ nm}$). Chl was determined by method described by Arnon (Arnon 1949).

Example 5 – Measurement of pigment content by HPLC

[0126] *Chlamydomonas* cultures were grown at low (50 µmol photons m^{-2} s^{-1}) and high (saturating) light (500 µmol photons m^{-2} s^{-1}) intensities for 5 days in HS media in shaker flasks. Cells were centrifuged at 3,000 rpm for 3 min and immediately frozen in liquid nitrogen and lyophilized. Carotenoids and chlorophylls were extracted with 100% acetone in the dark for 20 min. After incubation samples were centrifuged at 14,000 rpm for 2 min in a microfuge and the supernatant was transferred to a glass tube and dried under vacuum. The dried samples were re-suspended in 1 mL of acetonitrile:water:triethylamine (900:99:1, v/v/v) for HPLC analysis. Pigment separation and chromatographic analysis were performed on a Beckman HPLC equipped with a UV–Vis detector, using a C18 reverse phase column at a flow rate of 1.5 ml/min. Mobile phases were (A) acetonitrile/H_2O/triethylamine (900:99:1, v/v/v) and (B) ethyl acetate. Pigment detection was carried out at 445 nm with reference at 550 nm (Tian and DellaPenna 2001). Individual algal pigments were identified on the basis of their retention times and optical absorbance properties and quantified on the basis of their integrated absorbance peaks relative to known carotenoid standards. Carotenoid standards were purchased from DHI, Denmark. Pigments were standardized on the basis of dry weight of three replicates.

Example 7 – Transmission electron microscopy

[0127] Cells were prepared for electron microscopy by immobilizing cells in 3% sodium alginate (w/v) and the alginate beads were then solidified by incubation in cold 30mM CaCl2 for 30min. We used alginate encapsulated algal cells to keep cells intact as well as to protect from direct and harmful effect of chemicals during fixation processes. These cells were fixed using 2% glutaraldehyde for 1.5-2 hours and after fixation, these cells were post fixed in buffered 2% osmium tetroxide for 1.5 hours. After dehydration these cells were embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead

citrate. LEO 912 transmission electron microscope was used to view and collect images at 120kv and a Proscan digital camera.

Example 8 – Transcriptome analysis

[0128] Total RNA was extracted from 100 mg of cells/sample, flash frozen in liquid nitrogen, grown at high light (500 µmol photons m^{-2} s^{-1}) intensities for 5 days in HS media in shaker flasks) using the Direct-zol RNA-miniprep kit (ZYMO, P/N 2051) according to the manufacturer's instructions. Each total RNA sample was enriched for mRNA by hybridizing the poly(A) tail to oligo d(T)25 probes covalently coupled to magnetic beads, followed by elution (NEB, P/N S1419S). The enriched mRNA fractions were prepared for Illumina sequencing using the ScriptSeq V.2 RNA-seq Library Preparation Kit (Epicentre, P/N SSV21106) and sequenced on a Hi-Seq 2000 (2 X 150bp), multiplexed at 6 samples per lane. The resultant sequence reads were trimmed for quality and mapped to the coding sequences present in version 9 of the *Chlamydomonas reinhardtii* genome annotation at web address phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Creinhardtii using bowtie2. The relative transcript abundance of each gene (mean of 3 biological samples) was determined using RSEM and differential expression values (UV4 vs A4) were calculated using EdgeR. All genes identified as differentially expressed were mapped to KEGG biochemical maps using the v.9 annotation assignments.

Example 9- Identification of *Chlorella* spp. Phototropin coding sequence

[0129] Phototropin genes were identified in three *Chlorella* species (herein designated as strain 1412, strain 1228 and *Chlorella sorokiniana* UTEX1230) and a *Picochlorum soloecismus* (DOE101) by conducting homologous BLASTp searches against the annotations of *Chlorella* species using *Chlamydomonas reinhardtii* phototropin genes/proteins (NP_851210) and *Arabidopsis thaliana* protein sequences (Accession # AED97002.1 and AEE78073) as query proteins. The *Chlorella* spp. and *Picochlorum* phototropin homologs were aligned to other phototropin amino acid sequences using CLUSTALW, then truncated based on conserved sequence alignments and phylogenetically analyzed using a Maximum-Likelihood algorithm. Each *Chlorella* strain contains two paralogous copies of photoropin and *Picochlorum soloecismus*. (DOE101) was found to contain 1 homolog of phototropin. These sequences are provided as SEQ ID Nos. 1-14. Additional phototropin sequences and functional homologs are provided in Table 1 and SEQ ID NO 51-66.

Example 10 – Inducible control of phototropin expression in *Chlamydomonas reinhardtii*

[0130] One method to reduce expression of algal PHOT gene(s) is to use RNAi technology driving the expression of double stranded, fold-back RNA elements to reduce the PHOT expression. A strong gene promoter such as *psa*D or other strong constitutive gene promoters could be used to drive expression of the RNAi construct similar to methods used previously in *Chlamydmonas* for modulation of light harvesting antennae complex (Perrine, Negi et al. 2012).

Example 11 – Production of a *Chlorella* phototropin minus mutant

[0131] PHOT gene knockouts could be potentially generated by traditional mutagenesis approaches including chemical, UV, random insertional mutagenesis screened by TILLING (Comai, Young et al. 2004, Nieto, Piron et al. 2007), and by targeted knock outs using CRISPR/cas9 (Wang, Yang et al. 2013, Xiao, Wang et al. 2013, Dubrow 2014). Pooled PHOT-based PCR screening coupled with sequencing of PHOT PCR products could be used to screen for PHOT mutants.

Example 12 – Chemical mutagenesis for production of a phototropin K/O mutant in *Chlorella sorokiniana*

[0132] Classical chemical mutagenesis is carried out using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). This mutagen makes nucleotide changes in the DNA and these changes, depending on their position, can have effects that are either positive or negative in the use of the strain being treated. By careful observation of phenotypes produced, as well as implementation of selective pressure, one selects mutants with improved traits for the desired purpose. This method has been applied to algae previously (Yan, Aruga et al. 2000).

[0133] Identifying strains of algae that grow rapidly and produce high starch is used as a selection marker for PHOT K/O mutants. Because this approach does not involve adding foreign DNA (in fact is focused only on existing genetic potential of the strain being mutagenized), strains generated by chemical mutagenesis are not considered to be "genetically modified", allowing deployment in the field without additional government regulation.

[0134] N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was chosen based on its proven use for modifying blue-green algae, as well as its ability to eliminate toxicity by degradation in dilute acid. First, the conditions required to result in approximately 99% lethality for *Chlorella protothecoides* are determined; this degree of lethality generated optimal mutation frequency in blue-green algae (Chapman and Meeks 1987). Two treatments, exposure to 0.25 mg/mL MNNG for 30 minutes and 0.025 mg/mL MNNG for 60 minutes, result in approximately 99% lethality for this strain (unpublished data). Both treatments are used to generate mutagenized populations of *Chlorella* using enrichment strategies.

[0135] Approximately 10^8 cells are mutagenized with four concentrations of MNNG and incubated for three different durations. After rinsing out the mutagen, approximately 10^4 cells are spread plated on nutrient plates, and the number of colonies scored after 12 days. Treatments with approximately 100 surviving colonies, representing 99% lethality, are chosen as optimal for generating mutations.

[0136] PHOT K/O mutants are expected to be more rapidly growing and to produce excess sink molecules/material. In *C. protothecoides* the sink is lipid which could be used as a screen for selection of cells representing high lipid cells. Numerous methods are in the literature for such selection such as Nile red (Pick and Rachutin-Zalogin 2012) and BODIPY 493/503 (Ohsaki, Shinohara et al. 2010). High lipid cells are selected by flow cytometry and then placed in flask for cell culture. Rapid growing high lipid cells will dominate the culture and should be PHOT K/O as determined in this invention.

Example 13 – Genome editing using CRISPR/cas9 to reduce expression of phototropin in *Chlamydomonas reinhardtii.*

[0137] Recently, it has been demonstrated that CRISPR/cas9 genome editing techniques can be used to knock out genes of interest in Chlamydomonas when the Cas9 gene is expressed constitutively. By incorporating multiple guide RNA elements to specifically recognize the PHOT gene high efficiencies of gene mutagenesis can occur during miss-repair of the double stranded break in the target gene catalyzed by Cas/9 by the endogenous repair enzymes. By targeting repair of a recognized restriction endonuclease site, inhibition of the digestion of the PHOT-specific PCR product by the diagnostic restriction endonuclease can be used as an effective screen for PHOT mutants. Similarly, DNA repair mistakes that occur following double stranded DNA breaks in the PHOT gene generated by TALEN complexes can be used to generate PHOT-specific mutants.

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The following references and others cited herein, to the extent that they provide exemplary procedural and other details supplementary to those set forth herein, are specifically incorporated herein by reference and include US published patent applications and published patents: US 20130116165; US 20140249295; US 20130330718; US 8,859,232 and other patent related documents EP2682469; WO 2011133493; WO 201408626; and WO 2013056212 and other publications listed:

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CLAIMS

What is clamed is:

1. A method for increasing the productivity of an algal strain wherein the expression or function of the gene of the *Chlamydomnas reinhardtii* phototropin gene or genes that are substantially similar to the *Chlamydomnas reinhardtii* phototropin gene is reduced or eliminated.

2. The method of claim 1, wherein the gene has greater than 75% homology to the *Chlamydomnas reinhardtii* phototropin gene.

3. The method of claim 1, wherein the gene has greater than 80% homology to the *Chlamydomnas reinhardtii* phototropin gene.

4. The method of claim 1, wherein the biomass productivity of the algal strain is increased by greater than around 2-fold.

5. The method of claim 1, wherein the production of storage products in the algal strain is increase by greater than around 2-fold.

6. The method of claim 5, wherein the storage product is selected from starch, lipid,

7. The method of claim 1, wherein the productivity of biomass is increased by greater than around 2-fold.

8. The method of claim 1, wherein the productivity is increased of bioproducts chosen from lipids, waxes, polysaccharides (*e.g.*, starch, glycogen, mannans, glycans, cellulose, hemicellulose), pigments (*e.g.*, xanthophyll),

9. The method of claim 1, wherein the expression of the *Chlamydomnas reinhardtii* phototropin gene is reduced by chemical mutagenesis and selection.

10. The method of claim 1, wherein the expression of the *Chlamydomnas reinhardtii* phototropin gene is reduced by genome editing.

11. The method of claim 1, wherein the expression of the *Chlamydomnas reinhardtii* phototropin gene is reduced by trans acting elements (e.g., RNAi).

INCORPORATED BY REFERENCE (RULE 20.6)

12. An algal strain wherein relative to the wild-type parental line
- the expression of the phototropin gene or substantially similar gene is reduced,
- the photosynthetic pigments making up the antennae complex are reduced,
- and the content of sink compounds is increased.

13. The algal line of claim 12, wherein the phototropin gene or substantially similar gene been rendered to be non-functional.

14. The algal line of claim 12, wherein the phototropin gene or substantially similar gene been substantially deleted.

15. The algal line of claim 12, wherein the phototropin gene or substantially similar gene can be rendered to be non-functional on an inducible basis through a inducible promoter.

INCORPORATED BY REFERENCE (RULE 20.6)

CLAIMS

What is claimed is:

1. A method for increasing a biomass productivity of an algal strain wherein the expression or function of a *Chlamydomonas reinhardtii* phototropin gene, a gene substantially similar to the *Chlamydomonas reinhardtii* phototropin gene or a sequence substantially similar to SEQ ID NO 1-14 and 51-66 is reduced or eliminated as compared to the wild-type parental line.

2. The method of claim 1, wherein the gene substantially similar has greater than 75% homology to the *Chlamydomonas reinhardtii* phototropin gene or the sequence identified in SEQ ID NO 1-14 and 51-66.

3. The method of claim 1, wherein the gene substantially similar has greater than 80% homology to the *Chlamydomonas reinhardtii* phototropin gene or the sequence identified in SEQ ID NO 1-14 and 51-66.

4. The method of claim 1, wherein the biomass productivity of the algal strain is increased by greater than around 2-fold.

5. The method of claim 1, wherein the biomass production of storage product(s) in the algal strain is increased by greater than around 2-fold.

6. The method of claim 5, wherein the storage product(s) is selected from starch, lipid, pigments and other sink molecules.

7. The method of claim 4, wherein the productivity of biomass is increased by greater than around 2-fold.

8. The method of claim 1, wherein the biomass productivity is increased for bioproducts chosen from lipids, waxes, polysaccharides (*e.g.*, starch, glycogen, mannans, glycans, cellulose, hemicellulose), pigments (*e.g.*, xanthophyll).

9. The method of claim 1, wherein the expression of the *Chlamydomonas reinhardtii* phototropin gene, the gene substantially similar to the *Chlamydomonas reinhardtii* phototropin gene or the sequence substantially similar to SEQ ID NO 1-14 and 51-66 is reduced by chemical mutagenesis and selection.

10. The method of claim 1, wherein the expression of the *Chlamydomonas reinhardtii* phototropin gene, the gene substantially similar to the *Chlamydomonas reinhardtii* phototropin gene or the sequence substantially similar to SEQ ID NO 1-14 and 51-66 is reduced by genome editing.

11. The method of claim 1, wherein the expression of the *Chlamydomonas reinhardtii* phototropin gene, the gene substantially similar to the *Chlamydomonas reinhardtii* phototropin gene or the sequence substantially similar to SEQ ID NO 1-14 and 51-68 is reduced by trans acting elements (e.g., RNAi).

12. The method of claim 1 wherein the expression of the *Chlamydomonas reinhardtii* phototropin gene, the gene substantially similar to the *Chlamydomonas reinhardtii* phototropin gene or the sequence substantially similar to SEQ ID NO 1-14 and 51-66 is reduced on an inducible basis through an inducible promoter.

13. An algal strain wherein relative to the wild-type parental line
- the expression of the phototropin gene or a substantially similar gene is reduced,
- the photosynthetic pigments making up the antenna complex are reduced,
- and the content of sink molecules is increased.

14. The algal line of claim 13, wherein the phototropin gene or a substantially similar gene been rendered to be non-functional.

15. The algal line of claim 13, wherein the phototropin gene or a substantially similar gene been substantially deleted.

16. The algal line of claim 13, wherein the phototropin gene or a substantially similar gene can be rendered to be non-functional on an inducible basis through an inducible promoter.

17. The algal line of claim 13, wherein the phototropin gene deletion would generate sterile and stable diploid population of polyploid algae to avoid recombination of genetic material during sexual reproduction.

18. The algal line of claim 13, wherein the phototropin gene deletion would be used to generate stable transgene-stacking traits in polyploid algal strains.

19. The algal line of claim 13 wherein the photogropin gene or a substantially similar gene is selected from SEQ ID NO 1-14 and 51-66.

20. The method of claim 13, wherein the gene substantially similar has greater than 75% homology to the *Chlamydomonas reinhardtii* phototropin gene or the sequence identified in SEQ ID NO 1-14 and 51-66.

21. The method of claim 13, wherein the gene substantially similar has greater than 80% homology to the *Chlamydomonas reinhardtii* phototropin gene or the sequence identified in SEQ ID NO 1-14 and 51-66.

22. A method for increasing a biomass productivity of an algal strain wherein the expression or function of a *Chlamydomonas reinhardtii* NTR2 or NTRC gene, a gene substantially similar to a *Arabidopsis* NTR2 or NTRC gene or a sequence substantially similar to SEQ ID NO 35-50 and 67-68 is over expressed in the algal strain as compared to the wild-type parental line.

23. The method of claim 22, wherein the gene substantially similar has greater than 75% homology to the *Arabidopsis* NTR2 or NTRC gene or the sequence identified in SEQ ID NO 35-50 and 67-68.

24. The method of claim 22, wherein the gene substantially similar has greater than 80% homology to the *Arabidopsis* NTR2 or NTRC gene or the sequence identified in SEQ ID NO 35-50 and 67-68.

25. The method of claim 22, wherein the biomass productivity of the algal strain is increased by greater than around 2-fold.

26. The method of claim 22, wherein the biomass production of storage product(s) in the algal strain is increase by greater than around 2-fold.

27. The method of claim 26, wherein the storage product(s) is selected from starch, lipid, pigments and other sink molecules.

28. The method of claim 22, wherein the productivity of biomass is increased by greater than around 2-fold.

29. The method of claim 22, wherein the biomass productivity is increased for bioproducts chosen from lipids, waxes, polysaccharides (*e.g.*, starch, glycogen, mannans, glycans, cellulose, hemicellulose), pigments (*e.g.*, xanthophyll).

30. A method for increasing a productivity of an algal strain wherein the expression or function of a *Chlamydomonas reinhardtii* KIN10 or KIN11 gene, a gene substantially similar to a *Arabidopsis* KIN10 or

KIN11 gene or a sequence substantially similar to SEQ ID NO 15-34 is over expressed in the algal strain as compared to the wild-type parental line.

31. The method of claim 30, wherein the gene substantially similar has greater than 75% homology to the *Arabidopsis* KIN10 or KIN11 gene or the sequence identified in SEQ ID NO 15-34.

32. The method of claim 30, wherein the gene substantially similar has greater than 80% homology to the *Arabidopsis* KIN10 or KIN11 gene or the sequence identified in SEQ ID NO 15-34.

33. The method of claim 30, wherein the biomass productivity of the algal strain is increased by greater than around 2-fold.

34. The method of claim 30, wherein the biomass production of storage products in the algal strain is increase by greater than around 2-fold.

35. The method of claim 34, wherein the storage product is selected from starch, lipid, pigments and other sink molecules.

36. The method of claim 30, wherein the productivity of biomass is increased by greater than around 2-fold.

37. The method of claim 30, wherein the biomass productivity is increased for bioproducts chosen from lipids, waxes, polysaccharides (*e.g.*, starch, glycogen, mannans, glycans, cellulose, hemicellulose), pigments (*e.g.*, xanthophyll).

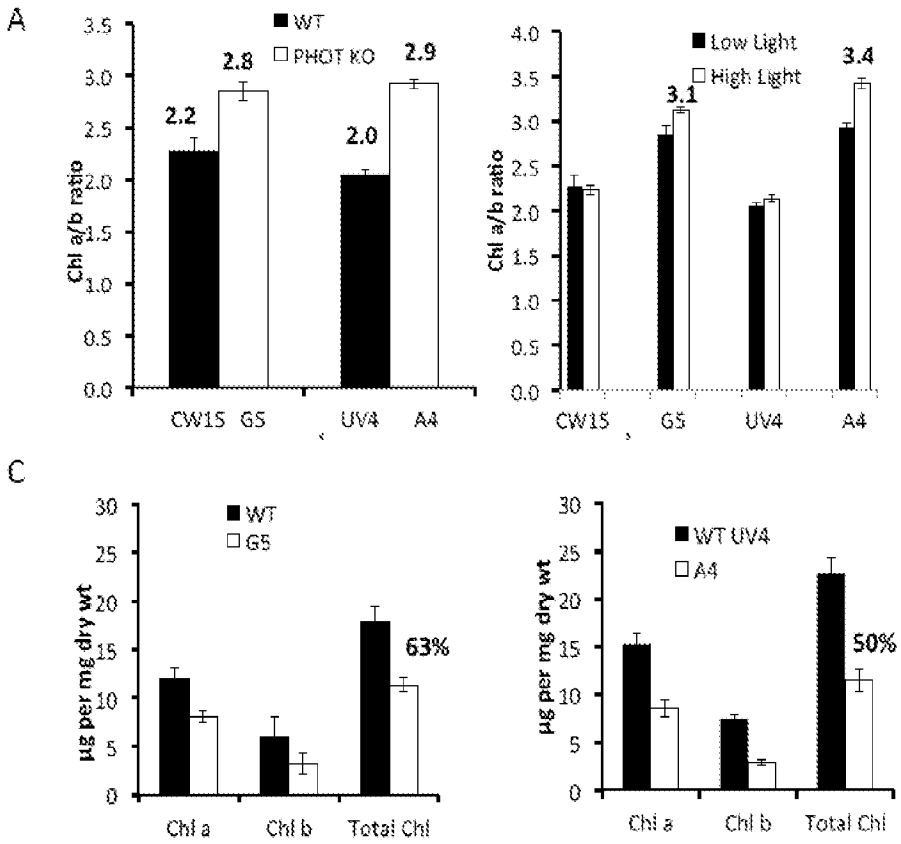

A
WT
PHOT KO
Chl a/b ratio
2.2
2.8
2.0
2.9
CW15
G5
UV4
A4
Low Light
High Light
3.1
3.4
C
WT
G5
µg per mg dry wt
63%
Chl a
Chl b
Total Chl
WT UV4
A4
50%

FIG. 1

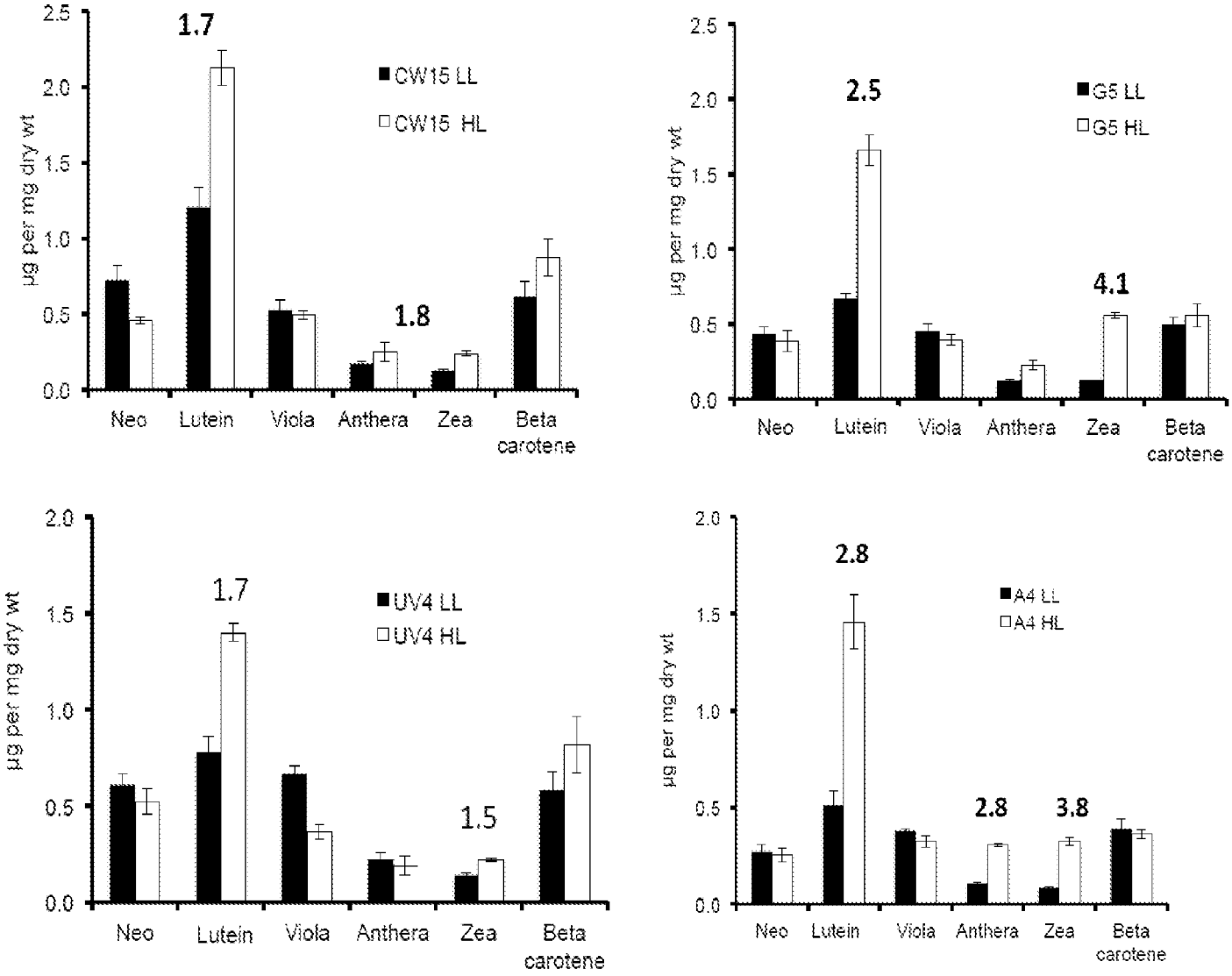

µg per mg dry wt
CW15 LL
CW15 HL
1.7
1.8
Neo
Lutein
Viola
Anthera
Zea
Beta carotene
G5 LL
G5 HL
2.5
4.1
UV4 LL
UV4 HL
1.7
1.5
A4 LL
A4 HL
2.8
2.8
3.8

FIG. 2

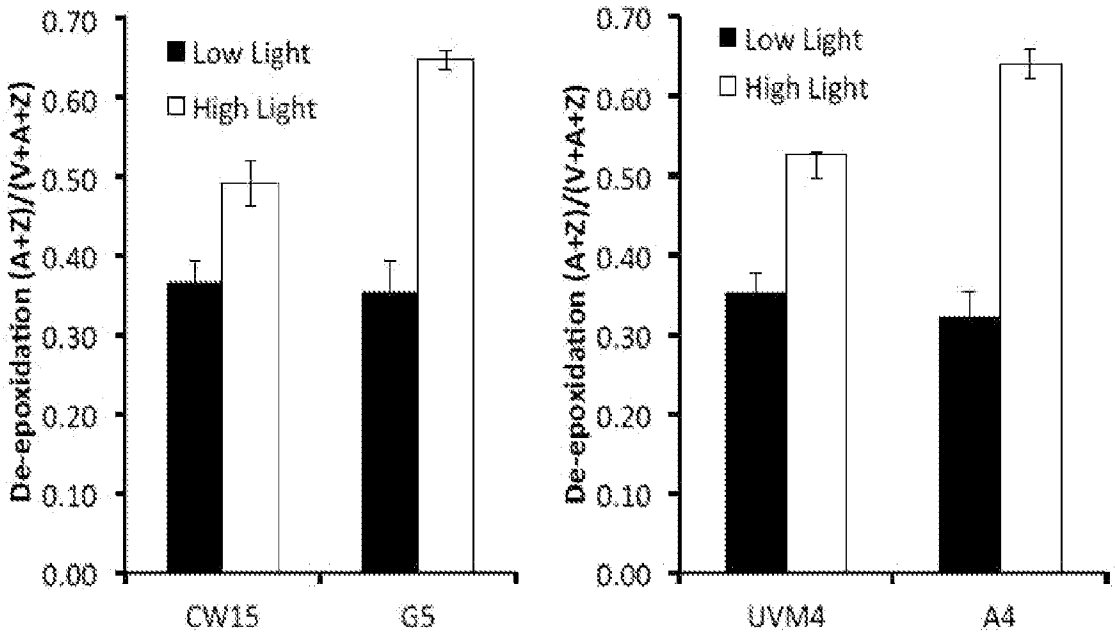

De-epoxidation (A+Z)/(V+A+Z)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Low Light
High Light
CW15
G5
UVM4
A4

FIG. 3

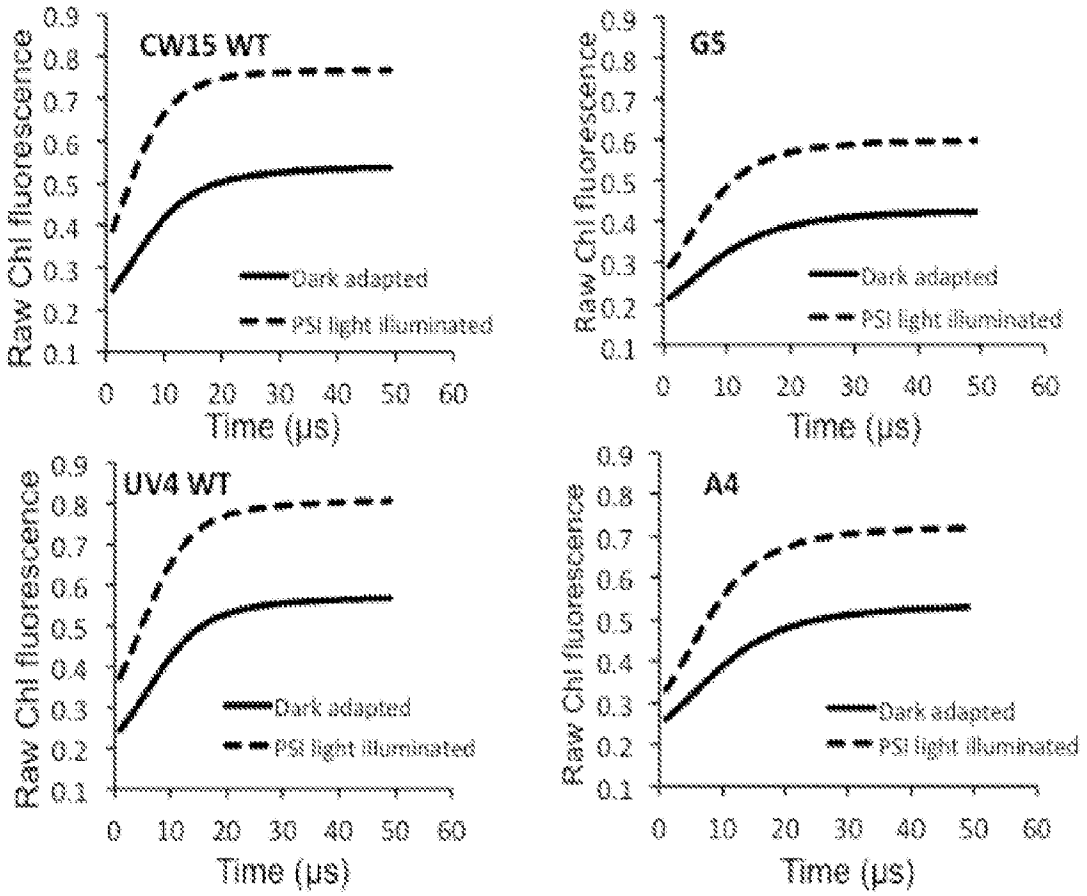
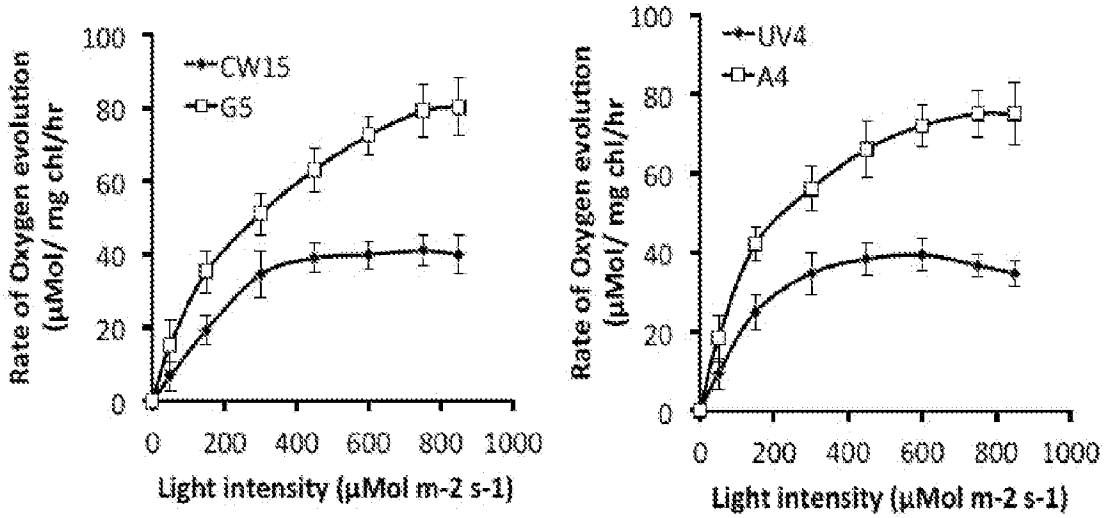

CW15 WT
G5
UV4 WT
A4
Raw Chl fluorescence
Time (µs)
Dark adapted
PSI light illuminated
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
10
20
30
40
50
60

FIG. 4



Low light grown cultures

FIG. 5

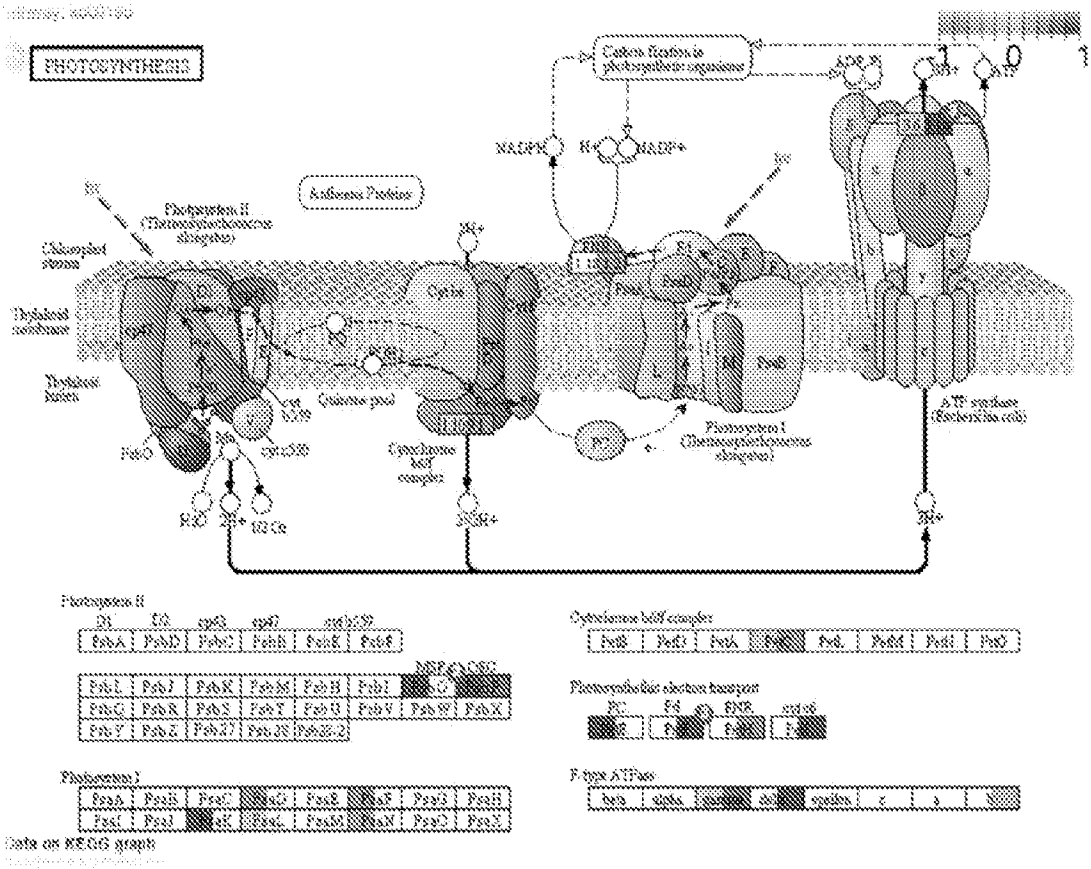

PHOTOSYNTHESIS
Antenna Proteins
Photosystem II
Photosystem I
Cytochrome b6f complex
ATP synthase
Calvin Cycle
Thylakoid membrane
Thylakoid lumen
Chloroplast stroma
Data on KEGG graph

FIG. 6

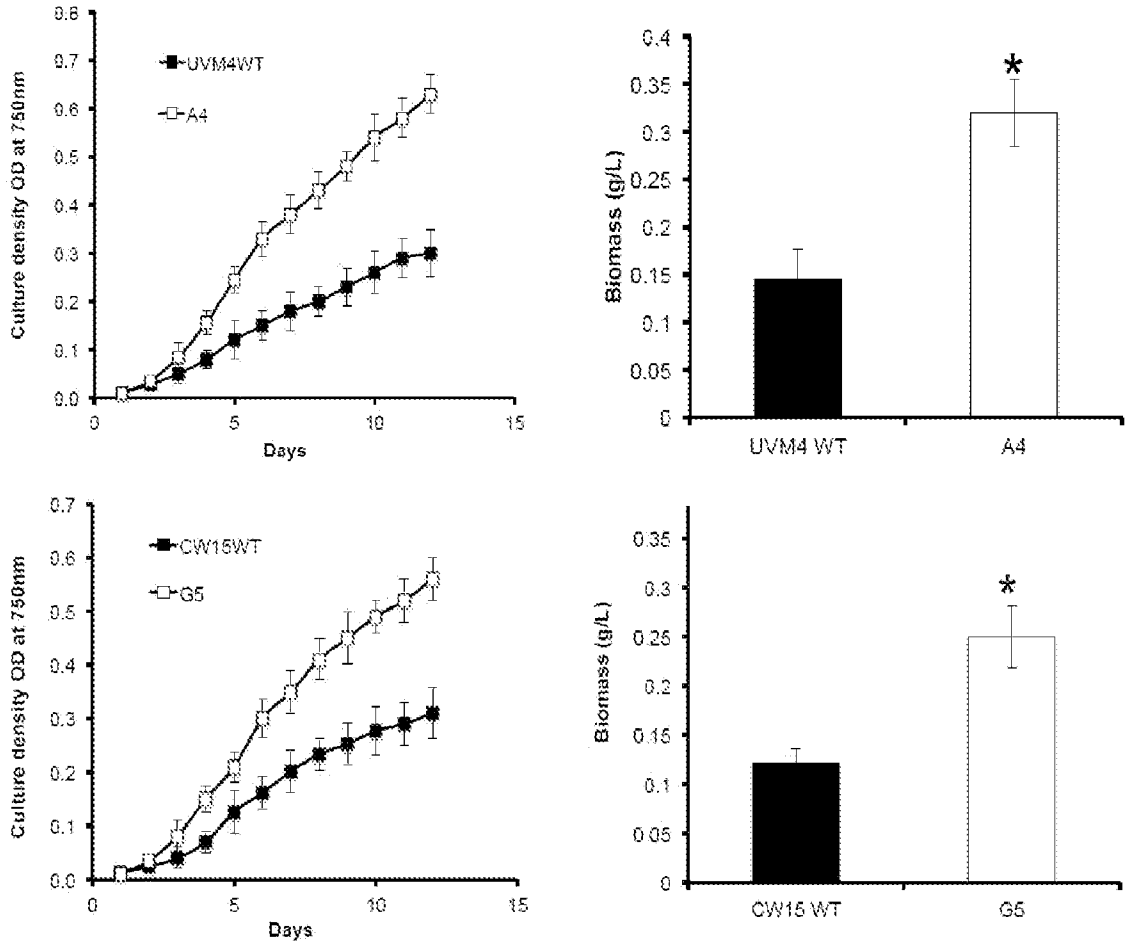

Culture density OD at 750nm
UVM4WT
A4
Days
Biomass (g/L)
UVM4 WT
A4
CW15WT
G5
CW15 WT
G5

FIG. 7

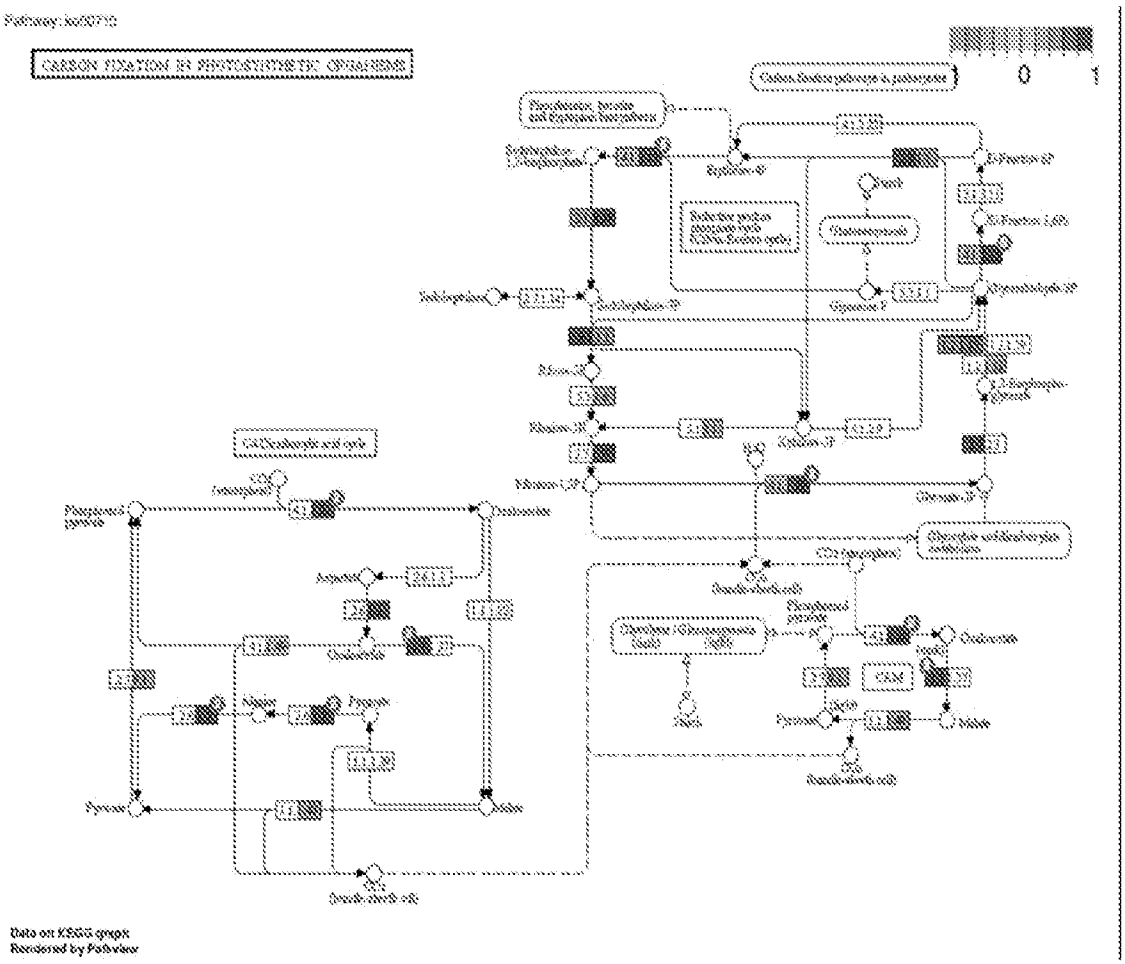


FIG. 8

INCORPORATED BY REFERENCE (RULE 20.6)

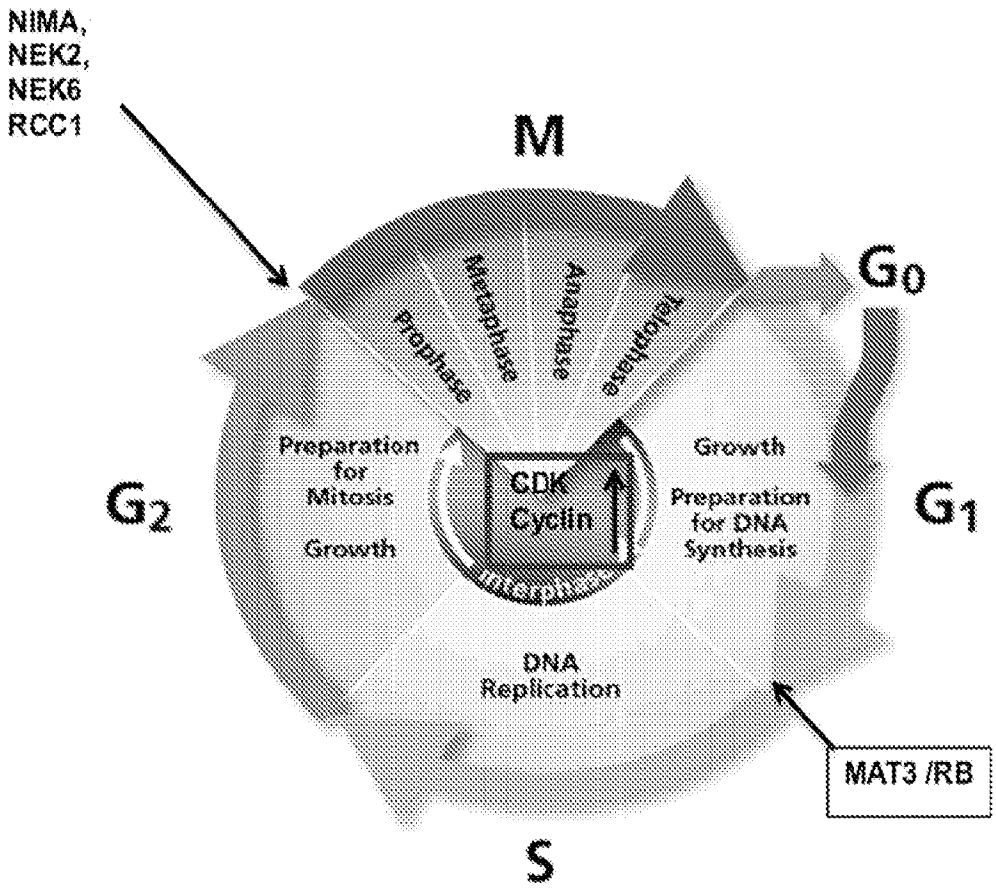
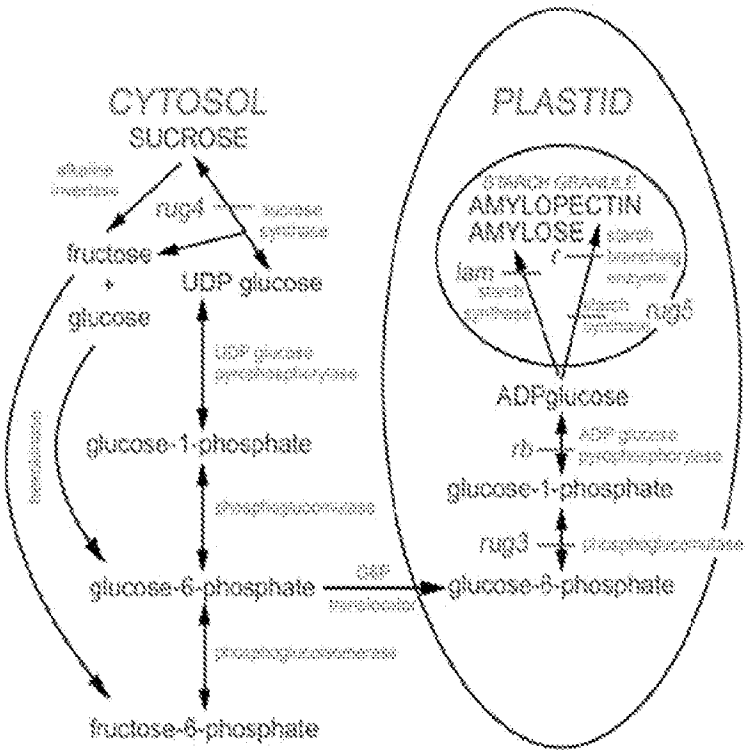


FIG. 9

INCORPORATED BY REFERENCE (RULE 20.6)



Starch synthase 2,3 and 4
Starch branching enzyme

FIG. 10

INCORPORATED BY REFERENCE (RULE 20.6)

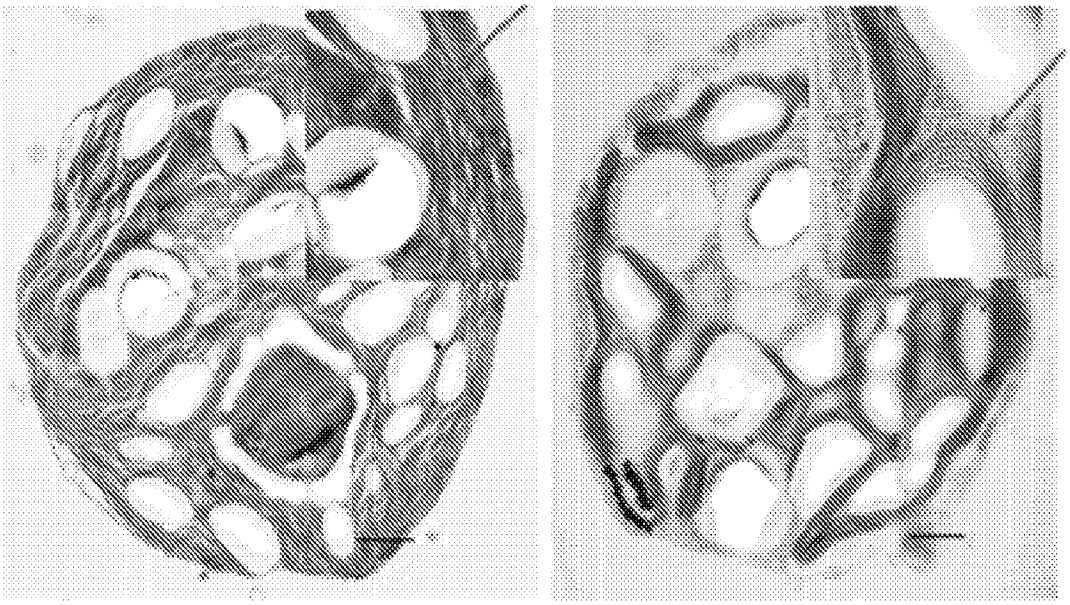


FIG. 11

INCORPORATED BY REFERENCE (RULE 20.6)

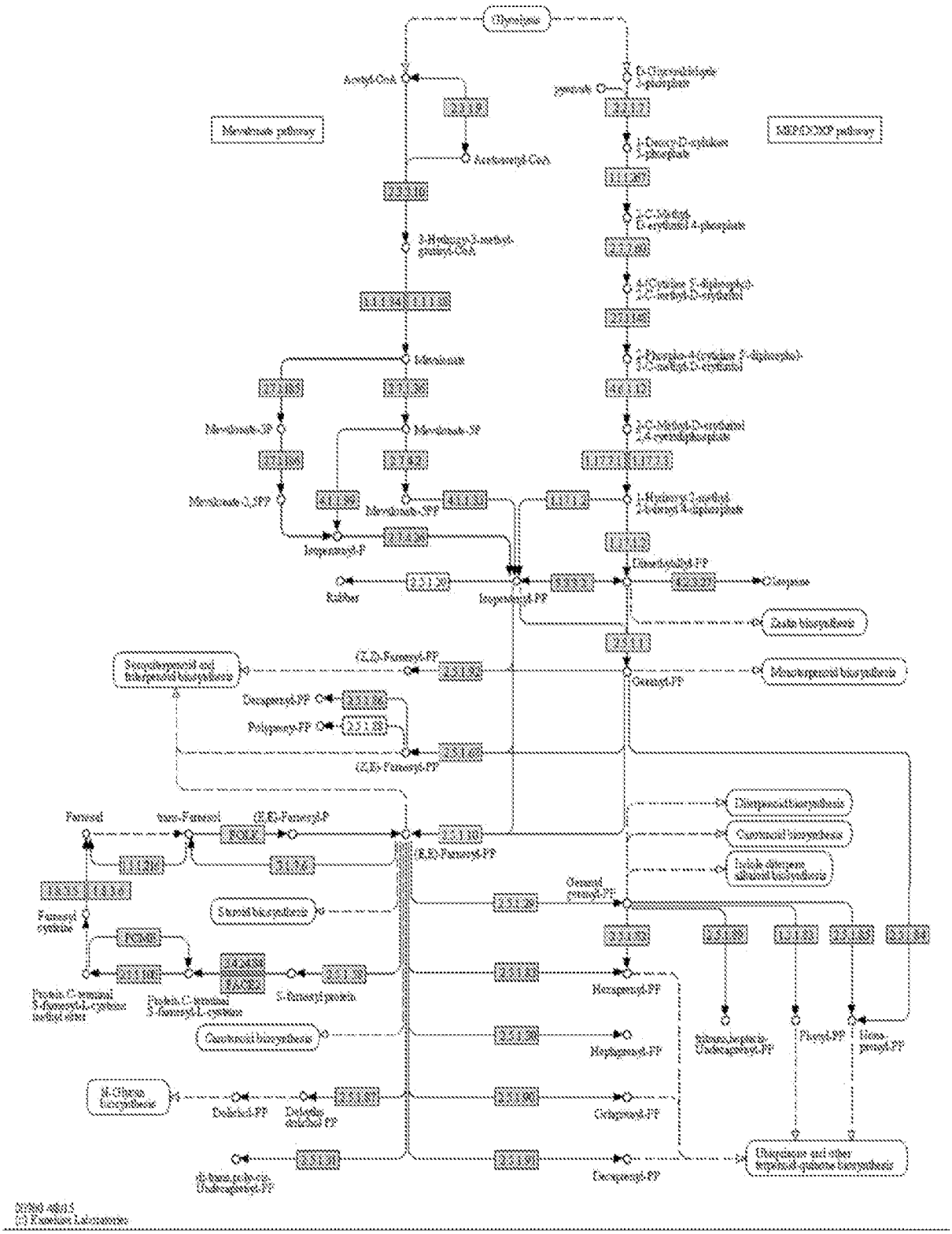

Glycolysis
Acetyl-CoA
Mevalonate pathway
Acetoacetyl-CoA
3-Hydroxy-3-methyl-glutaryl-CoA
Mevalonate
Mevalonate-5P
Mevalonate-5PP
Mevalonate-3-PP
Isopentenyl-P
Isopentenyl-PP
Rubber
Dimethylallyl-PP
Geranyl-PP
Geranyl-P
Geranyl-PP
Farnesol
trans-Farnesol
(2E,6E)-Farnesyl-PP
(2Z,6E)-Farnesyl-PP
Decaprenyl-PP
Polyprenyl-PP
Sesquiterpenoid and triterpenoid biosynthesis
Monoterpenoid biosynthesis
Carotenoid biosynthesis
Ubiquinone and other terpenoid-quinone biosynthesis
Steroid biosynthesis
Protein C-terminal S-farnesyl-L-cysteine
S-farnesyl protein
Protein C-terminal S-farnesyl-L-cysteine methyl ester
N-Glycan biosynthesis
Dolichol-P
Dolichyl-P
Dolichyl-PP
Undecaprenyl-PP
Heptaprenyl-PP
Octaprenyl-PP
Nonaprenyl-PP
Decaprenyl-PP
Phenyl-PP
Diterpenoid biosynthesis
Cysteine
Cutin biosynthesis
Diterpenoid biosynthesis
Monoterpenoid biosynthesis
Indole diterpene alkaloid biosynthesis

FIG. 12

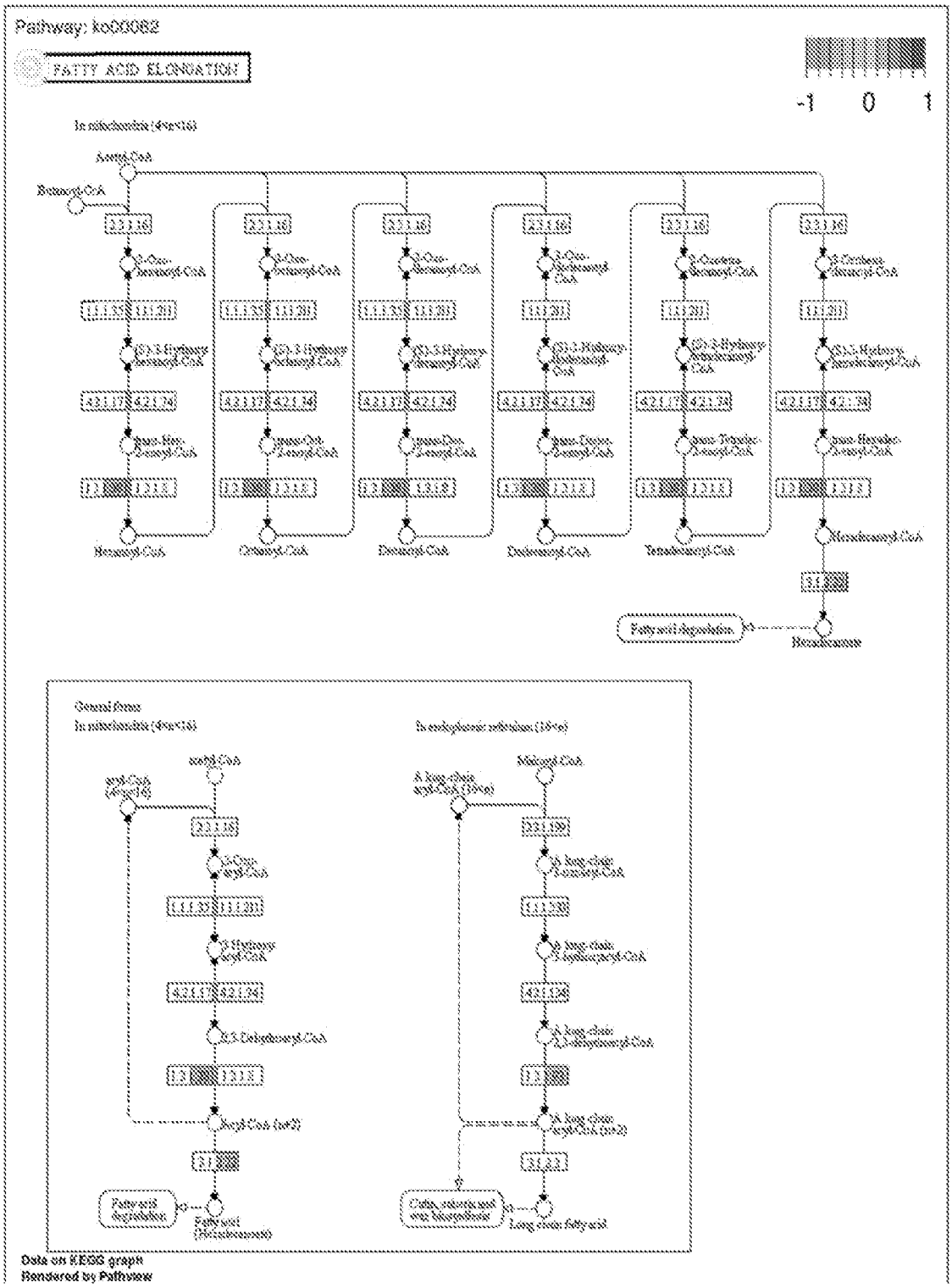

Pathway: ko00062
FATTY ACID ELONGATION
-1 0 1
Data on KEGG graph
Rendered by Pathview

FIG. 13

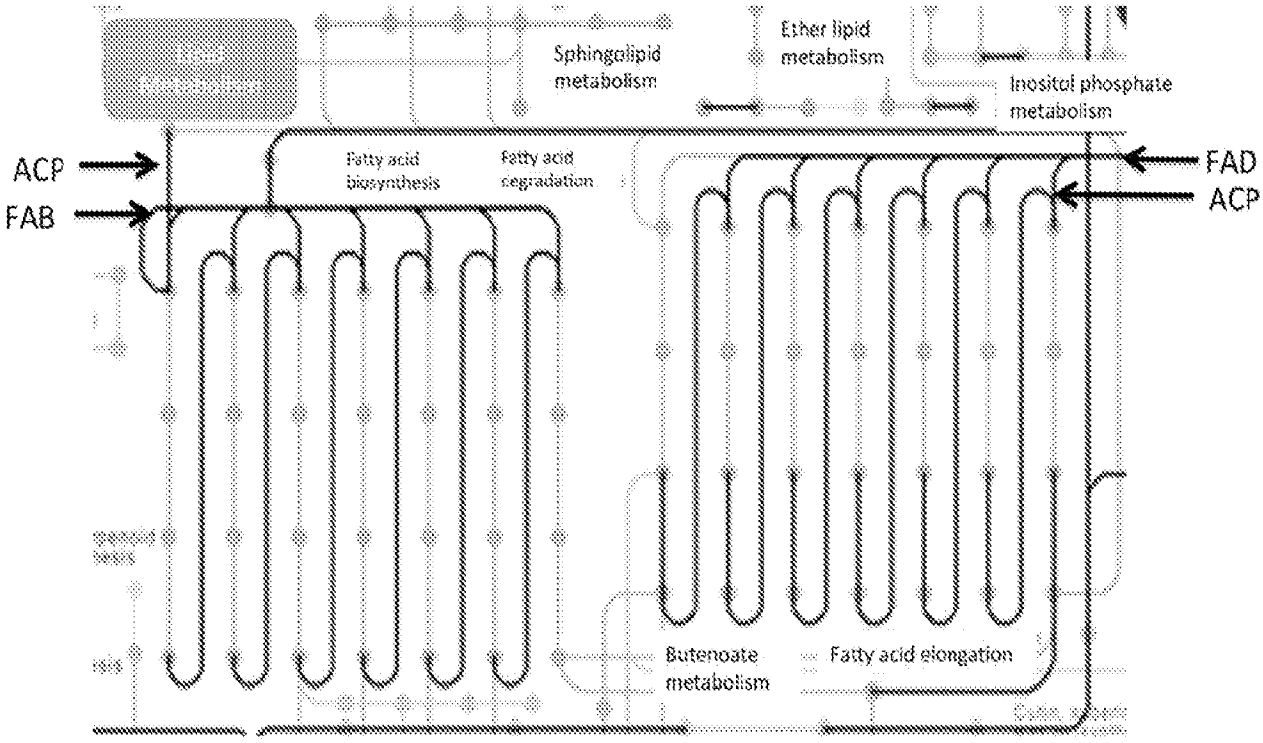


FIG. 14

INCORPORATED BY REFERENCE (RULE 20.6)

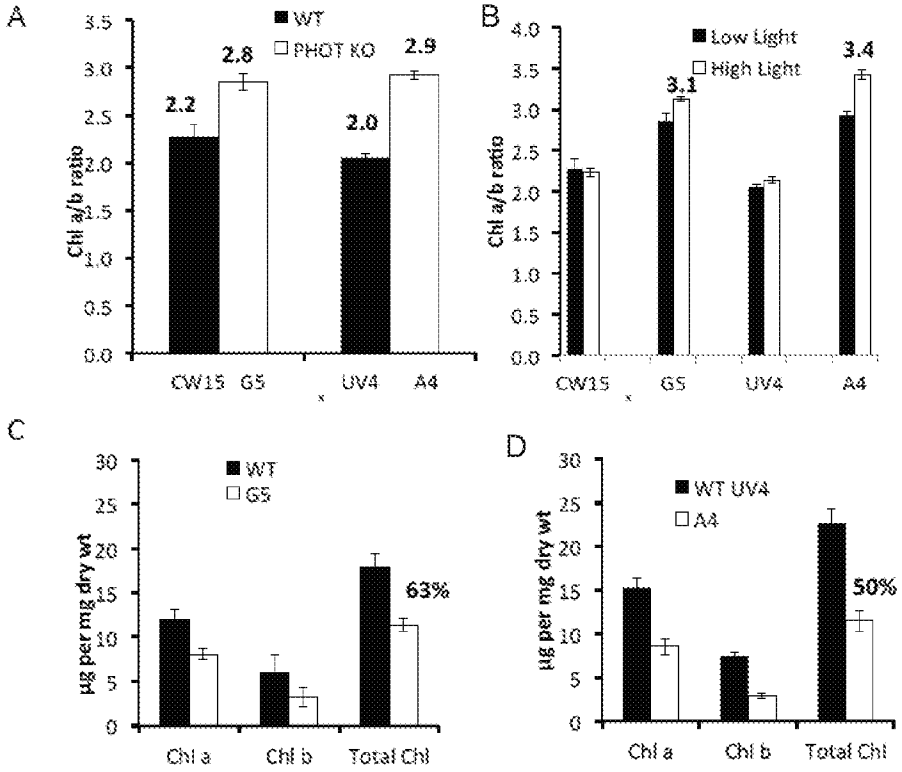

A
WT
PHOT KO
2.2
2.8
2.0
2.9
Chl a/b ratio
CW15
G5
UV4
A4
B
Low Light
High Light
3.1
3.4
Chl a/b ratio
CW15
G5
UV4
A4
C
WT
G5
63%
µg per mg dry wt
Chl a
Chl b
Total Chl
D
WT UV4
A4
50%
µg per mg dry wt
Chl a
Chl b
Total Chl

FIG. 1

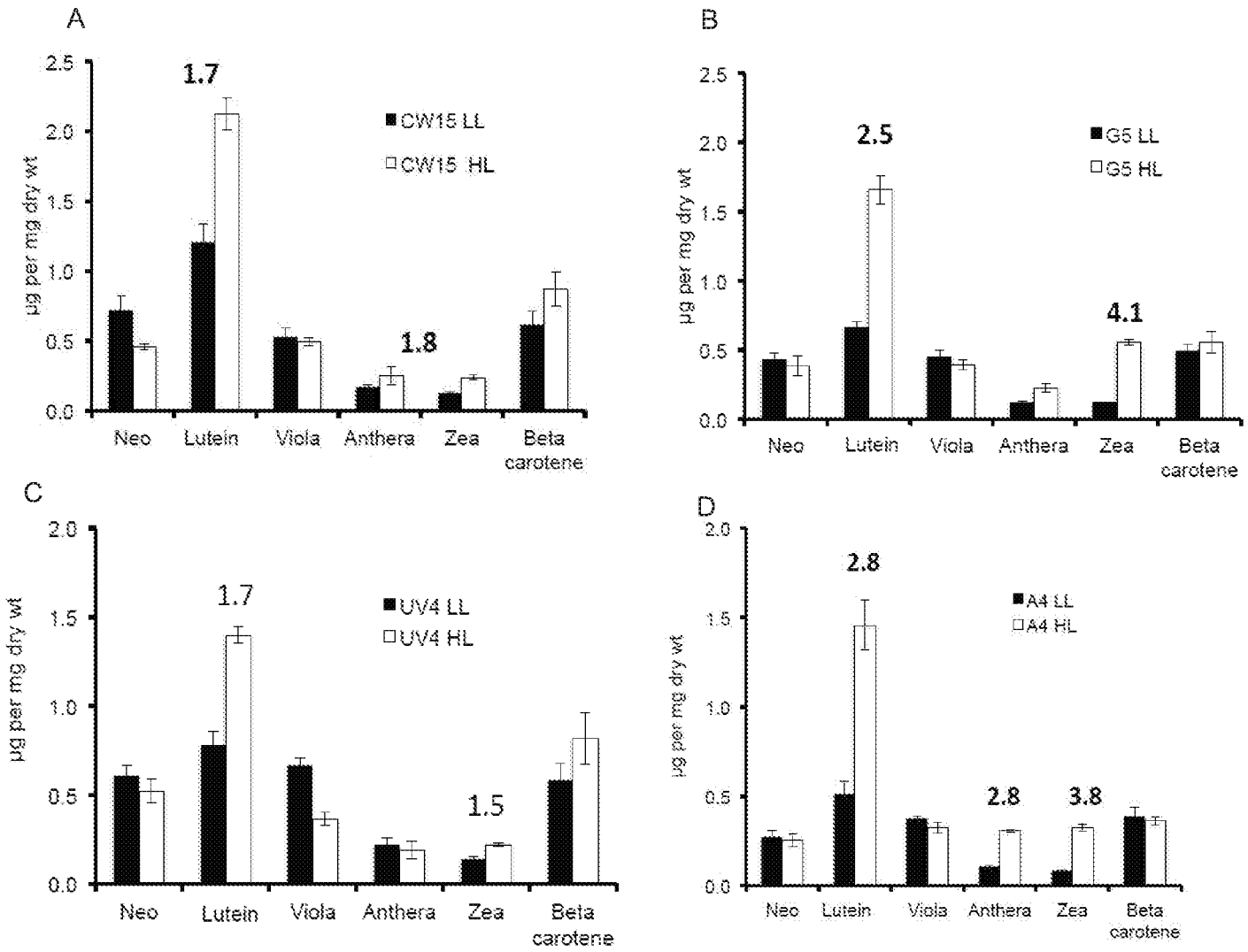

A
1.7
1.8
CW15 LL
CW15 HL
µg per mg dry wt
Neo
Lutein
Viola
Anthera
Zea
Beta carotene
B
2.5
4.1
G5 LL
G5 HL
C
1.7
1.5
UV4 LL
UV4 HL
D
2.8
2.8
3.8
A4 LL
A4 HL

FIG. 2

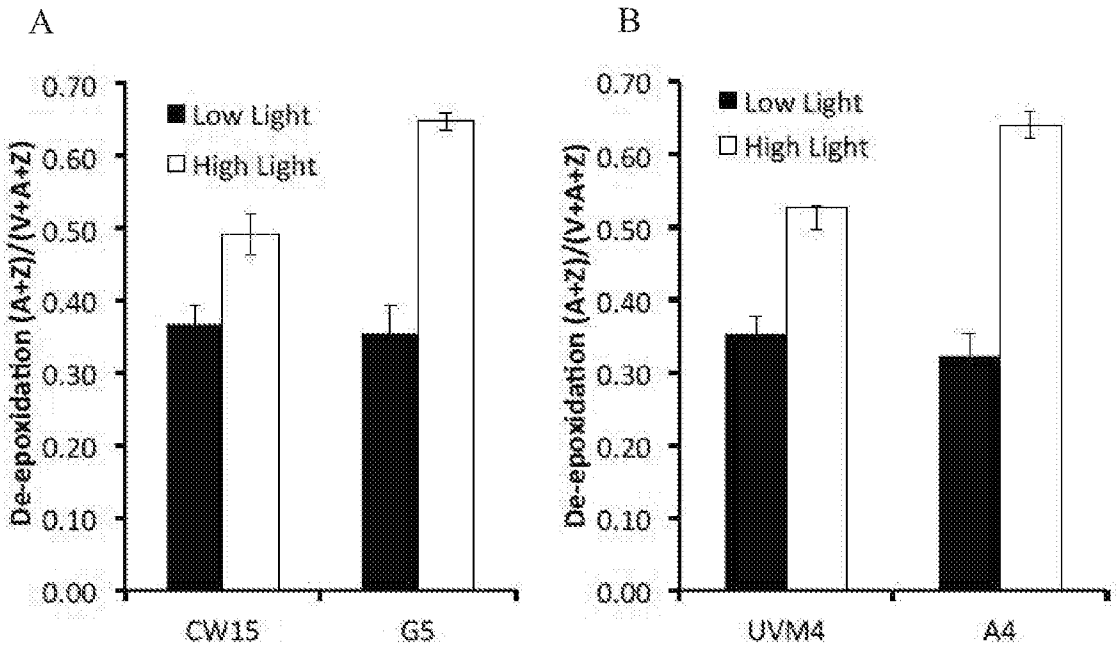

A
B
De-epoxidation (A+Z)/(V+A+Z)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Low Light
High Light
CW15
G5
UVM4
A4

FIG. 3

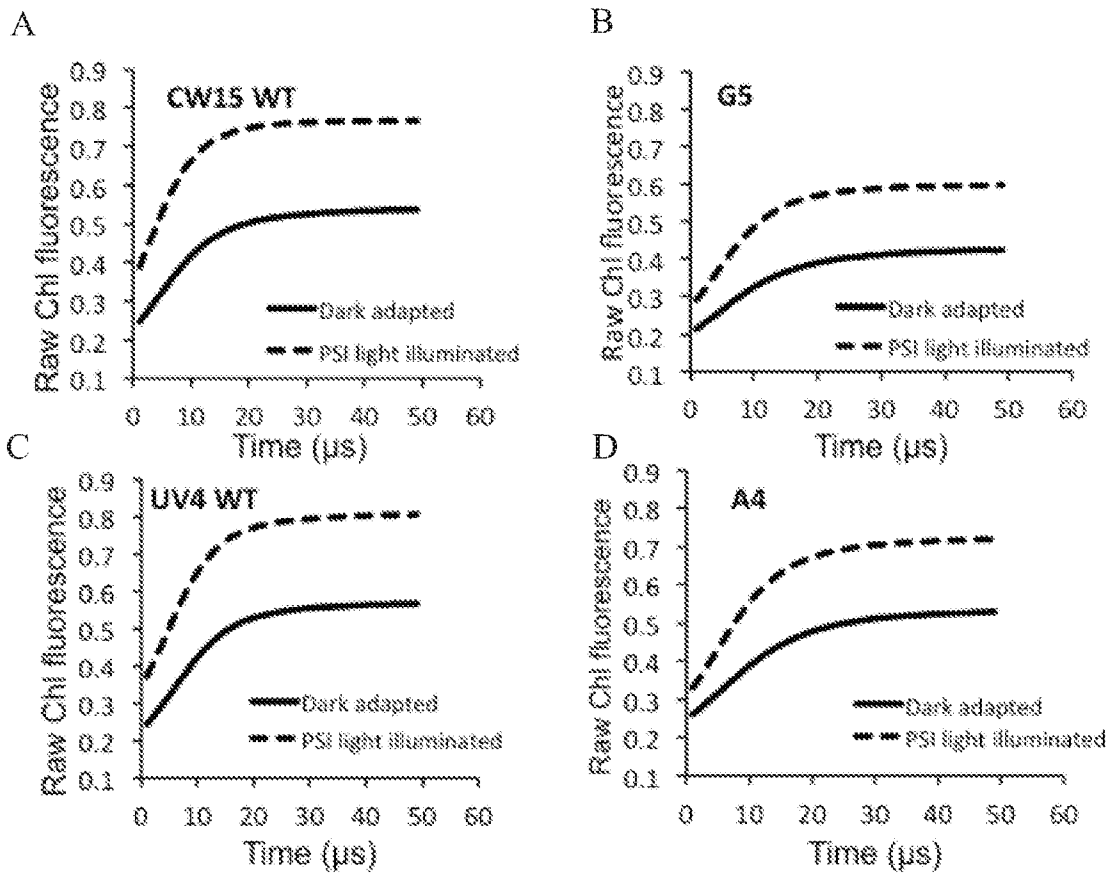

A
CW15 WT
Raw Chl fluorescence
Time (µs)
Dark adapted
PSI light illuminated
B
G5
C
UV4 WT
D
A4

FIG. 4

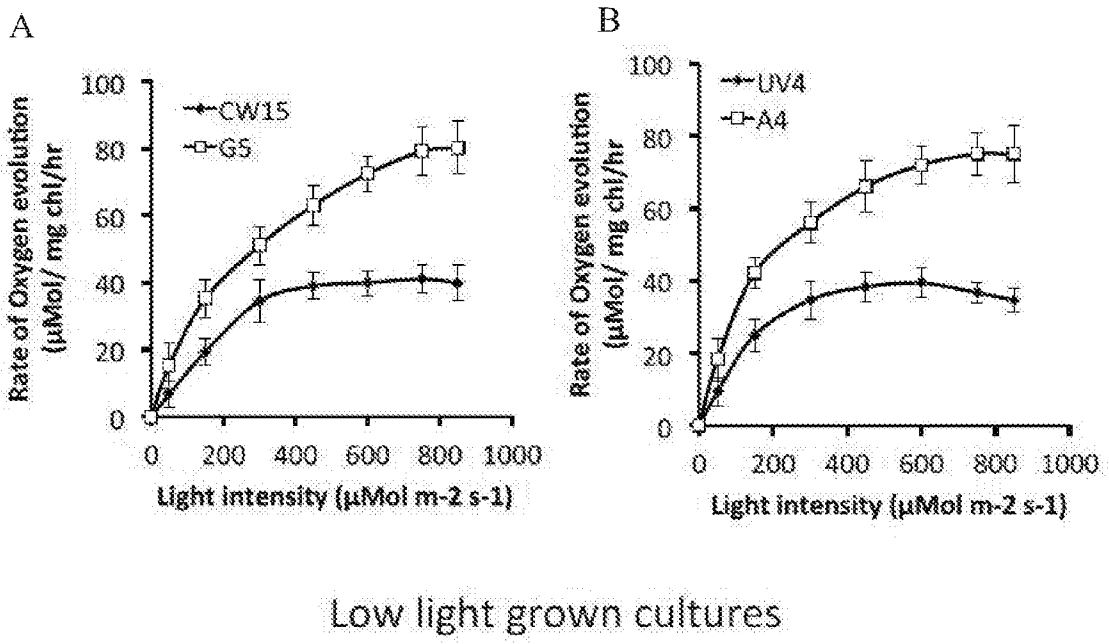

A
B
Rate of Oxygen evolution (µMol/ mg chl/hr
CW15
G5
UV4
A4
0
20
40
60
80
100
0
200
400
600
800
1000
Light intensity (µMol m-2 s-1)
Low light grown cultures

FIG. 5

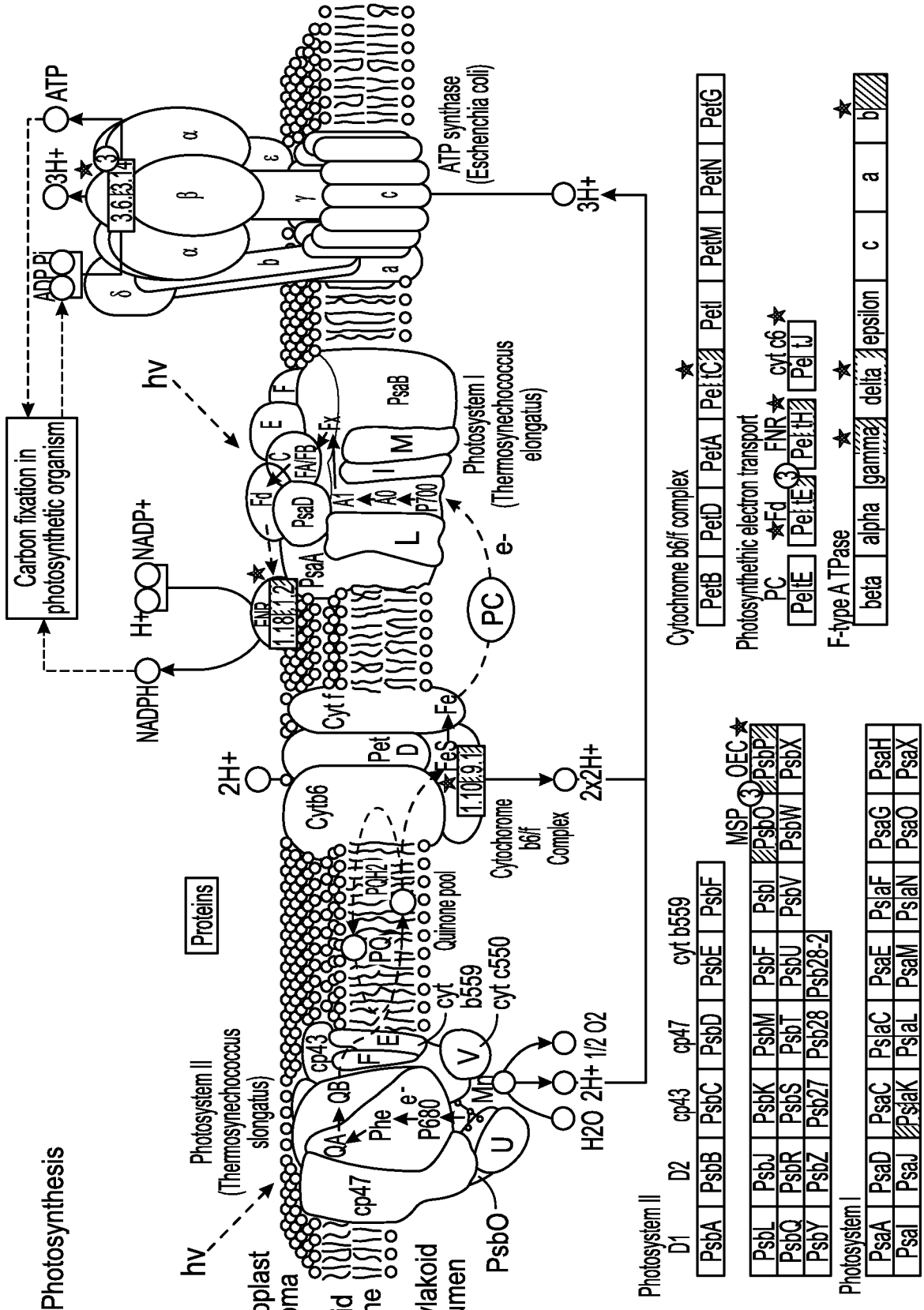

Photosynthesis
Carbon fixation in photosynthetic organism
ATP
3H+
ADP Pi
3.6.3.14
ATP synthase (Escherichia coli)
hv
NADP+
H+
NADPH
FNR
1.18.1.2
Fd
PsaA
PsaB
PsaD
PsaC
PsaE
PsaF
PsaG
PsaH
PsaI
PsaJ
PsaK
PsaL
PsaM
PsaN
PsaO
PsaX
Photosystem I (Thermosynechococcus elongatus)
PC
e-
Cytochrome b6/f Complex
1.10.99.1
Cytb6
Cyt f
PetD
FeS
2H+
2x2H+
Proteins
Photosystem II (Thermosynechococcus slongatus)
cp43
cp47
QA
QB
PQ
PQH2
Quinone pool
cyt b559
cyt c550
Phe
P680
Mn
H2O 2H+ 1/2 O2
PsbO
Chloroplast Stroma
Thylakoid Membrane
Thylakoid Lumen
Cytochrome b6/f complex
PetB
PetD
PetA
PetC
PetI
PetM
PetN
PetG
Photosynthethic electron transport
PC
PetE
Fd
PetF
cyt c6
PetJ
F-type ATPase
beta
alpha
gamma
delta
epsilon
c
a
b
Photosystem II
D1
D2
cp43
cp47
cyt b559
PsbA
PsbB
PsbC
PsbD
PsbE
PsbF
PsbL
PsbJ
PsbK
PsbM
PsbF
PsbI
PsbO
PsbP
PsbQ
PsbR
PsbS
PsbT
PsbU
PsbV
PsbW
PsbX
PsbY
PsbZ
Psb27
Psb28
Psb28-2
MSP
OEC

FIG. 6

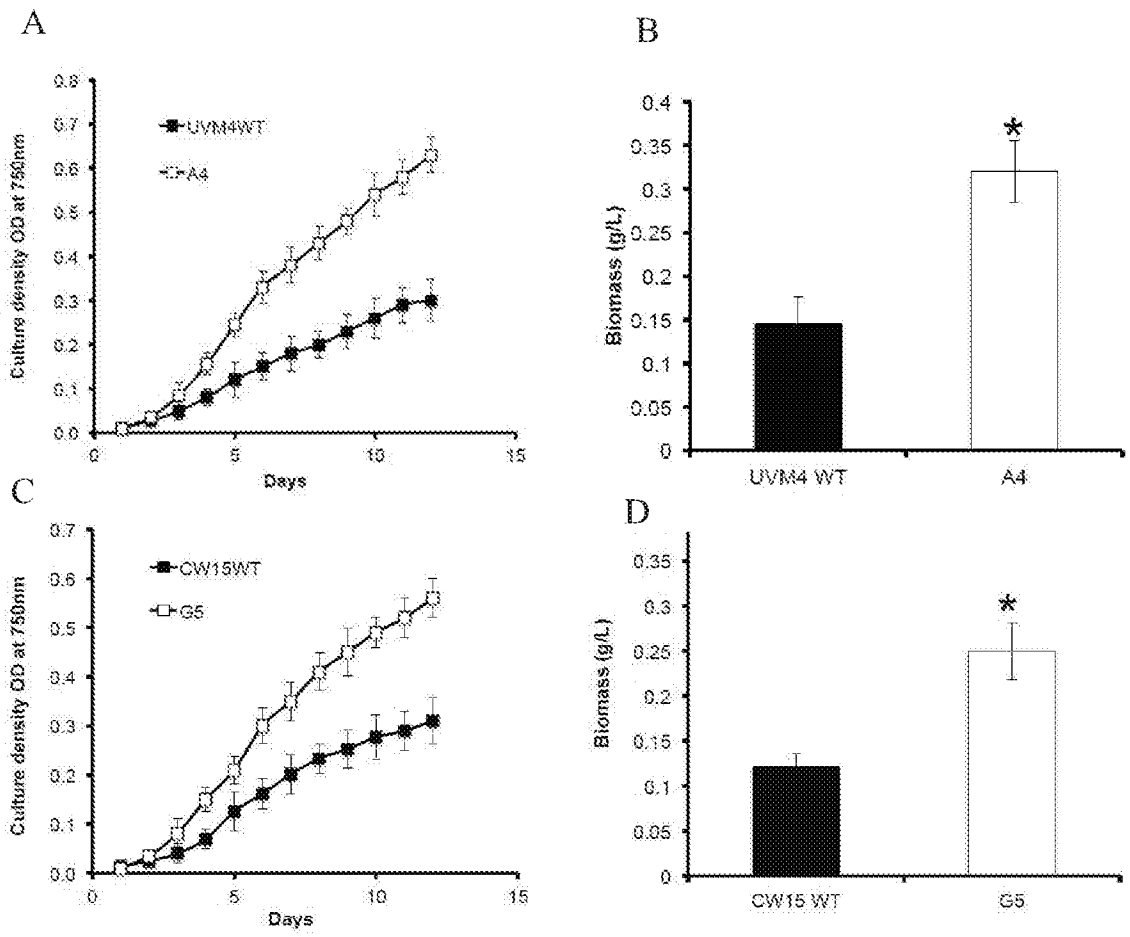

A
B
C
D
UVM4WT
A4
Culture density OD at 750nm
Days
Biomass (g/L)
UVM4 WT
A4
CW15WT
G5
CW15 WT
G5
*

FIG. 7

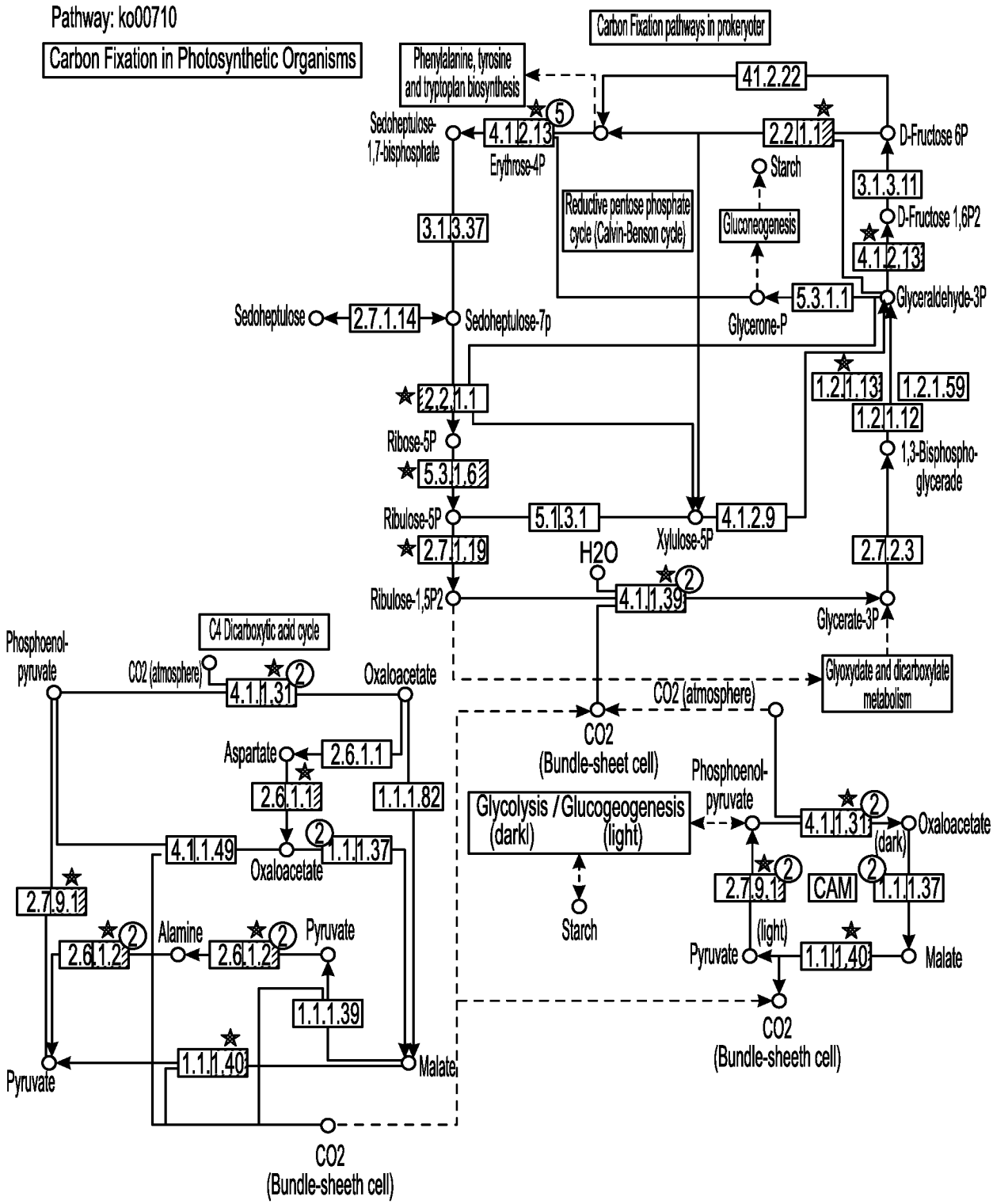

Pathway: ko00710
Carbon Fixation in Photosynthetic Organisms
Carbon Fixation pathways in prokeryoter
Phenylalanine, tyrosine and tryptoplan biosynthesis
41.2.22
Sedoheptulose-1,7-bisphosphate
4.1.2.13
Erythrose-4P
2.2.1.1
D-Fructose 6P
Starch
3.1.3.11
D-Fructose 1,6P2
Reductive pentose phosphate cycle (Calvin-Benson cycle)
Gluconeogenesis
3.1.3.37
4.1.2.13
Sedoheptulose
2.7.1.14
Sedoheptulose-7p
5.3.1.1
Glyceraldehyde-3P
Glycerone-P
1.2.1.13
1.2.1.59
1.2.1.12
2.2.1.1
Ribose-5P
1,3-Bisphospho-glycerade
5.3.1.6
Ribulose-5P
5.1.3.1
4.1.2.9
Xylulose-5P
2.7.1.19
H2O
2.7.2.3
Ribulose-1,5P2
4.1.1.39
Glycerate-3P
C4 Dicarboxylic acid cycle
Phosphoenol-pyruvate
CO2 (atmosphere)
4.1.1.31
Oxaloacetate
Glyoxydate and dicarboxylate metabolism
CO2 (atmosphere)
CO2
(Bundle-sheet cell)
Aspartate
2.6.1.1
2.6.1.1
1.1.1.82
Glycolysis / Glucogeogenesis
(dark) (light)
Phosphoenol-pyruvate
4.1.1.31
Oxaloacetate
(dark)
4.1.1.49
1.1.1.37
Oxaloacetate
2.7.9.1
CAM
1.1.1.37
Starch
Alamine
Pyruvate
2.6.1.2
2.6.1.2
(light)
Pyruvate
1.1.1.40
Malate
1.1.1.39
1.1.1.40
Malate
Pyruvate
CO2
(Bundle-sheeth cell)
CO2
(Bundle-sheeth cell)

FIG. 8

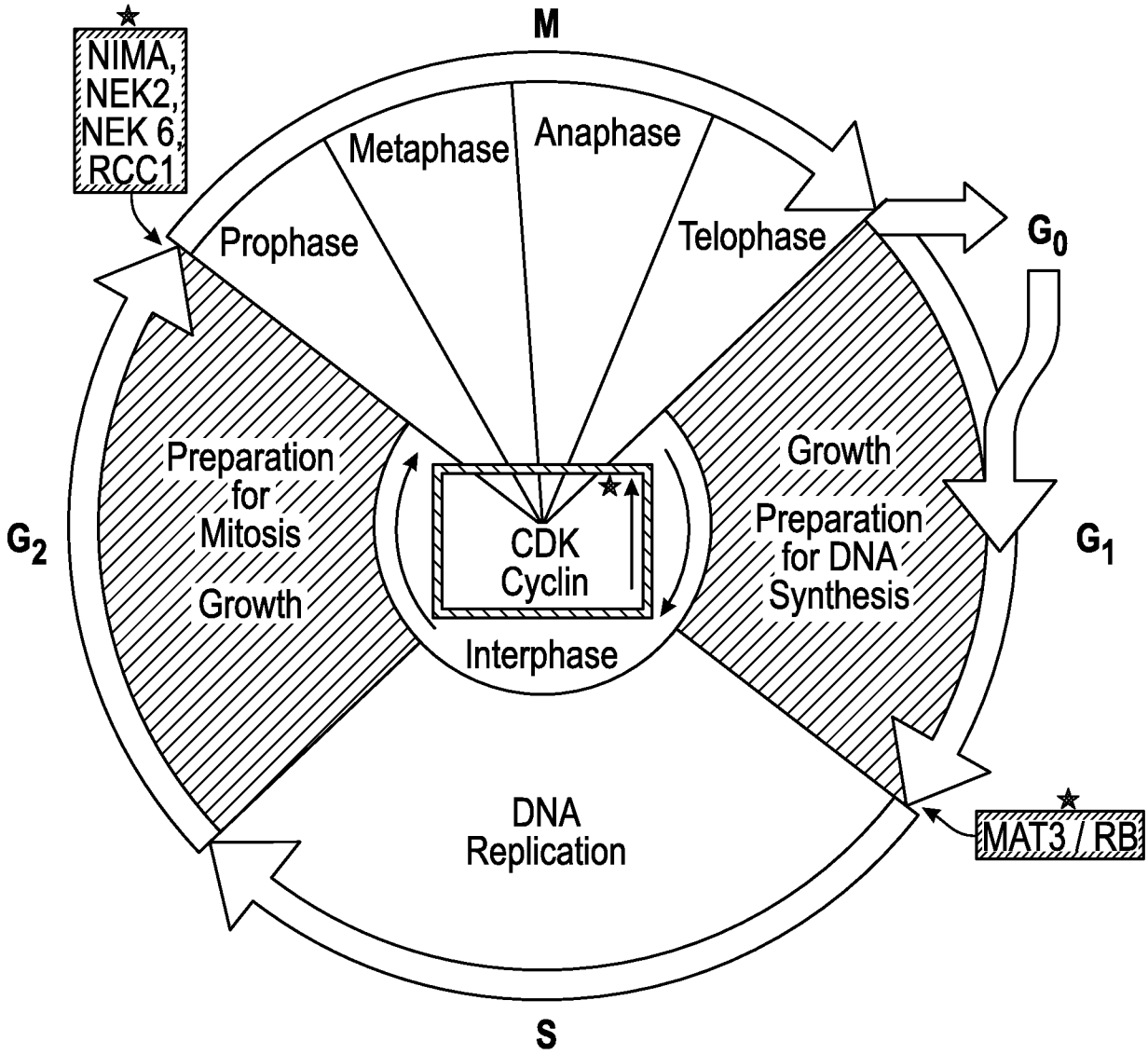

M
NIMA,
NEK2,
NEK 6,
RCC1
Metaphase
Anaphase
Prophase
Telophase
G0
Preparation
for
Mitosis
Growth
G2
CDK
Cyclin
Growth
Preparation
for DNA
Synthesis
G1
Interphase
DNA
Replication
MAT3 / RB
S

FIG. 9

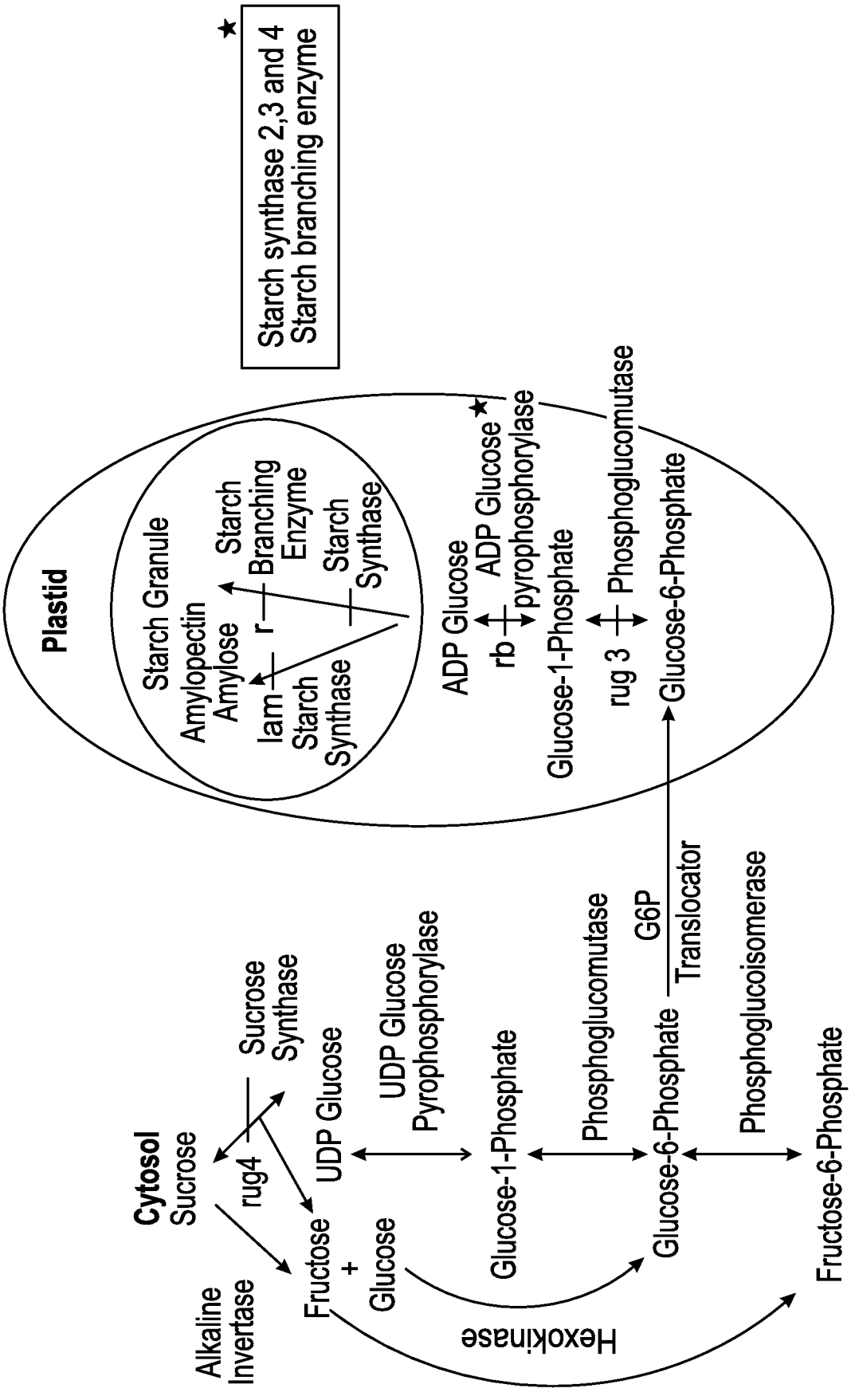

Starch synthase 2,3 and 4
Starch branching enzyme
Plastid
Starch Granule
Amylopectin
Amylose
Starch Branching Enzyme
Starch Synthase
Iam
Starch Synthase
r
ADP Glucose
ADP Glucose
rb
pyrophosphorylase
Glucose-1-Phosphate
rug 3
Phosphoglucomutase
Glucose-6-Phosphate
Cytosol
Sucrose
rug4
Sucrose Synthase
UDP Glucose
UDP Glucose Pyrophosphorylase
Glucose-1-Phosphate
Phosphoglucomutase
G6P
Translocator
Glucose-6-Phosphate
Phosphoglucoisomerase
Fructose-6-Phosphate
Alkaline Invertase
Fructose
+
Glucose
Hexokinase

FIG. 10

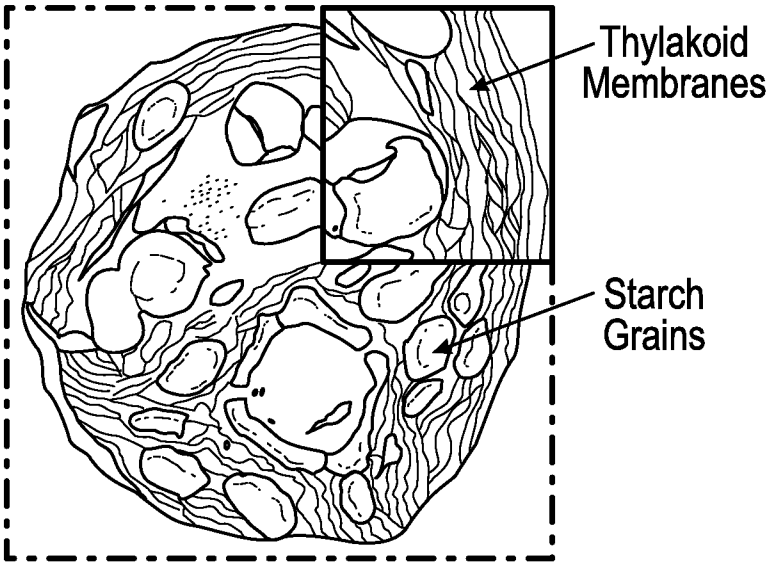


FIG. 11A

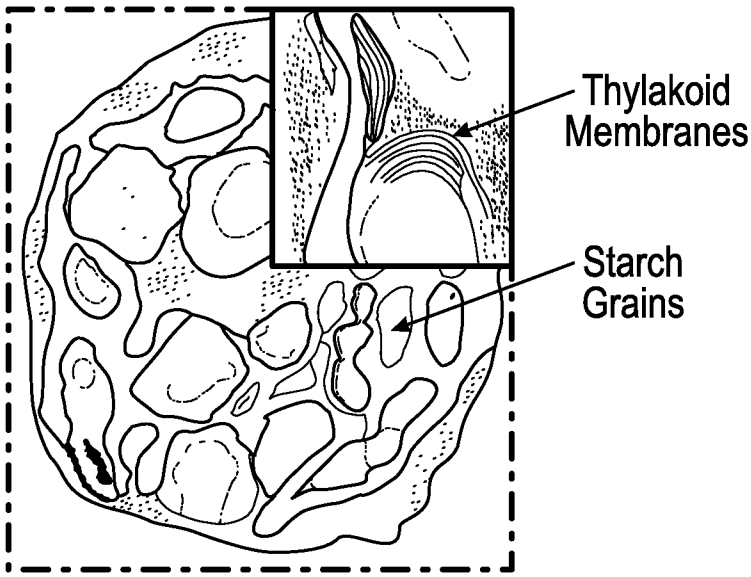


FIG. 11B

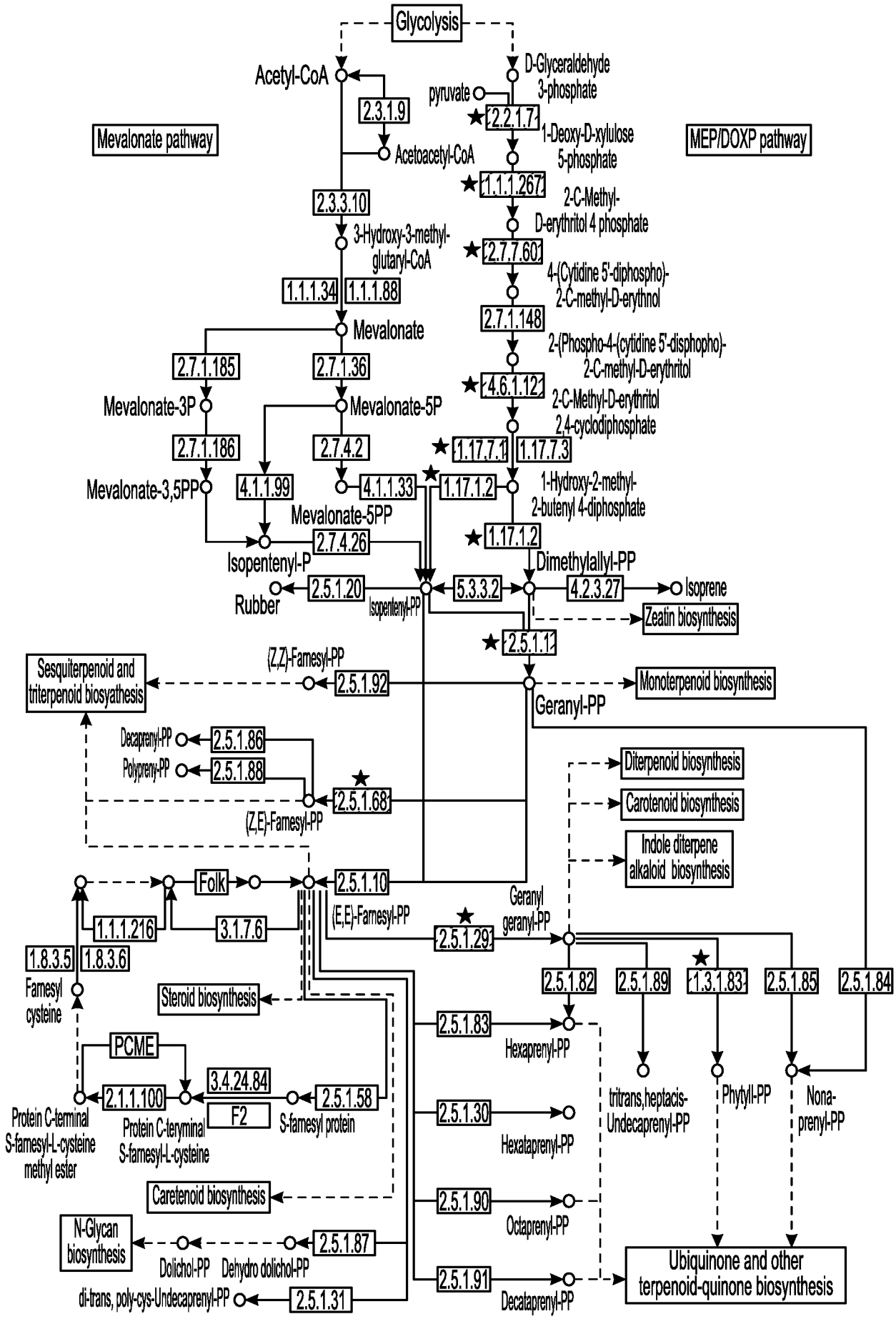

Glycolysis
Acetyl-CoA
pyruvate
D-Glyceraldehyde 3-phosphate
2.3.1.9
2.2.1.7
Mevalonate pathway
Acetoacetyl-CoA
1-Deoxy-D-xylulose 5-phosphate
MEP/DOXP pathway
1.1.1.267
2.3.3.10
2-C-Methyl-D-erythritol 4 phosphate
3-Hydroxy-3-methyl-glutaryl-CoA
2.7.7.60
4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythnol
1.1.1.34
1.1.1.88
2.7.1.148
Mevalonate
2-(Phospho-4-(cytidine 5'-disphopho)-2-C-methyl-D-erythritol
2.7.1.185
2.7.1.36
4.6.1.12
Mevalonate-3P
Mevalonate-5P
2-C-Methyl-D-erythritol 2,4-cyclodiphosphate
2.7.1.186
2.7.4.2
1.17.7.1
1.17.7.3
Mevalonate-3,5PP
4.1.1.99
4.1.1.33
1.17.1.2
1-Hydroxy-2-methyl-2-butenyl 4-diphosphate
Mevalonate-5PP
1.17.1.2
2.7.4.26
Isopentenyl-P
Dimethylallyl-PP
Rubber
2.5.1.20
Isopentenyl-PP
5.3.3.2
4.2.3.27
Isoprene
Zeatin biosynthesis
2.5.1.11
(Z,Z)-Farnesyl-PP
Sesquiterpenoid and triterpenoid biosyathesis
2.5.1.92
Monoterpenoid biosynthesis
Geranyl-PP
Decaprenyl-PP
2.5.1.86
Polyprenyl-PP
2.5.1.88
Diterpenoid biosynthesis
2.5.1.68
Carotenoid biosynthesis
(Z,E)-Farnesyl-PP
Indole diterpene alkaloid biosynthesis
Folk
2.5.1.10
Geranyl geranyl-PP
1.1.1.216
3.1.7.6
(E,E)-Farnesyl-PP
2.5.1.29
1.8.3.5
1.8.3.6
2.5.1.82
2.5.1.89
1.3.1.83
2.5.1.85
2.5.1.84
Farnesyl cysteine
Steroid biosynthesis
2.5.1.83
Hexaprenyl-PP
PCME
3.4.24.84
2.1.1.100
F2
2.5.1.58
tritrans,heptacis-Undecaprenyl-PP
Phytyl-PP
Nona-prenyl-PP
Protein C-terminal S-farnesyl-L-cysteine methyl ester
Protein C-teryminal S-farnesyl-L-cysteine
S-farnesyl protein
2.5.1.30
Hexataprenyl-PP
Caretenoid biosynthesis
2.5.1.90
Octaprenyl-PP
N-Glycan biosynthesis
2.5.1.87
Dolichol-PP
Dehydro dolichol-PP
Ubiquinone and other terpenoid-quinone biosynthesis
2.5.1.91
di-trans, poly-cys-Undecaprenyl-PP
2.5.1.31
Decataprenyl-PP

FIG. 12