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(54) **ALGAE AND METHOD FOR PRODUCING SAME, AND METHOD FOR PRODUCING BIOMASS USING SAID ALGAE**

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(51) **Int. Cl.**

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C12N 15/113	(2010.01)
C12P 1/00	(2006.01)
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C08B 30/00	(2006.01)
C12N 1/12	(2006.01)
C12N 15/79	(2006.01)

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CPC ***C12P 19/04*** (2013.01); ***C08B 30/00*** (2013.01); ***C12N 1/12*** (2013.01); ***C12N 15/09*** (2013.01); ***C12N 15/113*** (2013.01); ***C12N 15/79*** (2013.01); ***C12P 1/00*** (2013.01); ***C12P 7/64*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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Morell et al., Chap 10: Control of starch biosynthesis in vascular plants and algae. In Annual Plant Reviews, Control of Primary Metabolism in Plants, Edited by William C. Plaxton, Michael T. McManus Copyright © 2006 by Blackwell Publishing Ltd (pp. 258-289) Retrieved from the Internet:URL:http://epo.summon.serialssolutions.com/2.0.0/link/O/eLvHCXMwY2BQSUu2NEpONDXStUxNMdU1SbUwBbJME3XNUpMtk5MS08yTwde3RTqbhLibB7mb-.
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(57) **ABSTRACT**

The present invention provides a method of biomass production using a modified alga having suppressed expression of ATG8.

4 Claims, 7 Drawing Sheets

Specification includes a Sequence Listing.

FIG.1

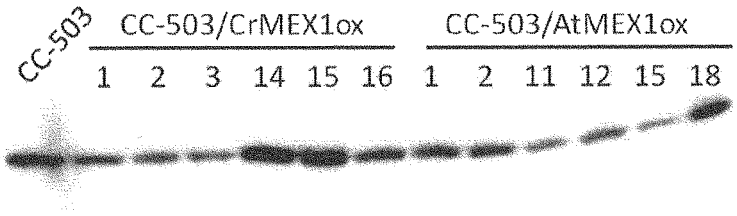

CC-503
CC-503/CrMEX1ox
1 2 3 14 15 16
CC-503/AtMEX1ox
1 2 11 12 15 18

FIG.2

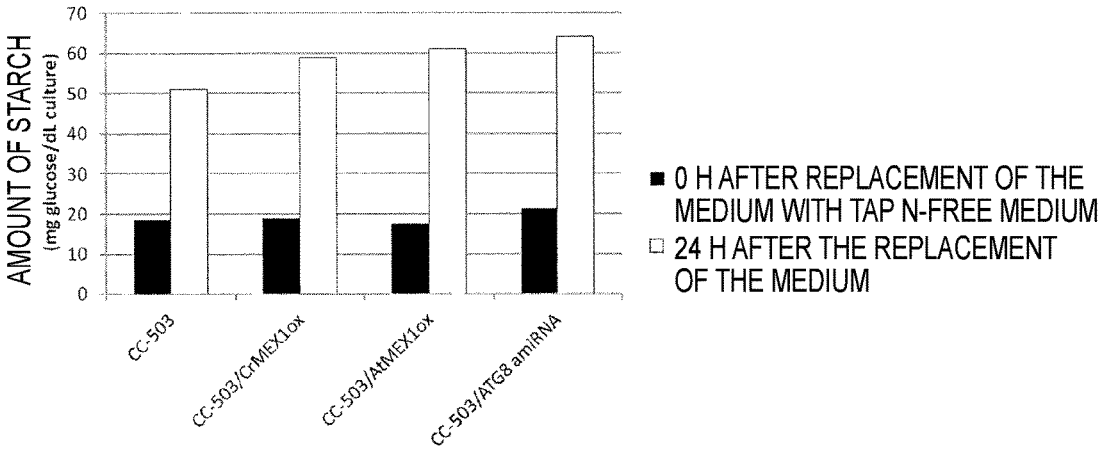

AMOUNT OF STARCH
(mg glucose/dL culture)
70
60
50
40
30
20
10
0
CC-503
CC-503/CrMEX1ox
CC-503/AtMEX1ox
CC-503/ATG8 amiRNA
0 H AFTER REPLACEMENT OF THE MEDIUM WITH TAP N-FREE MEDIUM
24 H AFTER THE REPLACEMENT OF THE MEDIUM

FIG.3

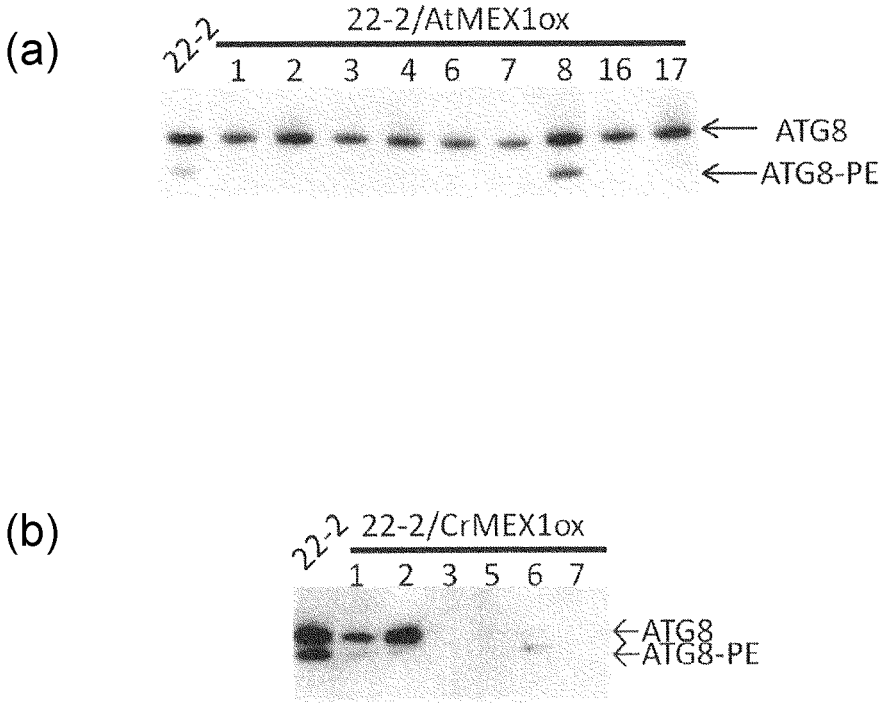

(a)
22-2
22-2/AtMEX1ox
1 2 3 4 6 7 8 16 17
ATG8
ATG8-PE
(b)
22-2
22-2/CrMEX1ox
1 2 3 5 6 7
ATG8
ATG8-PE

FIG.4

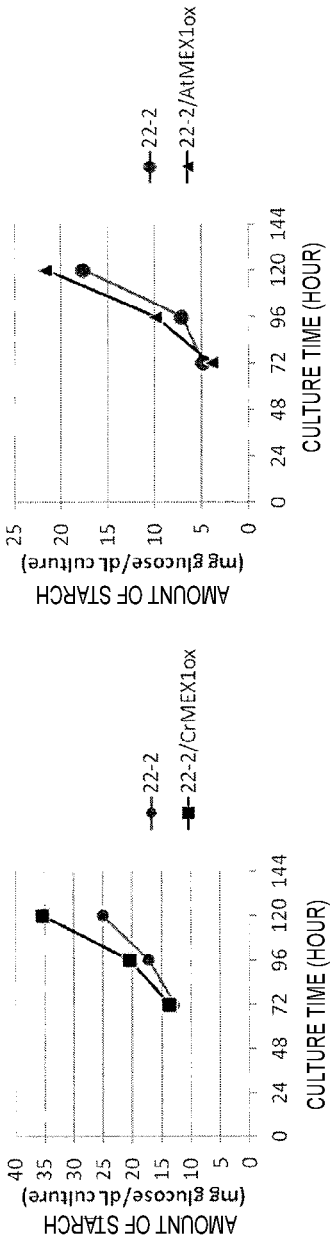
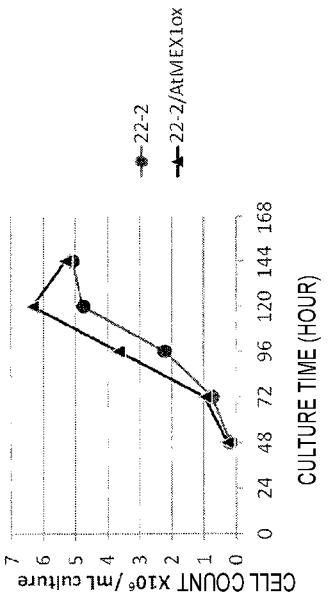
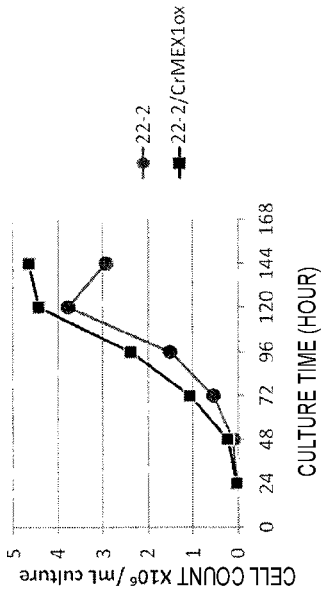

AMOUNT OF STARCH
(mg glucose/dL culture)
0
5
10
15
20
25
30
35
40
0 24 48 72 96 120 144
CULTURE TIME (HOUR)
22-2
22-2/CrMEX1ox
(a)
AMOUNT OF STARCH
(mg glucose/dL culture)
0
5
10
15
20
25
0 24 48 72 96 120 144
CULTURE TIME (HOUR)
22-2
22-2/AtMEX1ox
(b)

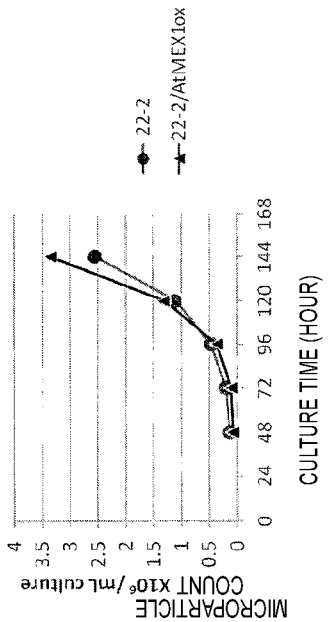
FIG.5



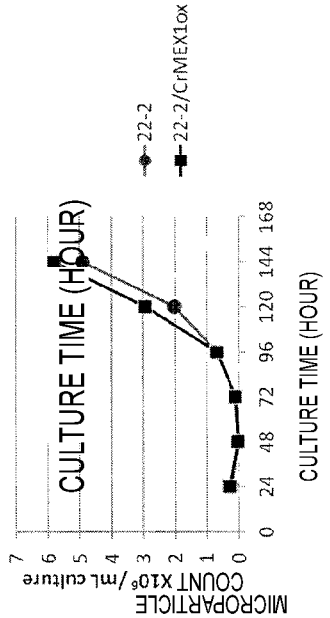
(a-2)



(a-1)



(b-2)



(b-1)

FIG.6

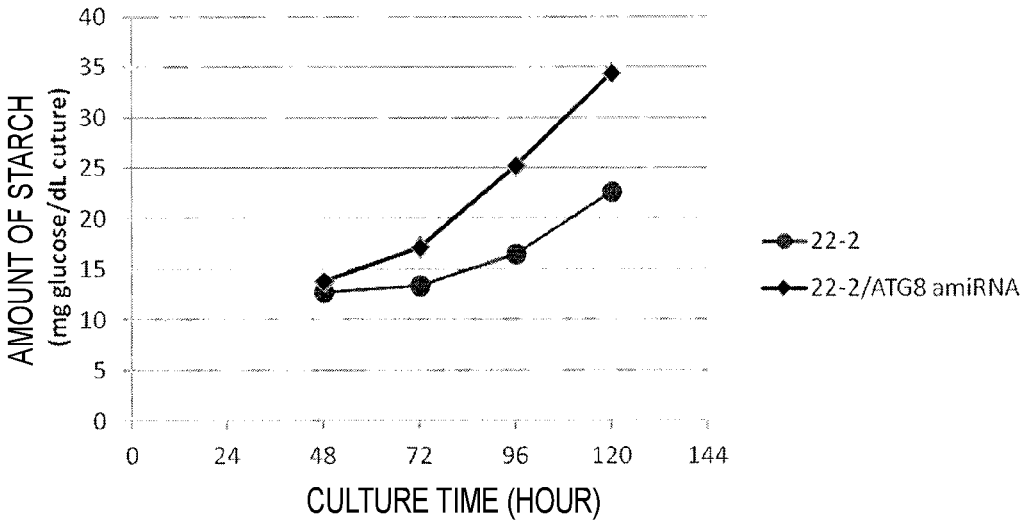
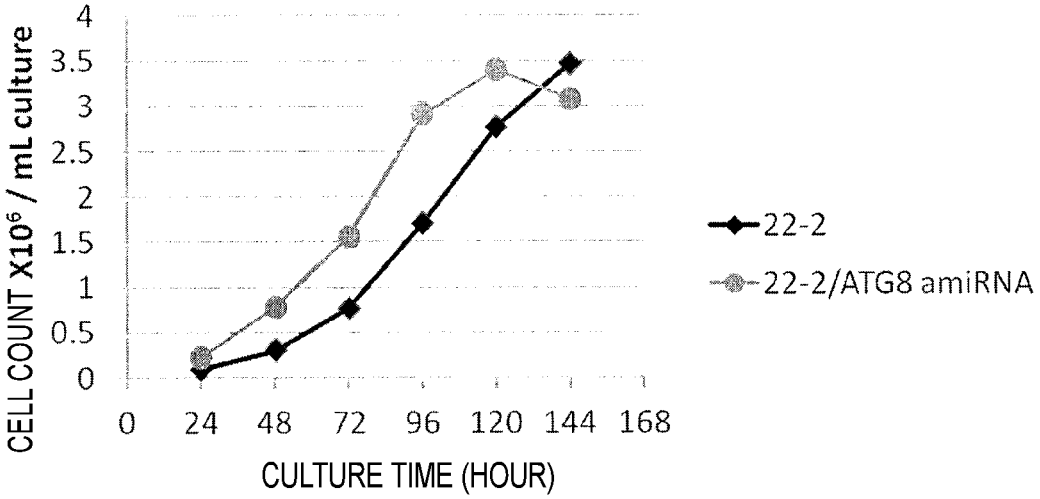
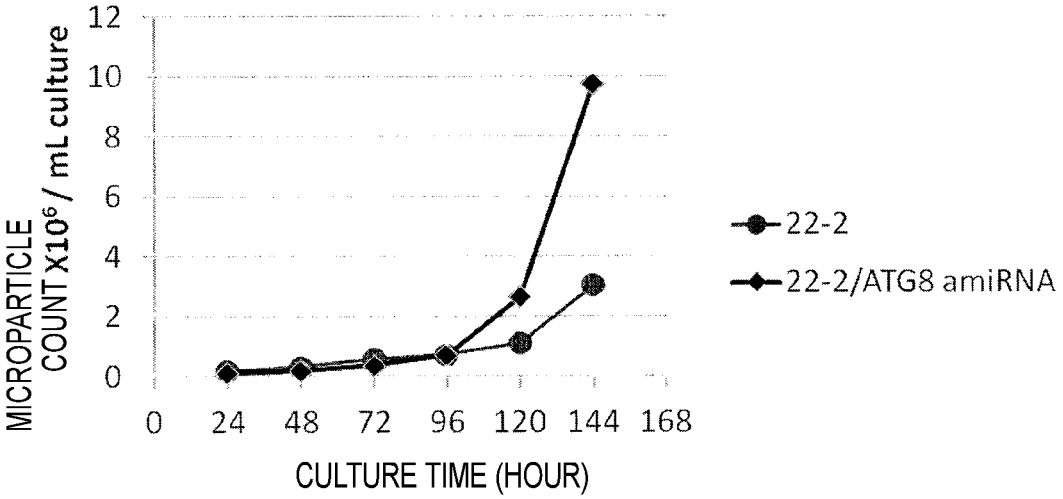

AMOUNT OF STARCH
(mg glucose/dL cuture)
40
35
30
25
20
15
10
5
0
0
24
48
72
96
120
144
CULTURE TIME (HOUR)
22-2
22-2/ATG8 amiRNA

FIG.7

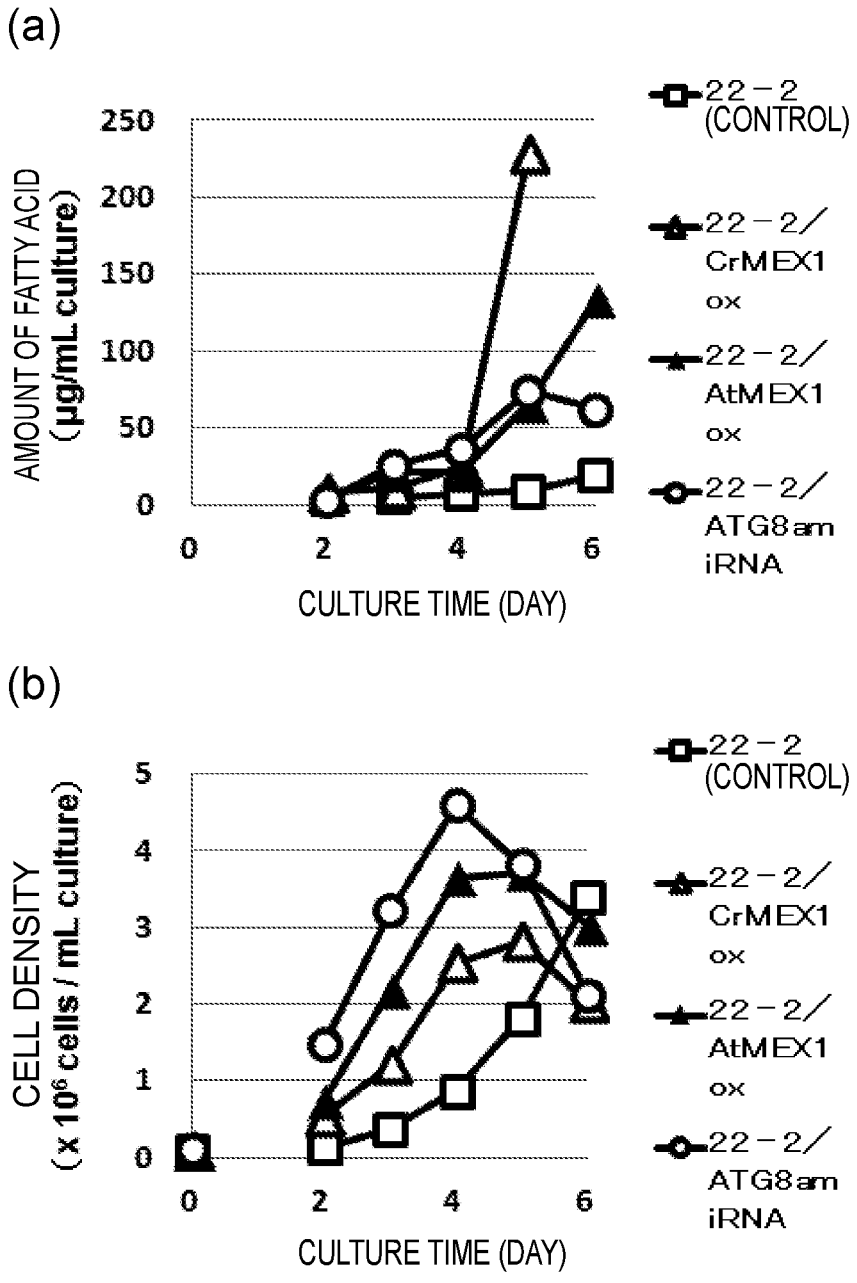

CELL COUNT X10⁶ / mL culture
4
3.5
3
2.5
2
1.5
1
0.5
0
0 24 48 72 96 120 144 168
CULTURE TIME (HOUR)
22-2
22-2/ATG8 amiRNA

(a)


MICROPARTICLE COUNT X10⁶ / mL culture
12
10
8
6
4
2
0
0 24 48 72 96 120 144 168
CULTURE TIME (HOUR)
22-2
22-2/ATG8 amiRNA

(b)

FIG.8


(a)
AMOUNT OF FATTY ACID (μg/mL culture)
250
200
150
100
50
0
0
2
4
6
CULTURE TIME (DAY)
22－2 (CONTROL)
22－2／CrMEX1 ox
22－2／AtMEX1 ox
22－2／ATG8am iRNA
(b)
CELL DENSITY (x 10⁶ cells / mL culture)
5
4
3
2
1
0
0
2
4
6
CULTURE TIME (DAY)
22－2 (CONTROL)
22－2／CrMEX1 ox
22－2／AtMEX1 ox
22－2／ATG8am iRNA

ALGAE AND METHOD FOR PRODUCING SAME, AND METHOD FOR PRODUCING BIOMASS USING SAID ALGAE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present invention is filed under 35 U.S.C. § 371 as the U.S. national phase of International Patent Application No. PCT/JP2015/002634, filed 26 May 2015, which designated the U.S. and claims the benefit of priority to Japanese Patent Application No. 2014-111577, filed 29 May 2014, each of which is hereby incorporated in its entirety including all tables, figures and claims.

TECHNICAL FIELD

The present invention relates to a modified alga, a method of producing the same, and a method of biomass production using the modified alga. More specifically, the invention relates to a modified alga with increased photosynthetic productivity.

BACKGROUND ART

Fuels from biomass, or so-called biofuels (such as bioethanol and biodiesel, for example) are promising alternatives to fossil fuels.

Biomass, a raw material for biofuel, includes saccharides (for example, starch) and oils and fats, and is produced by plants through photosynthesis. Accordingly, plants which are capable of active photosynthesis and intracellular accumulation of saccharides or oils and fats can be used as biomass sources. Corn and soybean are major plants that are currently used for biomass production. These crops are also consumed as food and forage, and dramatic increases in biofuel production would lead to soaring prices of food and forage, which has been disputed.

Under such circumstances, algae are attracting attention as alternative biomass sources to corn and soybean (see, for example, PTLs 1 and 2). Algal biomass production has advantages such as compatibility with food and forage supply and the massive algal growth.

For example, some mutants of *Chlamydomonas,* an alga, are known which lack a cell wall or have a thinner cell wall (cw15 and cw92, for example). These mutants have properties convenient for introduction of exogenous DNA into cells, and have been broadly used in gene transfer experiments. They are also helpful for increasing biomass productivity in that their cell is easily disrupted and facilitates recovery of contents thereof, and thus are reported to be used for biomass production. For example, PTL 3 discloses production of oils and fats using a cell-wall-deficient *Chlamydomonas* mutant. NPL 1 reports that a *Chlamydomonas* mutant with the cell wall mutation (cw15) and deficiency of a starch synthesis gene releases lipid droplets outside the cell. NPL 2 reports that a cell wall mutant of *Chlamydomonas* (cw15) further knocked out for a starch synthesis gene has increased productivity of oils and fats. NPL 3 is a known report on cell wall mutants of *Chlamydomonas.*

PTL 4 reports a technique involving recovery of starch produced and extracellularly released by an algal source, *Chlorella,* and subsequent fermentation of the starch to produce ethanol. PTL 5 discloses a technique of modifying an alga to have an increased chloroplastic glutathione concentration for increasing its starch productivity.

CITATION LIST

Patent Literature

[PTL 1] Japanese Unexamined Patent Application Publication No. 11-196885

[PTL 2] Japanese Unexamined Patent Application Publication No. 2003-310288

[PTL 3] WO 2009/153439

[PTL 4] Japanese Unexamined Patent Application Publication No. 2010-88334

[PTL 5] WO 2012/029727

Non Patent Literature

[NPL 1] Zi Teng Wang, Nico Ullrich, Sunjoo Joo, Sabine Waffenschmidt, and Ursula Goodenough (2009) Eukaryotic Cell Vol. (12): 1856-1868. Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless *Chlamydomonas reinhardtii.*

[NPL 2] Yantao Li, Danxiang Han, Guongrong Hu, David Dauvillee, Milton Sommerfeld, Steven Ball and Qiang Hu (2010) Metabolic Engineering Vol. 12 (4): 387-391. *Chlamydomonas* starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol.

[NPL 3] Jerry Hyams, D. Roy Davies (1972) Mutation Research 14 (4): 381-389. The induction and characterisation of cell wall mutants of *Chlamydomonas reinhardtii.*

SUMMARY OF INVENTION

Technical Problem

Unfortunately, the techniques of algal biomass production disclosed in PTLs 1 to 4 and NPLs 1 to 3 still need improvements in productivity. For example, biomass production involving culture of an alga under heterotrophic conditions where acetic acid serves as a carbon source requires a step of nutrient restriction, such as a step of providing a nitrogen-deficient condition, for inducing biomass production and accumulation in the alga. Algae are generally grown in a nitrogen-containing culture medium, and providing the nitrogen-deficient condition requires replacement of the culture medium with a nitrogen-free culture medium. It complicates the process, resulting in reduced productivity and increased costs. PTL 5 provides a solution to the issue, but the solution still has room for improvement in productivity.

The present invention was conceived as a solution to the existing issues, that is, an object of the invention is to provide a novel modified alga that can achieve increased biomass productivity, and use of the alga.

Solution to Problem

The inventors have made various studies mainly for the purpose of solving the issues, and have consequently found that suppression of ATG8 expression in algae can increase biomass productivity in algal cells. The invention was completed based on such finding.

The present invention involves the following aspects:

[1]. A modified alga having suppressed expression of ATG8 as compared to that of the reference strain.

[2]. The modified alga according to [1], wherein the alga overexpresses MEX1.

[3]. The modified alga according to [2], wherein the alga comprises an exogenous polynucleotide introduced therein, the exogenous polynucleotide encoding MEX1.

[4]. The modified alga according to [3], wherein the exogenous polynucleotide is one or more polynucleotides selected from the group consisting of:

(a) a polynucleotide encoding a polypeptide which comprises an amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3;

(b) a polynucleotide encoding a polypeptide which comprises an amino acid sequence derived from the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3 and maintaining the function of MEX1 wherein one or more amino acids are deleted, substituted, or added in the amino acid sequence; and

(c) a polynucleotide which is hybridizable with a polynucleotide comprising a base sequence complementary to that of the polynucleotide (a) or (b) under stringent conditions and encodes a polypeptide having the function of MEX1.

[5]. The modified alga according to [1], wherein the alga includes an ATG8 gene silenced.

[6]. The modified alga according to [5], wherein the alga includes a miRNA introduced therein, the miRNA having a base sequence set forth in SEQ ID NO: 5.

[7]. The modified alga according to any one of [1] to [6], wherein the alga has an increased chloroplastic glutathione concentration as compared to that of the reference strain.

[8]. A method of producing a modified alga, the method involving an ATG8 expression suppressing step to suppress expression of ATG8.

[9]. The method according to [8], wherein the method involves a glutathione enrichment step to increase a chloroplastic glutathione concentration.

[10]. A method of biomass production using a modified alga that has a suppressed expression of ATG8 as compared to that of the reference strain.

[11]. The method according to [10], wherein the method involves a photoirradiation step to irradiate the alga with light.

[12]. The method according to [10] or [11], wherein the alga has an increased chloroplastic glutathione concentration as compared to that of the reference strain.

[13]. The method according to [12], wherein the photoirradiation step is carried out under conditions substantially without nitrogen deficiency.

[14]. The method according to [13], wherein the method involves no cell lysis step to disrupt algal cells.

[15]. Starch produced using a modified alga that has suppressed expression of ATG8 as compared to that of the reference strain.

[16]. The starch according to [15], wherein the alga has an increased chloroplastic glutathione concentration as compared to that of the reference strain.

As used herein, the term "reference strain" refers to an algal strain before an inventive modification. Specifically, "reference strain" refers to an algal strain before a treatment for suppression of ATG8 expression, more specifically to that before overexpression of MEX1 or silencing of ATG8. If a wild-type algal strain is subjected to an inventive modification, the term "reference strain" refers to the wild-type strain or an algal strain of the same species. If an algal strain produced by a preliminary modification (for example, modification to increase the chloroplastic glutathione concentration) is further subjected to an inventive modification, the term "reference strain" refers to the algal strain produced by the preliminary modification or an algal strain of the same species.

Advantageous Effects of Invention

The inventive alga has suppressed expression of ATG8, and thus can achieve increased intracellular photosynthetic productivity. Accordingly, the inventive method of biomass production provides algal biomass production with lower costs and higher efficiency than traditional methods.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** illustrates the results of measurement of the ATG8 protein expressions in some of the "modified strains with suppressed expression of ATG8" produced in Example 1.

FIG. **2** illustrates the results of measurement of starch productions in the "modified strains with suppressed expression of ATG8" produced in Example 1.

FIG. **3** illustrates the results of measurement of the ATG8 protein expressions in some of the "modified strains with overexpression of GSH1 and suppressed expression of ATG8" produced in Example 1.

FIG. **4** illustrates the results of measurement of starch productions in some of the "modified strains with overexpression of GSH1 and suppressed expression of ATG8" produced in Example 1 and then cultured.

FIG. **5** illustrates the results of measurement of (a) cell counts and (b) microparticle counts in some of the "modified strains with overexpression of GSH1 and suppressed expression of ATG8" produced in Example 1 and then cultured.

FIG. **6** illustrates the results of measurement of starch productions in one of the "modified strains with overexpression of GSH1 and suppressed expression of ATG8" produced in Example 1 and then cultured.

FIG. **7** illustrates the results of measurement of (a) cell counts and (b) microparticle counts in the one of the "modified strains with overexpression of GSH1 and suppressed expression of ATG8" produced in Example 1 and then cultured.

FIG. **8** illustrates the results of measurement of (a) fatty acid levels and (b) cell counts in the "modified strains with overexpression of GSH1 and suppressed expression of ATG8" produced in Example 1 and then cultured.

DESCRIPTION OF EMBODIMENTS

Embodiments of the present invention will now be described in details, but should not be construed to limit the invention. The invention can be practiced in embodiments with various modifications to the embodiments below within the scope of the disclosure. All of the academic and patent documents mentioned herein are incorporated by reference in its entirety. As used herein, the expression "A to B" indicating a numerical range indicates "from A to B, inclusive of A and B", unless otherwise specified.

1. Inventive Alga

The inventive alga may have any structure having suppressed intracellular expression of ATG8, and should preferably be an alga with increased photosynthetic productivity.

"ATG8" is an abbreviation for "autophagy-related protein 8" and is also referred to as APG8. ATG8 is a ubiquitin-like protein, and is known to form a conjugate with phosphatidylethanolamine (hereinafter, the conjugate is referred to as "ATG8-PE") and to be involved in the formation of autophagosomal membranes (see Nakatogawa Het al .

(2007) Cell 130: 165-178 (non-patent literature)). As used herein, "suppressed expression of ATG8" refers to reduced intracellular expression of ATG8 as compared to that of a reference strain. The reduction of intracellular expression of ATG8 is preferably determined if the intracellular ATG8 expression in an alga is 0.9 times or less, more preferably with at least 5% significant difference as determined by t-test, as compared to that of a reference strain cultured under the same conditions. The expression of ATG8 by the alga of the invention is, for example, 90% or less, 80% or less, 70% or less, 60% or less, 50% or less, 40% or less, 30% or less, 20% or less, 10% or less, or 0% (less than the lower limit of detection), of that of a reference strain (see Examples 2 and 4).

Intracellular expression of ATG8 in a reference strain is preferably measured at the same time and by the same method as an inventive alga, but may be determined based on accumulated background data. Intracellular expression of ATG8 in an alga can be determined by any conventional technique such as Western blotting.

As used herein, "increased photosynthetic productivity" refers to an increased production of photosynthate as compared to that of a reference strain. The increase in photosynthetic productivity is preferably determined if the production of photosynthate in an alga is 1.1 times or more, more preferably with at least 5% significant difference determined by t-test, as compared to that of a reference strain cultured under the same conditions. The productivity can be evaluated from various viewpoints including conditions of photoirradiation (for example, quantity of light, intensity and duration of irradiation), nutrients applied, productivity per unit time, whether or not a step of providing a nutrient-deficient condition is essentially required, and culture temperature.

As used herein, the term "photosynthate" refers to substances produced by algae through photosynthetic carbon fixation, specifically to biomass such as saccharides (for example, starch) and oils and fats and derivatives (such as metabolites) thereof. As used herein, "photosynthetic carbon fixation" is a general term for metabolism of carbon compounds using chemical energy derived from light energy. Accordingly, the origin of carbon to be incorporated in a metabolic pathway includes not only inorganic compounds such as carbon dioxide but also organic compounds such as acetic acid.

As used herein, the "alga" may be any alga that is capable of photosynthesis, that is, biosynthesis of photosynthate. Such an alga include, for example, microalgae belonging to the class Chlorophyceae within the phylum Chlorophyta. More specifically, examples of the alga include: species belonging to the genus *Chlamydomonas* within the class Chlorophyceae, such as *Chlamydomonas reinhardtii, Chlamydomonas moewusii, Chlamydomonas eugametos,* and *Chlamydomonas segnis;* species belonging to the genus *Scenedesmus* within the class Chlorophyceae, such as *Scenedesmus acumunatus, Scenedesmus dimorphus, Scenedesmus disciformis,* and *Scenedesmus ovaltermus;* species belonging to the genus *Dunaliella* within the class Chlorophyceae, such as *Dunaliella salina, Dunaliella tertiolecta,* and *Dunaliella primolecta;* species belonging to the genus *Chlorella* within the class Chlorophyceae, such as *Chlorella vulgaris* and *Chlorella pyrenoidosa;* species belonging to the genus *Haematococcus* within the class Chlorophyceae, such as *Haematococcus pluvialis;* species belonging to the genus *Chlorococcum* within the class Chlorophyceae, such as *Chlorococcum littorale;* species belonging to the genus *Botryococcus* within the class Chlorophyceae or *Xanthophyceae,* such as *Botryococcus braunii;* species belonging to the genus *Choricystis* within the class Chlorophyceae, such as *Choricystis minor;* species belonging to the genus *Pseudochoricystis* within the class Chlorophyceae, such as *Pseudochoricystis ellipsoidea;* species belonging to the genus *Amphora* within the class Diatomophyceae (e.g., *Amphora* sp.); species belonging to the genus *Nitzschia* within the class Diatomophyceae, such as *Nitzschia alba, Nitzschia closterium,* and *Nitzschia laevis;* species belonging to the genus *Crypthecodinium* within the class Dinophyceae, such as *Crypthecodiniumcohnii;* species belonging to the genus *Euglena* within the class Euglenophyceae, such as *Euglena gracilis* and *Euglena proxima;* species belonging to the genus *Paramecium* within the phylum Ciliophora, such as *Paramecium bursaria;* species belonging to the genus *Synechococcus* within the phylum Cyanobacteria, such as *Synechococcus aquatilis* and *Synechococcus elongatus;* species belonging to the genus *Spirulina* within the phylum Cyanobacteria, such as *Spirulina platensis* and *Spirulina subsalsa;* species belonging to the genus *Prochlorococcus* within the phylum Cyanobacteria, such as *Prochlorococcus marinus;* and species belonging to the genus *Oocystis* within the phylum Cyanobacteria, such as *Oocystis polymorpha.*

An alga with suppressed intracellular expression of ATG8 may be produced by any method that will be described in detail in a section explaining an inventive method of producing a modified alga.

In one embodiment, the inventive alga preferably has increased intracellular expression and/or activity of MEX1 (maltose transporter gene) as compared to that of a reference strain. MEX1 is known to serve in a cell as a transporter to deliver maltose from chloroplast to cytoplasm (see Niittyla T et al. (2004), Science 303 (5654): 87-89 (non-patent literature)). Overexpression of MEX1 in algae results in decreased expression of ATG8.

The inventive alga may include a polynucleotide that has been expressibly introduced therein and encodes an MEX1 protein. Introduction of such an exogenous polynucleotide suppresses intracellular expression of ATG8 in the alga through overexpression of MEX1.

In other words, the invention provides a transformed (modified) alga which includes a polynucleotide introduced therein and encoding an MEX1 protein and has suppressed intracellular expression of ATG8. Such a transformed alga achieves increased photosynthetic productivity as compared to a reference strain, as will be described below.

The MEX1 protein to be expressed in the alga or the polynucleotide encoding the MEX1 protein maybe from any origin if it can be introduced or expressed to exert its action in a host alga. Examples of such an MEX1 protein or a polynucleotide encoding the MEX1 protein include MEX1 proteins from the host alga, algae of other species than the host alga, and other plants, and polynucleotides encoding the MEX1 proteins. An MEX1 protein from other plant or a polynucleotide encoding the MEX1 protein is preferably derived from a plant belonging to the genus *Arabidopsis* within the class Dicotyledoneae, for example.

Specific examples of a polynucleotide encoding an MEX1 protein to be expressed include (a) a polynucleotide encoding an MEX1 protein which comprises an amino acid sequence set forth in SEQ ID NO: 1 and is derived from *Arabidopsis thaliana* (the base sequence of such a polynucleotide is set forth in SEQ ID NO: 2); and a polynucleotide encoding an MEX1 protein which comprises an amino acid sequence set forth in SEQ ID NO: 3 and is derived from

Chlamydomonas reinhardtii (the base sequence of such a polynucleotide is set forth in SEQ ID NO: 4).

Specific examples of a polynucleotide encoding an MEX1 protein to be expressed also include:

(b) a polynucleotide encoding a polypeptide which comprises an amino acid sequence derived from the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3 and maintaining the function of MEX1 wherein one or more amino acids are deleted, substituted, or added in the amino acid sequence; and

(c) a polynucleotide which is hybridizable with a polynucleotide comprising a base sequence complementary to that of the polynucleotide (a) or (b) under stringent conditions and encodes a polypeptide having the function of MEX1. Such polynucleotides (a) to (c) will be described below in more details.

Other specific examples of a polynucleotide encoding an MEX1 protein include a polynucleotide encoding an MEX1 protein which comprises an amino acid sequence set forth in SEQ ID NO: 17 and is derived from *Arabidopsis thaliana* (the base sequence of such a polynucleotide is set forth in SEQ ID NO: 18); and a polynucleotide encoding an MEX1 protein which comprises an amino acid sequence set forth in SEQ ID NO: 19 and is derived from *Chlamydomonas reinhardtii* (the base sequence of such a polynucleotide is set forth in SEQ ID NO: 20).

In another embodiment, the inventive alga preferably includes an ATG8 gene silenced. As used herein, the term "silencing" refers to decreasing the amount of a specific messenger RNA. "Silencing" encompasses transcriptional gene silencing and post-transcriptional gene silencing. Examples of the transcriptional gene silencing include epigenetic silencing, genomic imprinting, paramutation, transposon silencing, transgene silencing, and position effect. Examples of the post-transcriptional gene silencing include silencing using microRNA (miRNA), RNA interference, and nonsense mediated decay. The reduction of intracellular messenger RNA level is preferably determined if the intracellular messenger RNA level in an alga is 0.9 times or less, more preferably with at least 5% significant difference determined by t-test, as compared to that of a reference strain cultured under the same conditions.

Intracellular messenger RNA level in a reference strain is preferably measured at the same time and by the same method as that of an inventive alga, but may be determined based on accumulated background data. Intracellular messenger RNA level in an alga can be determined by any conventional technique such as real-time RT-PCR technique.

Examples of silencing applicable to the invention include silencing using miRNAs, i.e. low-molecular-weight RNAs produced from genes coding for primary transcripts of various sizes. A primary transcript (referred to as "pri-miRNA") is subjected to various nucleolytic steps to be processed into a shorter precursor miRNA or "pre-miRNA". The pre-miRNA is present in a folded form, and a resulting final (mature) miRNA is present in a form of duplex. The strands of the duplex are referred to as miRNAs (one of which eventually form a base pair with a target). The pre-miRNA is a substrate for a dicer that processes the precursor to generate a miRNA duplex. As with siRNA, one of the two strands of the miRNA duplex can be then incorporated into an RNA-induced splicing complex (RISC). A miRNA can be expressed by gene transfer. It binds to a target transcript sequence that is only partially complementary to the miRNA (see, for example, Zeng Y et al. (2002), Mol. Cell 9: 1327-1333) to inhibit translation of the target without affecting levels of non-target RNAs in the steady state (see, for example, Lee R C et al . (1993), Cell 75: 843-854; and Wightman B et al. (1993), Cell 75: 855-862). Examples of a miRNA applicable to the invention include artificial microRNAs (amiRNAs), i.e. molecules designed to induce silencing via the same mechanism as miRNAs. One of specific examples thereof is an amiRNA having the base sequence set forth in SEQ ID NO: 5.

An inventive alga with suppressed expression of ATG8 has increased production and/or accumulation of photosynthate as compared to that of a reference strain without suppression in expression of ATG8, and thus can allows algal biomass production with lower costs and higher efficiency than traditional methods.

The inventive alga preferably has both suppressed expression of ATG8 and increased chloroplastic glutathione concentration.

As used herein, "increased chloroplastic glutathione concentration" refers to a higher glutathione concentration in chloroplast as compared to that of a reference strain. The increase in chloroplastic glutathione concentration is preferably determined if the chloroplastic glutathione concentration in an alga is 1.1 times or more, more preferably with at least 5% significant difference determined by t-test, as compared to that of a reference strain cultured under the same conditions. The chloroplastic glutathione concentration of a reference strain is preferably measured at the same time and by the same method as that of an inventive alga, but may be determined based on accumulated background data.

The chloroplastic glutathione concentration of an alga can be directly measured by expressing roGFP2, i.e. a molecular probe that visualizes the redox state via redox-responsive changes in its fluorescence color, in chloroplast (see, for example, Meyer A J et al. (2007), Plant Journal 52: 973-986. Redox-sensitive GFP in *Arabidopsis thaliana* is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer; and Gutscher M et al. (2009), Nat Methods 5: 553-559. Real-time imaging of the intracellular glutathione redox potential). Alternatively, the increase in glutathione concentration can be indirectly determined based on an increase in expression level of a protein involved in biosynthesis of glutathione or a polynucleotide encoding such a protein. Expression level of such a protein or polynucleotide can be appropriately measured by any conventional technique.

The "glutathione" includes reduced glutathione (hereinafter, referred to as "GSH") and oxidized glutathione (hereinafter, referred to as "GSSG"). In a method of the present invention, GSH and GSSG concentrations may be increased either alone or together.

The inventive alga preferably has increased chloroplastic expression and/or activity of at least one protein selected from the group consisting of γ-glutamylcysteine synthetase (hereinafter, also referred to as "GSH1"), glutathione synthetase (hereinafter, also referred to as "GSH2"), ATP sulfurylase, adenosine 5'-phosphosulfate reductase, sulfite reductase, cysteine synthase, and serine acetyltransferase. These proteins are involved in chloroplastic biosynthesis of glutathione, and an increased expression thereof indicate an increased chloroplastic glutathione concentration.

An inventive alga may further include a polynucleotide introduced therein and encoding at least one protein selected from the group consisting of GSH1, GSH2, ATP sulfurylase, adenosine 5'-phosphosulfate reductase, sulfite reductase, cysteine synthase, and serine acetyltransferase, in addition to a polynucleotide encoding MEX1 and/or a nucleic acid for silencing ATG8. An alga which includes such an exogenous

polynucleotide introduced and expressed (overexpressed) therein has an increased chloroplastic glutathione concentration.

In other words, the present invention provides a transformed alga which includes a polynucleotide introduced therein and encoding at least one protein selected from the group consisting of GSH1, GSH2, ATP sulfurylase, adenosine 5'-phosphosulfate reductase, sulfite reductase, cysteine synthase, and serine acetyltransferase, in addition to a polynucleotide encoding MEX1 and/or a nucleic acid for silencing ATG8, and has increased chloroplastic glutathione concentration. Such a transformed alga naturally has an increased photosynthetic productivity.

Such a protein to be expressed in an alga or the polynucleotide encoding the protein may be from any origin if the protein or polynucleotide can be introduced or expressed to exert its action in the host alga, and may be either derived from the host alga, or from algae of other species than the host alga or from other plants. Preferred examples of the aforementioned proteins from other plant or polynucleotides encoding the proteins include those from a plant belonging to the genus *Arabidopsis* within the class Dicotyledoneae.

Specific examples of a polynucleotide encoding γ-glutamylcysteine synthetase to be expressed include (a) a polynucleotide encoding a γ-glutamylcysteine synthetase which comprises an amino acid sequence set forth in SEQ ID NO: 6 and is derived from *Chlamydomonas reinhardtii* (the base sequence of such a polynucleotide is set forth in SEQ ID NO: 7).

Specific examples of a polynucleotide encoding γ-glutamylcysteine synthetase to be expressed also include:

(b) a polynucleotide encoding a polypeptide which comprises an amino acid sequence derived from the amino acid sequence set forth in SEQ ID NO: 6 and maintaining the activity of γ-glutamylcysteine synthetase wherein one or more amino acids are deleted, substituted, or added in the amino acid sequence; and

(c) a polynucleotide which is hybridizable with a polynucleotide comprising a base sequence complementary to that of the polynucleotide (a) or (b) under stringent conditions and encodes a polypeptide having the activity of γ-glutamylcysteine synthetase. Such polynucleotides (a) to (c) will be described below in more details.

Introduction of such a polynucleotide in the cell of the inventive alga can be confirmed by any conventional technique such as PCR, southern hybridization, or northern hybridization. Alternatively, the introduction of the polynucleotide may also be confirmed by measuring expression of a protein encoded by the polynucleotide by any conventionally known immunological technique, or by measuring enzymatic activity of the protein encoded by the polynucleotide by any conventional biochemical technique.

An inventive alga with suppressed expression of ATG8 can achieve increased production and/or accumulation of photosynthate as compared to that of a reference strain. An inventive alga with both suppressed expression of ATG8 and increased chloroplastic glutathione concentration can achieve more increase in the production and/or accumulation of photosynthate as compared to that of a reference strain.

An alga with both suppressed expression of ATG8 and increased chloroplastic glutathione concentration can produce and/or accumulate photosynthate in a nitrogen-sufficient medium without requiring a nitrogen-deficient medium, which saves time and effort in replacement of the medium. Such an alga is also advantageous in that production and/or accumulation of photosynthate thereof can be readily enhanced by a mere slight improvement in light conditions.

Such an alga with both suppressed expression of ATG8 and increased chloroplastic glutathione concentration can cause extracellular transfer of photosynthate accumulated in the algal cell, which facilitates recovery of photosynthate. For example, if a photosynthate is starch, starch accumulated during photosynthesis can be transferred outside the cell as starch granules without requiring disruption of the algal cell. Accordingly, biomass production using an alga with suppressed expression of ATG8 and increased chloroplastic glutathione concentration enables relatively easy purification of starch.

The inventive alga increases facility and efficiency in induction of accumulation of photosynthate and/or recovery thereof, as compared to traditional techniques. Accordingly, use of the inventive alga in a method of biomass production that will be described below provides algal biomass production with lower costs and higher efficiency than traditional methods.

2. Starch Produced by an Inventive Alga

Starch granules produced by an inventive alga are characterized by a minute particle size. For example, general starch granules produced by plants such as corn, potato, and wheat have a mean particle size of 10 to 50 μm. In contrast, starch granules produced by the inventive alga are minute and uniform in size, with a mean particle size of 1.3 μm (S.D.: 0.181) in a major diameter and 1.0 μm (S.D.: 0.204) in a minor diameter. Starch granules produced by plants such as rice and quinoa are also minute, with a mean particle size of about 2 to 3 μm, but form an endosperm tissue via adhesion to each other, as in starch granules produced by plants such as corn, potato, and wheat. Accordingly, a costly process such as grinding is required for preparation of minute starch granules in a form of disaggregated particles from a raw material such as corn, potato, wheat, rice, or quinoa. Such minute starch granules are useful in production of pharmaceutical products. In other words, use of an inventive alga allows massive production of starch granules that are minute and uniform in size, and also enables a relatively easy purification thereof, via transfer of the produced starch granules outside the algal cell. Further, use of the inventive alga provides starch granules in a disaggregated form without requiring a process such as grinding.

As explained above, starch granules produced by the inventive alga are minute as compared to general starch granules produced by plants such as corn, potato, or wheat. Such minute starch granules are useful in production of pharmaceutical products. Specifically, such minute starch granules have a particle size smaller than a diameter of bronchioles in lungs, and will be a promising carrier for delivering a therapeutic agent for a lung disease to the bronchioles (dry powder inhaler containing a therapeutic agent combined with minute starch granules).

Such minute starch granules are also expected to be used in a form of so-called "edible vaccine", i.e. a peptidic antigen associated with the starch granule surface (for example, Dauvillee D et al. (2010) PLoS ONE 5(12): e15424; and Japanese Unexamined Patent Application Publication (Translation of PCT Application) No. 2003-500060 (Japanese Patent Application No. 2000-620111) disclose genetically engineered starch granules produced in *Chlamydomonas* and containing a malarial antigen associated on their surface.

3. Inventive Method of Producing Modified Alga

An inventive method of producing an alga produces the aforementioned modified alga with suppressed intracellular expression of ATG8 and increased photosynthetic productivity as compared to that of a reference strain (i.e. the inventive alga). The inventive method involves at least an ATG8 expression suppressing step to suppress expression of ATG8 in the algal cell, and may involve any other step and may be practiced under a variety of conditions.

The inventive method of producing a modified alga will now be described in detail.

(1) ATG8 Expression Suppressing Step

The ATG8 expression suppressing step suppresses the expression of ATG8 in an alga.

The expression "suppress the expression of ATG8" refers to reducing the expression of ATG8 in an alga as compared to that of a reference strain. In other words, an alga after the ATG8 expression suppressing step has decreased expression of ATG8 as compared to that of a reference strain. The reduction in expression of ATG8 in an alga as compared to that of a reference strain can be determined by a method described in the section explaining the inventive alga.

In the ATG8 expression suppressing step, expression of ATG8 may be suppressed by any method that can produce an alga with decreased expression of ATG8. Examples of such a method include (i) random mutagenesis in the alga and (ii) introduction of a substance which suppresses the expression of ATG8 into the algal cell (or into the algal genome, in some cases).

The methods (i) and (ii) are now described in detail.

(i) Random Mutagenesis in an Alga

Random mutation may be introduced in an alga by any method appropriately selected from known techniques. Specific examples of the method for random mutagenesis include chemical treatment of an alga (with EMS or NTG, for example), radioactive mutagenesis, transposon mutagenesis, T-DNA mutagenesis, mutagenesis using prokaryotic-eukaryotic cell conjugation, and physical gene transfer using a gene gun, for example. For example, mutation may be introduced into an ATG8 gene or a gene encoding a protein which positively controls the expression of ATG8 by such a method, so as to decrease the expression of ATG8. Alternatively, mutation may be introduced into a gene encoding a protein which negatively controls the expression of ATG8 by any of the methods described above, so as to increase the activity of the protein to decrease the expression of ATG8.

Algae with desired mutation may be screened by any known method. Examples of the screening method include selection of mutant algae with suppressed expression of ATG8 based on the direct measurement of intracellular expression of ATG8 as described above, and selection of mutant algae with increased expression and/or activity of MEX1 protein.

(ii) Introduction of Substance which Suppresses Expression of ATG8 into Cell

A modified alga with decreased intracellular expression of ATG8 can also be produced by introducing the "substance which suppresses intracellular expression of ATG8" as described above, such as (A) a polynucleotide encoding a protein which suppresses expression of ATG8 or (B) a polynucleotide having a function of silencing the ATG8 gene, for example. Such polynucleotides (A) and (B) may be used either alone or in combination.

As used herein, the term "polypeptide" is interchangeable with the terms "peptide" or "protein". As used herein, the term "polynucleotide" is interchangeable with the term "gene", "nucleic acid" or "nucleic acid molecule" and refers to a nucleotide polymer.

The "introduction of a polynucleotide" refers to any process that allows an intended polynucleotide to be included in the algal cell, and include insertion (introduction) of the intended polynucleotide in an algal genome. Successful introduction of a polynucleotide in an algal cell can be confirmed by any conventional technique such as PCR, southern hybridization, or northern hybridization.

Introduction of at least one polynucleotide (A) into an algal cell increases the expression of a protein which suppresses intracellular expression of ATG8, resulting in suppressed expression of ATG8 in the cell.

Preferred examples of such a polynucleotide include a polynucleotide encoding MEX1 (hereinafter, also referred to as "MEX1 gene"). Such a polynucleotide is preferably from a plant, more preferably from the host alga, but polynucleotides from algae other than the host alga or from higher plants may also be suitably used.

The "MEX1 gene" is not limited to specific genes. Examples of MEX1 genes preferred in the invention include the MEX1 gene of *Chlamydomonas* used by the inventors in the Examples. *Chlamydomonas* MEX1 has the amino acid sequence set forth in SEQ ID NO: 1, and a gene (full-length cDNA) encoding it has the base sequence set forth in SEQ ID NO: 2.

Another example of MEX1 genes preferred in the invention is the MEX1 gene of *Arabidopsis thaliana* used by the inventors in the Examples. MEX1 of *Arabidopsis thaliana* has the amino acid sequence set forth in SEQ ID NO: 3, and a gene (full-length cDNA) encoding it has the base sequence set forth in SEQ ID NO: 4.

In summary, preferred examples of the nucleotide to be introduced in an alga in the invention include the following polynucleotides (a) to (c):

(a) a polynucleotide encoding a polypeptide which comprises an amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3;

(b) a polynucleotide encoding a polypeptide which includes an amino acid sequence derived from the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3 and maintaining the function of MEX1 wherein one or more amino acids are deleted, substituted, or added in the amino acid sequence; and

(c) a polynucleotide which is hybridizable with a polynucleotide comprising a base sequence complementary to that of the polynucleotide (a) or (b) under stringent conditions and encodes a polypeptide having the function of MEX1.

The expression "deletion, substitution, or addition of one or more amino acids" refers to deletion, substitution, or addition of any number of amino acids that can be involved in deletion, substitution, or addition by a known technique of producing a mutant peptide, such as site-specific mutagenesis (preferably ten or less, more preferably seven or less, yet more preferably five or less amino acids). Such a mutant protein includes not only proteins having mutation artificially introduced by a known technique of producing a mutant polypeptide but also proteins isolated and purified from naturally-occurring proteins.

It is well-known in the art that one or more amino acids in an amino acid sequence of a protein may be readily modified without any significant influence on its original structure or function. It is also well-known that mutant proteins include not only artificially modified proteins but also naturally-occurring mutant proteins that substantially maintain the structure and function of the original protein.

Preferred mutants have conservative or non-conservative amino acid substitution, deletion, or addition, preferably silent substitution, addition, or deletion. Conservative substitution is particularly preferred. Such mutation does not change the polypeptide activity in the present invention.

Typical conservative substitutions include the following substitutions: one substitution among aliphatic amino acids, i.e. Ala, Val, Leu, and Ile; substitution among hydroxyl residues, i.e. Ser and Thr; substitution among acidic residues, i.e. Asp and Glu; substitution among amido residues, i.e. Asn and Gln; substitution among basic residues, i.e. Lys and Arg; and substitution among aromatic residues, i.e. Phe and Tyr.

As used herein, the term "stringent conditions" refers to conditions where polynucleotides hybridize with each other only if they have sequence identity of at least 90%, preferably at least 95%, most preferably at least 97%. Specific examples of such conditions include conditions involving overnight incubation in a hybridization solution (containing 50% formamide, 5×SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH: 7.6), 5× Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA) at 42° C., followed by washing of the filter in 0.1×SSC at about 65° C.

Hybridization can be carried out by any well-known technique such as the method described in Sambrook J et al. (2001), Molecular Cloning, A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory. In general, a higher temperature or lower salt concentration increases stringency (i.e. decreases the probability of hybridization). As a result, hybridization under higher stringency provides a polynucleotide with higher homology.

The identity of amino acid sequences or base sequences can be determined using the BLAST algorithm as described by Karlin and Altschul (Karlin S, Altschul S F (1990), Proc. Natl. Acad. Sci. USA 87: 2264-2268; and Karlin S, Altschul S F (1993), Proc. Natl. Acad. Sci. USA, 90: 5873-5877). Programs based on the BLAST algorithm, such as BLASTN and BLASTX, have also been developed (Altschul S F et al. (1990), J. Mol. Biol. 215: 403-410).

MEX1 genes from other plants than *Chlamydomonas* may also be suitably used in the invention. Known examples of such MEX1 genes include those from *Arabidopsis thaliana* (TAIR Accession Gene: 2157491; Name: AT5G17520.1), *Glycine max* (NCBI Reference Sequence: XP_003539988), *Oryza sativa* (Genbank accession: AGR54532.1), and *Malus domestica* (Genbank accession: DQ648082.1).

Alternatively, introduction of the aforementioned polynucleotide (B) into an algal cell can decrease expression of ATG8 in the cell. Examples of such a polynucleotide include double-stranded RNAs (dsRNAs), small interfering RNAs (siRNAs), template DNAs for these RNAs, and microRNAs (miRNAs), as used in a conventional RNA interference (RNAi) technique. Such polynucleotides inhibit transcription and/or translation of an ATG8 gene, for example.

Preferred examples of the miRNA usable in the invention include, but are not limited to, the miRNA of SEQ ID NO: 5.

"The polynucleotide" used in the inventive method of producing a modified alga may be a DNA from a genomic DNA or a cDNA, a chemically synthesized DNA, or an RNA, and may be appropriately selected depending on the purpose.

If a polynucleotide used in the inventive method of producing a modified alga is, for example, an MEX1 gene, examples of a method of preparing the polynucleotide include isolating and cloning a DNA fragment encoding MEX1 by a known technique. In such a case, a probe is prepared which can specifically hybridize with a partial base sequence of a DNA encoding *Chlamydomonas* MEX1, and a genomic DNA library or cDNA library is screened with the probe.

Alternatively, a polynucleotide used in the inventive method of producing a modified alga may be prepared by an amplification technique such as PCR. For example, an MEX1 gene may be prepared by designing a set of primers based on 5'- and 3'-terminal sequences (or complementary sequences thereof), respectively, of a cDNA encoding *Chlamydomonas* MEX1; and carrying out a process such as PCR with the primer set using a genomic DNA (or cDNA) as template, to amplify a DNA region between the primers. Such a method allows large-scale preparation of MEX1-encoding DNA fragments (MEX1 gene) used in the invention.

The polynucleotide used in the inventive method of producing a modified alga may be derived from a desired algal or plant source.

In the inventive method of producing a modified alga, a polynucleotide may be introduced in an alga by any process. For example, a polynucleotide may be introduced in an algal cell by introduction of an expression vector including the polynucleotide. Such an expression vector may be constructed by any conventional technique. For example, "Japanese Unexamined Patent Application Publication No. 2007-43926" and "Japanese Unexamined Patent Application Publication No. 10-0570868" disclose methods for construction of an expression vector and transformation of algae. In accordance with such methods, a recombinant expression vector can be constructed by ligating a promoter and a terminator that act in an algal cell to the upstream and downstream, respectively, of a polynucleotide to be introduced, and then introduced into the algal cell.

Preferred examples of such a "promoter" include the Hsp70A/RBc_S2 promoter that has been broadly used for gene expression in algae and provides high constitutive expression of transcripts or proteins encoded by a polynucleotide to be introduced.

(2) Glutathione Enriching Step

The inventive method of producing a modified alga preferably further involves a glutathione enriching step to increase the chloroplastic glutathione concentration of the alga.

As used herein, the expression "to increase the chloroplastic glutathione concentration" refers to increasing a chloroplastic glutathione concentration in an alga as compared to that of a reference strain. In other words, an alga after the glutathione enriching step has a higher glutathione concentration than that of a reference strain. The increase in the chloroplastic glutathione concentration of the alga as compared to that of the reference strain can be determined by a method described in the section explaining the inventive alga.

In the glutathione enriching step, a chloroplastic glutathione concentration maybe increased by any process that can provide the resulting alga with an increased chloroplastic glutathione concentration. Examples of such a method include the same methods as in the ATG8 expression suppressing step and the method described in PTL 5, including (i) random mutagenesis in an alga of interest by a known mutagenesis technique; and (ii) introduction of a substance which increases the chloroplastic glutathione concentration into the algal cell (or into the algal genome, in some cases).

(i) Random Mutagenesis in an Alga

Random mutation may be introduced in an alga by any one appropriately selected from known techniques. Specific examples of the technique for random mutagenesis include chemical treatment of an alga (with EMS or NTG, for example), radioactive mutagenesis, transposon mutagenesis, T-DNA mutagenesis, mutagenesis using prokaryotic-eukaryotic cell conjugation, and physical gene transfer using a gene gun, for example. For example, mutation is introduced into a polynucleotide encoding a protein involved in the glutathione biosynthetic pathway in the chloroplast, such as GSH1, GSH2, ATP sulfurylase, adenosine 5'-phosphosulfate reductase, sulfite reductase, cysteine synthase, or serine acetyltransferase, so as to increase the expression and/or activity of the protein, which provides the resulting alga with an increased chloroplastic glutathione concentration.

Algae with desired mutation may be screened by any known method. Examples of the screening method include selection of mutant algae with an increased glutathione concentration based on the direct measurement of the chloroplastic glutathione concentration as described above, and selection of mutant algae with increased expression and/or activity of a protein such as GSH1, GSH2, ATP sulfurylase, adenosine 5'-phosphosulfate reductase, sulfite reductase, cysteine synthase, or serine acetyltransferase.

(ii) Introduction of Substance which Increases a Chloroplastic Glutathione Concentration into Cell

A modified alga with an increased chloroplastic glutathione concentration can also be produced by introducing the "substance which increases a chloroplastic glutathione concentration" as described above, such as (A) a polynucleotide encoding a protein which increases a chloroplastic glutathione concentration in an alga; or (B) a polynucleotide having a function of suppressing expression of a protein which decreases a chloroplastic glutathione concentration in an alga. Such polynucleotides (A) and (B) may be used either alone or in combination.

Introduction of at least one polynucleotide (A) into an algal cell increases expression of a protein which increases a chloroplastic glutathione concentration, resulting in an increased chloroplastic glutathione concentration.

Preferred examples of such a polynucleotide include a polynucleotide encoding γ-glutamylcysteine synthetase (hereinafter, also referred to as "GSH1 gene"), a polynucleotide encoding glutathione synthetase (hereinafter, also referred to as "GSH2 gene"), a polynucleotide encoding ATP sulfurylase, a polynucleotide encoding adenosine 5'-phosphosulfate reductase, a polynucleotide encoding sulfite reductase, a polynucleotide encoding cysteine synthase, and a polynucleotide encoding serine acetyltransferase. Such polynucleotides are preferably from a plant, more preferably from the host alga, but polynucleotides from algae other than the host alga or from higher plants may also be suitably used.

The "γ-glutamylcysteine synthetase (GSH1)" is an enzyme that catalyzes combination of cysteine with glutamate at the γ-position of glutamate through an amido bond, to synthesize γ-glutamylcysteine. The "glutathione synthetase (GSH2)" is an enzyme that catalyzes binding of glycine to γ-glutamylcysteine to synthesize glutathione.

The "GSH1 gene" is not limited to specific genes. Examples of GSH1 gene preferred in the invention include the GSH1 gene of *Chlamydomonas* (CHLREDRAFT_181975) used by the inventors in the Examples. The *Chlamydomonas* GSH1 has the amino acid sequence set forth in SEQ ID NO: 6, and a gene (full-length cDNA) encoding it has the base sequence set forth in SEQ ID NO: 7. Translation products of the GSH1 gene of *Chlamydomonas* include a chloroplast targeting signal peptide in the N-terminal region. Thus, the translation products of the GSH1 gene from *Chlamydomonas,* i.e. *Chlamydomonas* GSH1, are normally present in the chloroplast.

In summary, preferred examples of the nucleotide to be introduced in an alga in the invention include the following polynucleotides (a) to (d):

(a) a polynucleotide encoding a polypeptide which comprises an amino acid sequence set forth in SEQ ID NO: 6;

(b) a polynucleotide encoding a polypeptide which comprises an amino acid sequence derived from the amino acid sequence set forth in SEQ ID NO: 6 and maintaining the γ-glutamylcysteine synthetase activity wherein one or more amino acids are deleted, substituted, or added in the amino acid sequence; and

(c) a polynucleotide which is hybridizable with a polynucleotide comprising a base sequence complementary to that of the polynucleotide (a) or (b) under stringent conditions and encodes a polypeptide having γ-glutamylcysteine synthetase activity.

As used herein, the "γ-glutamylcysteine synthetase activity" refers to activity of catalyzing a reaction that forms an amido bond between cysteine and the γ-position of glutamate. The "γ-glutamylcysteine synthetase activity" can be measured by the following method, for example: algal cell lysate is centrifuged and the supernatant is collected as a sample; the sample is added to a reaction solution containing cysteine, glutamate, and ATP; and the amount of γ-glutamylcysteine synthesized in a certain period of time is measured. Such measurement is carried out under conditions with an anti-oxidant measure, such as purging the solution with nitrogen. Alternatively the γ-glutamylcysteine synthetase activity may be measured by determining the amount of phosphoric acid generated the reaction.

GSH1 genes from other plants than *Chlamydomonas* may also be suitably used in the invention. Known examples of such GSH1 genes include those from *Arabidopsis thaliana* (TAIR Accession Gene: 2127172; Name AT4G23100.1), *Zinnia elegans* (Genbank accession: AB158510), *Oryza sativa* (Genbank accession: AJ508915), and *Nicotiana tabacum* (Genbank accession: DQ444219). Translation products of these genes also include a chloroplast targeting signal peptide in the N-terminal region, as in the case of *Chlamydomonas.*

Alternatively, introduction of the aforementioned polynucleotide (B) into an algal cell can decrease expression of a protein which decreases a chloroplastic glutathione concentration, resulting in an increased chloroplastic glutathione concentration. Examples of such a polynucleotide include double-stranded RNAs (dsRNAs), small interfering RNAs (siRNAs), and template DNAs for these RNAs, as used in a conventional RNA interference (RNAi) technique.

Preferred examples of the "protein which decreases a chloroplastic glutathione concentration in an alga" include CLT1. The "CLT1" is a transporter that delivers glutathione from the chloroplast to the cytoplasm. The transporter was first found in *Arabidopsis thaliana* and designated "CLT1" (see Maughan S C et al. (2010), Proc. Natl. Acad. Sci. USA 107 (5): 2331-2336). Accordingly, examples of the polynucleotide (B) include polynucleotides intended to decrease expression of a glutathione transporter, such as CLT1.

(3) Additional Steps

The inventive method of producing a modified alga may involve a screening step to screen modified algae with suppressed intracellular expression of ATG8, in addition to the "ATG8 expression suppressing step" described above.

The inventive method of producing a modified alga may also involve a screening step to screen modified algae with an increased chloroplastic glutathione concentration in addition to the "glutathione enriching step" described above.

For example, transformed algae after introduction of a gene of interest may be first screened by a conventional chemical screening test based on expression of a drug-resistant marker, such as kanamycin-resistant or hygromycin-resistant marker. The screened algae may be then tested to confirm whether the gene of interest has been successfully introduced therein, by a technique such as PCR, southern hybridization, or northern hybridization. For example, successful transformation can be confirmed in the following steps of: preparing DNA from a transformed alga; designing primers specific to the DNA that has been introduced into the alga; carrying out PCR; subjecting the amplification products to electrophoresis, such as agarose gel electrophoresis, polyacrylamide gel electrophoresis, or capillary electrophoresis; and staining the resulting gel with ethidium bromide, for example, to detect the amplification product of interest.

Transformed algae with suppressed intracellular expression of ATG8 may be screened by the measurement of intracellular expression of ATG8 as described above, for example. Individuals with an increased chloroplastic glutathione concentration may be screened by the measurement of chloroplastic glutathione concentration as described above, for example.

4. Inventive Method of Biomass Production

The inventive method of biomass production produces biomass using a modified alga produced by the aforementioned method of producing a modified alga which has suppressed intracellular expression of ATG8 or which has both suppressed expression of ATG8 and increased chloroplastic glutathione concentration, as compared to a reference strain.

The modified alga and the method of producing a modified alga according to the invention have been explained above in the sections "Inventive alga" and "Inventive method of producing modified alga", respectively, and redundant description will be eliminated.

As used herein, the term "biomass" refers to substances produced by algae through photosynthetic carbon fixation, including saccharides (for example, starch) and oils and fats, for example, and is interchangeable with "photosynthate".

According to the inventive method of biomass production, production or accumulation of photosynthate in the algal cell maybe induced by any method. For example, the inventive method of biomass production may involve a photoirradiation step to irradiate the alga with light for inducing production or accumulation of photosynthate in the algal cell.

(1) Photoirradiation Step

(i) Modified Alga with Suppressed Expression of ATG8

A modified alga which has suppressed expression of ATG8 but does not have an increased chloroplastic glutathione concentration (modified alga with suppressed ATG8 expression) is preferably cultured under a nitrogen-deficient condition for inducing accumulation of photosynthate in the algal cell. The term "nitrogen-deficient condition" refers to culture in a culture medium having an inorganic nitrogen content of less than 0.001% by weight (calculated as nitrogen atom concentration). The term "inorganic nitrogen" refers to nitrogen such as ammonia nitrogen, nitrite nitrogen, and nitrate nitrogen, for example. A non-limiting example of a nitrogen-free culture medium preferred in the invention is a TAP N-free medium. It has substantially the same composition as a known TAP medium, but does not contain a nitrogen source. The TAP culture medium is mainly composed of tris(hydroxymethyl)aminomethane (Tris), acetate, and phosphate, and contains ammonium chloride (0.4 g/l) as a nitrogen source. Its detailed composition is described in Fukuzawa H and Kubo T (2009), Teion Kagaku (Low Temperature Science) 67: 17-21. The TAP N-free culture medium has substantially the same composition as the TAP culture medium, except that it contains 0.4 g/l of potassium chloride instead of ammonium chloride.

If a polynucleotide having a function of silencing an ATG8 gene is used for suppressing expression of ATG8, such a culture medium preferably contain the polynucleotide. The concentration of the polynucleotide in the culture medium is preferably 10 μg/ml, more preferably 4 μg/ml, for example.

The sequence of the polynucleotide having a function of silencing an ATG8 gene can be designed by a known technique. For example, the sequence set forth in SEQ ID NO: 5 can be used suitably. The polynucleotide having a function of silencing an ATG8 gene can be incorporated in a genome of a target alga in combination with a suitable promoter, so that it is expressed and acts in the target alga. Alternatively, the polynucleotide may be added to a culture medium under suitable conditions to exert the function thereof.

Induction of accumulation of photosynthate does not require adjustment of light intensity for irradiation of an alga, but the light intensity may be 40 $E/m^2/sec$ or more, for example, preferably 50 $\mu E/m^2/sec$ or more, more preferably 60 $\mu E/m^2/sec$ or more, yet more preferably 70 $\mu E/m^2/sec$ or more, 80 $\mu E/m^2/sec$ or more, 90 $\mu E/m^2/sec$ or more, or 100 $\mu E/m^2/sec$ or more; and is preferably 1000 $\mu E/m^2/sec$ or less, for example, more preferably 500 $\mu E/m^2/sec$ or less, yet more preferably 100 $\mu E/m^2/sec$ or less.

Irradiation with light within such a range requires no special photoirradiation device. Examples of the light include sunlight; sunlight qualitatively and quantitatively adjusted with a mirror, optical fiber, filter, or mesh, for example; artificial light such as light from an incandescent lamp, fluorescent lamp, mercury lamp, or light-emitting diode. The light for irradiation may have a wavelength in a region suitable for photosynthesis in general algae, preferably 400 nm to 700 nm, for example.

Alternatively, the photoirradiation step may be carried out under an autotrophic condition. As used herein, the "autotrophic condition" refers to a condition of culturing without supply of a carbon source other than carbon dioxide. In detail, the photoirradiation step may be carried out under an autotrophic condition by irradiating an inventive alga with light in an HSM culture medium under supply of atmospheric air, for example. The source of carbon dioxide is not limited to the atmospheric air. Carbon dioxide contained in flue of thermal power stations or ironworks can also be supplied to a culture medium at a higher concentration than in the atmospheric air, which allows increased productivity.

Under an autotrophic condition, carbon dioxide or gas containing carbon dioxide is transmitted from near the bottom of a culture vessel (i.e. passed through the solution). Carbon dioxide diffuses in water at a rate much lower than in the atmospheric air, which requires stirring the culture medium to achieve uniform irradiation of the alga. Carbon dioxide dissolves in water to form anions. A culture medium with low buffer capacity is rendered more acidic by the bubbling to decrease the solubility of carbon dioxide, resulting in a lower consumption rate of carbon dioxide in photosynthesis. Thus, the culture medium preferably has buffer capacity sufficient for maintaining its pH value within

a neutral to alkaline pH range. Preferred examples of such a medium include conventional HSM medium.

(ii) Modified Alga with Suppressed Expression of ATG8 and Increased Glutathione Concentration

A modified alga which has both suppressed expression of ATG8 and increased chloroplastic glutathione concentration (modified alga with suppressed ATG8 expression and increased glutathione concentration) is not required to be cultured under the nitrogen-deficient condition for inducing accumulation of photosynthate in the algal cell. Thus, the photoirradiation step described above may be carried out under a condition without nitrogen deficiency. As used herein, the "condition without nitrogen deficiency" refers to culture in a culture medium having an inorganic nitrogen content necessary for growth of algae. The inorganic nitrogen content in a culture medium necessary for growth of algae is 0.001% to 0.1% by weight (calculated as nitrogen atom concentration), preferably 0.005% to 0.05% by weight. The TAP medium used in the Examples below has an inorganic nitrogen content of approximately 0.01% by weight (calculated as nitrogen atom concentration).

Non-limiting examples of a culture medium having an inorganic nitrogen content necessary for the growth of an alga with suppressed expression of ATG8 and increased glutathione concentration include culture media which are normally used in culturing algae, such as traditional TAP medium, HSM medium, and ATCC897 medium.

Induction of accumulation of photosynthate does not require adjustment of light intensity for irradiation of the alga, but the light intensity may be 1000 $\mu E/m^2/sec$ or less, for example, preferably 500 $\mu E/m^2/sec$ or less, more preferably 400 $\mu E/m^2/sec$ or less, 300 $\mu E/m^2/sec$ or less, 200 $\mu E/m^2/sec$ or less, 150 $\mu E/m^2/sec$ or less, 100 $\mu E/m^2/sec$ or less, or 80 $\mu E/m^2/sec$ or less. A lower light intensity for irradiation increases energy efficiency, resulting in increased productivity. The modified alga with suppressed expression of ATG8 and increased glutathione concentration is advantageous in that it can produce photosynthate inside and outside the cell under a lower light intensity than traditional wild-type algae. The lower limit of the light intensity for irradiation may be any value, for example, may be 40 $\mu E/m^2/sec$, which can be set as a practical value.

Examples of a photoirradiation device for irradiation with light within such a range include those described above in case (i).

In one embodiment, the modified alga with suppressed ATG8 expression and increased glutathione concentration may be cultured in a TAP medium under irradiation with light of 45 $\mu E/m^2/sec$ to induce accumulation of starch in the cell. In another embodiment, the modified alga with suppressed ATG8 expression and increased glutathione concentration may be cultured in a TAP medium under irradiation with light of 80 $\mu E/m^2/sec$ to induce accumulation of starch in the cell.

The photoirradiation step of the modified alga with suppressed expression of ATG8 and increased glutathione concentration may be carried out under the nitrogen-deficient condition explained above in case (i). In one embodiment, the modified alga with suppressed expression of ATG8 and increased glutathione concentration may be cultured in the nitrogen-deficient condition (i.e. in the TAP N-free medium) under photoirradiation with light of 80 $\mu E/m^2/sec$ to induce accumulation of starch in the cell.

As described above, the modified alga with suppressed expression of ATG8 and increased glutathione concentration is characterized in that it does not require any step of nutrient restriction, such as step of providing nitrogen-deficient condition, for inducing production of photosynthate. In other words, in one embodiment of the invention using the modified alga with suppressed expression of ATG8 and increased glutathione concentration, the method may be practiced substantially without carrying out a nutrient restriction step, such as a step of providing a nitrogen-deficient condition (i.e. such a method involves substantially no nutrient restriction step). Such a feature simplifies the process, resulting in increased photosynthetic productivity.

Alternatively, the photoirradiation step may be carried out under the autotrophic condition explained above in case (i).

(2) Additional Step

The inventive method of biomass production may further involve a step of recovering photosynthate in addition to the aforementioned "photoirradiation step".

(i) Modified Alga with Suppressed Expression of ATG8

If a modified alga which has suppressed expression of ATG8 but does not have increased chloroplastic glutathione concentration (modified alga with suppressed ATG8 expression) is used in the inventive method of biomass production, starch granules may be recovered by lysing the cell and then subjecting the lysate to a separation procedure, such as leaving the lysate to stand for spontaneous precipitation, centrifugation, or sieving. The separation procedure may be selected based on physical properties of the starch granules and the algal cell lysate, such as particle size and/or specific gravity.

(ii) Modified Alga with Suppressed Expression of ATG8 and Increased Glutathione Concentration

If a modified alga which has both suppressed expression of ATG8 and increased chloroplastic glutathione concentration (modified alga with suppressed expression of ATG8 and increased glutathione concentration) is used in the inventive method of biomass production, the cell can be induced to export the starch accumulated therein outside the cell in the form of starch granules. If the photosynthate is starch, the step of recovering photosynthate may be practiced by separating the exported starch granules from the alga and recovering the separated starch granules. The exported starch granules may be separated from the alga by any method, including a separation procedure such as standing for spontaneous precipitation, centrifugation, or sieving. The separation procedure may be selected based on physical properties of the starch granules and the algal cell lysate, such as particle size and/or specific gravity.

In the inventive method of biomass production, in either of cases (i) and (ii), use of the inventive alga or an alga produced by the inventive method of producing a modified alga increases the facility and efficiency in both inducing accumulation of photosynthate and recovery of the photosynthate, as compared to traditional techniques. Accordingly, the inventive method of biomass production provides algal biomass production with lower costs and higher efficiency than traditional methods.

The invention should not be construed to the aforementioned embodiments. Various modifications are possible within the scope of the claims, and embodiments formed by appropriately combining technical features disclosed in different embodiments are also encompassed within the technical scope of the invention.

EXAMPLES

The present invention will now be described in more details by way of Examples, but should not be construed to be limited to the Examples.

Example 1

Production of Modified Algae

<Production of Modified Strain with Suppressed Expression of ATG8: 1>

The MEX1 gene (SEQ ID NO: 4) encoding the MEX1 protein from *Chlamydomonas reinhardtii* (SEQ ID NO: 3; hereinafter, referred to as "CrMEX1") was ligated to the downstream of the Hsp70A/RBc_S2 promoter to prepare a plasmid.

Specific procedure was as follows: A circular DNA vector for *Chlamydomonas,* pChlamyl (available from Life Technologies Corporation) was sequentially treated with restriction enzymes Kpn I and Not I to be cleaved (DNA fragment 1). A polynucleotide (about 1.7 kbp) consisting of nucleotides 163 to 1830 of the sequence set forth in SEQ ID NO: 8 was prepared by the following method.

Chlamydomonas reinhardtii CC-503 strain (supplied from *Chlamydomonas* Genetics Center at Duke University, US) was cultured in a TAP medium for four days at 24° C. and under irradiation with light with an intensity of 50 $\mu E/m^2/sec$. The cells were then collected from the culture, and a cDNA mixture was prepared from the cells using a cDNA synthesis reagent kit (Solid phase cDNA synthesis kit; available from TAKARA BIO INC.). The cDNA mixture was used as a template, and was subjected to PCR with oligonucleotides having sequences of SEQ ID NO: 9 and SEQ ID NO: 10, respectively, in accordance with a known technique (annealing temperature: 68° C.). A *Chlamydomonas* MEX1 gene was thereby collected as a polynucleotide of about 1.7 kbp including the ORF followed by the 3'UTR region. The polynucleotide was further treated with the restriction enzymes Kpn I and Not I to process its terminal structure (DNA fragment 2).

The DNA fragments 1 and 2 were ligated or recircularized to each other. The circular DNA was amplified with *Escherichia coli,* and the amplification products were extracted from *E. coli* and were purified, in accordance with a known technique.

This procedure generated the base sequence set forth in SEQ ID NO: 8 in the circular DNA molecule. In the base sequence of SEQ ID NO: 8, nucleotides 1 to 3 form the start codon, and nucleotides 1286 to 1288 form the stop codon. In other words, the *Chlamydomonas* MEX1 gene includes nucleotides 1 to 1288 of the base sequence set forth in SEQ ID NO: 8 as the open reading frame (ORF). Nucleotides 9 to 153 of the base sequence form an intron sequence.

The resulting plasmid including the polynucleotide for Hsp70A-Rbc_S2 promoter-CrMEX1 was linearized with a restriction enzyme Sca I, and the linearized plasmid was introduced in the *Chlamydomonas reinhardtii* CC-503 strain by glass beads technique (see Kindle K L (1990), Proc. Natl. Acad. Sci. USA 87: 1228-1232). The transformed strain was screened to select a strain which included the polynucleotide inserted in the genomic DNA and exhibited stable inheritance of the polynucleotide to the next generation through the cell replication. The screening was based on the exhibition of hygromycin resistance by the transformed CC-503 strain. Insertion of the plasmid DNA including the polynucleotide into the genomic DNA was confirmed by PCR technique.

The produced algal strain with overexpression of CrMEX1 is referred to as "modified strain with suppressed expression of ATG8 (CC-503/CrMEX1ox)".

<Production of Modified Strain with Suppressed Expression of ATG8: 2>

An MEX1 gene (SEQ ID NO: 2) encoding the MEX1 protein from *Arabidopsis thaliana* (SEQ ID NO: 1; hereinafter, referred to as "AtMEX1") was ligated to the downstream of the Hsp70A/RBc_S2 promoter to prepare a plasmid.

Specific procedure was as follows: A circular DNA vector for *Chlamydomonas,* pChlamy3 (available from Life Technologies Corporation) was sequentially treated with restriction enzymes Kpn I and Not I to be cleaved (DNA fragment 3). A polynucleotide (about 1.3 kbp) consisting of nucleotides 163 to 1442 of the sequence set forth in SEQ ID NO: 11 was prepared by the following method.

Arabidopsis thaliana, Columbia strain was grown for three weeks at 22° C. under diurnal conditions with a light period (100 $\mu E/m^2/sec$) of 16 hours and a dark period of eight hours. A cDNA mixture was prepared from the plant as in <Production of modified strain with suppressed expression of ATG8: 1>. The cDNA mixture was used as a template, and was subjected to PCR with oligonucleotides having sequences of SEQ ID NO: 12 and SEQ ID NO: 13 in accordance with a known technique (annealing temperature: 68° C.). The ORF of the *Arabidopsis thaliana* MEX1 gene was thereby collected as a polynucleotide of about 1.3 kbp. The polynucleotide was further treated with the restriction enzymes Kpn I and Not I to process its terminal structure (DNA fragment 4).

The DNA fragments 3 and 4 were ligated or recircularized to each other. The circular DNA was amplified in *Escherichia coli,* and the amplification products were extracted from *E. coli* and were purified, in accordance with a known technique.

This procedure generated the base sequence set forth in SEQ ID NO: 11 in the circular DNA molecule. In the base sequence of SEQ ID NO: 11, nucleotides 1 to 3 form the start codon, and nucleotides 1412 to 1414 form the stop codon. In other words, the *Arabidopsis thaliana* MEX1 gene includes nucleotides 1 to 1414 of the base sequence set forth in SEQ ID NO: 11 as the open reading frame (ORF). Nucleotides 9 to 153 of the base sequence form an intron sequence.

The prepared plasmid including the polynucleotide for Hsp70A-Rbc_S2 promoter-AtMEX1 was linearized with a restriction enzyme Sca I, and the linearized plasmid was introduced in the *Chlamydomonas reinhardtii* CC-503 strain by glass beads technique (see Kindle K L (1990), Proc. Natl. Acad. Sci. USA 87: 1228-1232). The transformed strain was screened to select a strain which included the polynucleotide inserted in the genomic DNA and exhibited stable inheritance of the polynucleotide to the next generation through the cell replication. The screening was based on the exhibition of hygromycin resistance by the transformed CC-503 strain. Insertion of the plasmid DNA including the polynucleotide into the genomic DNA was confirmed by PCR technique.

The produced algal strain with overexpression of AtMEX1 is referred to as "modified strain with suppressed expression of ATG8 (CC-503/AtMEX1ox)".

<Production of Modified Strain with Suppressed Expression of ATG8: 3>

A DNA sequence complementary to the silencing construct (SEQ ID NO: 5; hereinafter, referred to as "ATG8-amiRNA") which specifically suppresses expression of the endogenous ATG8 gene of *Chlamydomonas* was ligated to the downstream of the PSAD promoter, to prepare a plasmid.

Specific procedure was as follows: A circular DNA vector for *Chlamydomonas,* pChlamiRNA3 (Molnar A et al. (2009), Plant Journal 58: 165-174) was treated with a restriction enzyme Spe I to be cleaved (DNA fragment 5). An oligonucleotide (134 bp) consisting of the sequence set forth in SEQ ID NO: 14 was prepared by the following method. Two single-stranded oligonucleotides consisting of the sequences of SEQ ID NO: 15 and SEQ ID NO: 16, respectively, were chemically synthesized. These two oligonucleotides were mixed and heated to 100° C., and then the temperature was gradually decreased to prepare a double-stranded oligonucleotide with a 5'-terminal protruding single-stranded DNA sequence consisting of four bases (5'-CTAG) (DNA fragment 6).

The DNA fragments 5 and 6 were ligated or circularized to each other using DNA Ligase (available from TAKARA BIO INC.). The circular DNA was amplified in *Escherichia coli,* and the amplification products were extracted from *E. coli* and were purified, in accordance with a known technique.

This procedure generated the base sequence set forth in SEQ ID NO: 14 in the circular DNA molecule. In the base sequence of SEQ ID NO: 14, nucleotides 6 to 28 and nucleotides 71 to 93 form base sequences which provide specificity for silencing of ATG8 in *Chlamydomonas.* In other words, the ATG8-amiRNA was designed so that the template DNA, i.e. the base sequence set forth in SEQ ID NO: 5 including the sequence of SEQ ID NO: 14, was transcribed to RNA under regulation by the PSAD promoter in the cell.

The prepared plasmid including the polynucleotide for PSAD promoter-ATG8-amiRNA was linearized with a restriction enzyme Sca I, and the linearized plasmid was introduced in the *Chlamydomonas reinhardtii* CC-503 strain by glass beads technique (see Kindle K L (1990), Proc. Natl. Acad. Sci. USA 87: 1228-1232). The transformed strain was screened to select a strain which included the polynucleotide inserted in the genomic DNA and exhibited stable inheritance of the polynucleotide to the next generation through the cell replication. The screening was based on the exhibition of paromomycin resistance by the transformed CC-503 strain. Insertion of the plasmid DNA including the polynucleotide into the genomic DNA was confirmed by PCR technique.

The produced algal strain with suppressed expression of ATG8 is referred to as "modified strain with suppressed expression of ATG8 (CC-503/ATG8amiRNA)".

<Production of Strain with Overexpression of GSH1>

A strain with overexpression of GSH1 (referred to as 22-2) was produced in accordance with the method described in PTL5 in Example 1.

<Production of Modified Strain with Overexpression of GSH1 and Suppressed Expression of ATG8: 1>

A transformed strain was produced by introducing a polynucleotide of the cell strain 22-2 with overexpression of CrGSH1 in the genome of the polynucleotide of Hsp70A-Rbc$_{_S}$2 promoter-CrMEX1, as in <Production of modified strain with suppressed expression of ATG8: 1>.

The produced algal strain with suppressed expression of ATG8 is referred to as "modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2/CrMEX1ox)".

<Production of Modified Strain with Overexpression of GSH1 and Suppressed Expression of ATG8: 2>

A transformed strain was produced by introducing a polynucleotide of the cell strain 22-2 with overexpression of CrGSH1 in the genome of the polynucleotide of Hsp70A-Rbc_S2 promoter-AtMEX1, as in <Production of modified strain with suppressed expression of ATG8: 2>.

The produced algal strain with suppressed expression of ATG8 is referred to as "modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2/AtMEX1ox)".

<Production of Modified Strain with Overexpression of GSH1 and Suppressed Expression of ATG8: 3>

A transformed strain was produced by introducing a polynucleotide of the cell strain 22-2 with overexpression of CrGSH1 in the genome of the polynucleotide of PSAD promoter-ATG8-amiRNA, as in <Production of modified strain with suppressed expression of ATG8: 3>.

The produced algal strain with suppressed expression of ATG8 is referred to as "modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2/ATG8-amiRNA)".

Example 2

Influences on the Expression of ATG8 in the Wild-type Strain by the Overexpression of MEX1

The modified strains with suppressed expression of ATG8 produced in Example 1 (CC-503/CrMEX1ox and CC-503/AtMEX1ox) were cultured and analyzed to determine the expression of ATG8. In detail, each strain with suppressed expression of ATG8 was cultured in a TAP medium under shaking, and the cells were collected after 72 hours. The cells were then lysed and analyzed by western blotting. The wild-type *Chlamydomonas* strain CC-503 (hereinafter, referred to as "parent strain (wild-type strain)") was used as a control.

The results are shown in FIG. **1** illustrating the expressions of the ATG8 protein in the clones produced in the Example, and indicating that the expression of ATG8 was suppressed in the cells with overexpression of MEX1 (see CC-503/CrMEX1ox clones 1, 2, 3, and 16; and CC-503/AtMEX1ox clones 1, 2, 11, 12, 15, and 18). The expression level of the ATG8 protein in each clone is shown in the table below. Each expression level is a relative value (%) to that of the parent strain (wild-type strain).

TABLE 1

	CC-503/CrMEX1ox						CC-503/AtMEX1ox					
CC-503	1	2	3	14	15	16	1	2	11	12	15	18
100%	58%	49%	40%	111%	115%	71%	71%	63%	31%	42%	22%	79%

Example 3

Influences on the Starch Production in the Wild-type Strain by Overexpression of MEX1 and Silencing of ATG8

The modified strains with suppressed expression of ATG8 produced in Example 1 (CC-503/CrMEX1ox and CC-503/AtMEX1ox) were transferred into a nitrogen-sufficient TAP medium at a cell density of 0.5×10^4 cells/ml, and then were cultured under shaking and continuous irradiation with a light intensity of 100 $\mu E/m^2/sec$ (preliminary culture). When the cells during the preliminary culture reached the logarithmic phase, the culture was centrifuged to collect the cells. The cells were then resuspended in a nitrogen-deficient TAP medium (TAP N-free medium) at a cell density of 5.0×10^6 cells/ml (replacement of medium). Cells were collected to determine the amount of starch therein, just after the replacement of the medium and after shaking culture in the nitrogen-deficient TAP medium (TAP N-free medium) for 24 hours. The determined value is an amount of starch per culture (calculated as glucose level). The wild-type strain CC-503 was used as a control.

The strain genetically modified to provide a cell with the ATG8-targeting RNA silencing construct (ATG8-amiRNA) was transferred into a nitrogen-sufficient TAP medium at a cell density of 0.5×10^4 cells/ml, and then were cultured under shaking and continuous irradiation with a light intensity of 100 $\mu E/m^2/sec$ (preliminary culture). When the cells during the preliminary culture reached the logarithmic phase, the culture was centrifuged to collect the cells. The cells were then resuspended in a nitrogen-deficient TAP medium (TAP N-free medium) at a cell density of 5.0×10^6 cells/ml (replacement of medium). Cells were collected to determine the amount of starch therein, just after the replacement of the medium and after shaking culture in the nitrogen-deficient TAP medium (TAP N-free medium) for 24 hours. The determined value is an amount of starch per culture (calculated as glucose level). The wild-type strain CC-503 was used as a control.

The results are shown in FIG. **2** illustrating amounts of starch measured at 0 h and 24 h after the replacement of the medium with the TAP N-free medium. The results for CC-503/CrMEX1ox clone 3 and CC-503/AtMEX1ox clone 15 are shown as representative. The amount of starch was calculated as glucose level per culture (mg glucose/dL culture).

FIG. **2** demonstrates that the MEX1-overexpressing modified cells (CC-503/CrMEX1ox and CC-503/AtMEX1ox) and the ATG8-silenced modified cells (CC-503/ATG8-amiRNA) had an increased amount of starch (starch accumulation) per culture after 24 hours, as compared to that of the wild-type strain. The results indicate that the amount of starch was increased by indirect suppression of ATG8 expression through overexpression of MEX1, or direct suppression of ATG8 expression.

Example 4

Influences on ATG8 Expression in the Strain with Overexpression of GSH1 by Overexpression of MEX1

The modified strains with overexpression of GSH and suppressed expression of ATG8 produced in Example 1 (22-2/CrMEX1ox and 22-2/AtMEX1ox) were cultured and analyzed to determine the expression of ATG8. In detail, each strain with suppressed expression of ATG8 was cultured in a TAP medium under shaking, and the cells were collected after 96 hours. The cells were then lysed and analyzed by western blotting. The strain with overexpression of GSH1 (22-2) was used as a control.

The results are shown in FIG. **3**. FIG. **3**(*a*) illustrates the expressions of the ATG8 protein in clones of a strain with overexpression of GSH and suppressed expressions of ATG8 (22-2/CrMEX1ox). FIG. **3**(*b*) illustrates the expressions of the ATG8 protein in clones of another strain with overexpression of GSH and suppressed expression of ATG8 (22-2/AtMEX1ox). The leftmost lane in each figure represents the control (22-2). The results shown in FIG. **3** indicate that the cells with overexpression of GSH1 and MEX1 exhibited suppressed expression of ATG8 and ATG-8PE (see 22-2/CrMEX1ox clones 1, 3, 5, 6, 7; and 22-2/AtMEX1ox clones 1, 2, 3, 4, 6, 7, and 16). The expressions of the ATG8 protein in the individual clones are shown in the table below. Each expression level is a relative value (%) to that of the parent strain (wild-type strain).

TABLE 2

	22-2/CrMEX1ox						22-2/AtMEX1ox								
22-2	1	2	3	5	6	7	1	2	3	4	6	7	8	16	17
100%	71%	118%	0	0	2%	0	64%	88%	58%	77%	57%	48%	121%	80%	102%

Example 5

Influences on Starch Production in Strains with Overexpression of GSH1 by Overexpression of MEX1

The strains with overexpression of GSH1 and suppressed expression of ATG8 produced in Example 1 (22-2/CrMEX1ox and 22-2/AtMEX1ox) were cultured and analyzed to determine the amount of starch. In detail, the cells were cultured in a TAP agar medium, and a small amount of the culture was then removed and transferred to a TAP liquid medium with a plastic inoculation loop. The cells were cultured under shaking and continuous irradiation with a light intensity of 10 $\mu E/m^2/sec$ (preliminary culture). When the cells during the preliminary culture reached the stationary phase, the culture was centrifuged to collect the cells. The cells were then diluted in a fresh TAP liquid medium at a cell density of 1.0×10^4 cells/ml, and were cultured under shaking and continuous irradiation with an intensity of light of 100 $\mu E/m^2/sec$ (main culture). The strain with overexpression of GSH1 (22-2) was used as a control.

The results are shown in FIG. **4**. FIG. **4**(*a*) illustrates the transition of the amount of starch in the modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2/CrMEX1ox), and FIG. **4**(*b*) illustrates the transition of the amount of starch in another modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2/AtMEX1ox). The results for 22-2/CrMEX1ox

clone 3 and 22-2/AtMEX1ox clone 7 are shown as representative. In each figure, the vertical axis represents amounts of starch per culture calculated as glucose level (mg glucose/dL culture), and the horizontal axis represents culture time in the main culture process.

FIG. **4** demonstrates that the modified strains with overexpression of GSH1 and suppressed expression of ATG8 (22-2/CrMEX1ox and 22-2/AtMEX1ox) had an increased amount of starch per culture after 96 hours from the initiation of the main culture, as compared to that of the strain with overexpression of GSH1 (22-2). Such results indicate that the overexpression of MEX1 and the suppression of ATG8 expression increase an amount of starch.

Example 6

Influences on the Cell Proliferation in the Strain with Overexpression of GSH1 by the Overexpression of MEX1

The modified strains with overexpression of GSH1 and suppressed expression of ATG8 produced in Example 1 (22-2/CrMEX1ox clone 3 and 22-2/AtMEX1ox clone 7) were cultured and analyzed to determine the amount of starch. In detail, the cells were cultured in a TAP agar medium, and a small amount of the cells was then removed and transferred to a TAP liquid medium with a plastic inoculation loop. The cells were cultured under shaking and continuous irradiation with an intensity of light of 10 $\mu E/m^2/sec$ (preliminary culture). When the cells during the preliminary culture reached the stationary phase, the culture was centrifuged to collect the cells. The cells were then diluted in afresh TAP liquid medium at a cell density of 1.0×10^4 cells/ml, and were cultured under shaking and continuous irradiation with an intensity of light of 100 $\mu E/m^2/sec$ (main culture). The strain with overexpression of GSH1 (22-2) was used as a control.

The results are shown in FIG. **5**. FIG. **5**(*a*-**1**) illustrates the transition of the cell count of the modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2/CrMEX1ox), and FIG. **5**(*a*-**2**) illustrates the transition of the cell count of another modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2/AtMEX1ox). FIG. **5**(*b*-**1**) illustrates the transition of the microparticle count of the modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2/CrMEX1ox), and FIG. **5**(*b*-**2**) illustrates the transition of the microparticle count of another modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2/AtMEX1ox). In the graphs shown in FIG. **5**(*a*), the vertical axis represents the cell counts per culture (mL culture), and the horizontal axis represents the culture time in the main culture process. In the graphs FIG. **5**(*b*), the vertical axis represents the counts of non-cellular microparticles per culture (mL culture), and the horizontal axis represents the culture time in the main culture process.

FIGS. **5**(*a*-**1**) and **5**(*a*-**2**) demonstrate that the cell proliferation was promoted in the modified strains with overexpression of GSH1 and suppressed expression of ATG8 (22-2/CrMEX1ox and 22-2/AtMEX1ox) as compared to the control, i.e. the strain with overexpression of GSH1 (22-2). FIGS. **5**(*b*-**1**) and **5**(*b*-**2**) demonstrate that the modified strains with overexpression of GSH1 and suppressed expression of ATG8 (22-2/CrMEX1ox and 22-2/AtMEX1ox) had a higher microparticle count after about 96 hours from the initiation of the culture, as compared to the strain with overexpression of GSH1 (22-2). The microparticles are presumed to be starch granules leaking from dead cells into the medium. Accordingly, the results suggest that extracellular release of starch was promoted.

Example 7

Influences on the Starch Production in the Strain with Overexpression of GSH1 by ATG8 Silencing

The modified strain with overexpression of GSH1 and suppressed expression of ATG8 produced in Example 1 (22-2/ATG8amiRNA) was cultured and analyzed to observe the cell proliferation and the amount of starch. In detail, the cells were cultured in a TAP agar medium, and a small amount of the cells was then removed and transferred to a TAP liquid medium with a plastic inoculation loop. The cells were cultured under shaking and continuous irradiation with an intensity of light of 10 $\mu E/m^2/sec$ (preliminary culture). When the cells during the preliminary culture reached the stationary phase, the culture was centrifuged to collect the cells. The cells were then diluted in a fresh TAP liquid medium at a cell density of 1.0×10^4 cells/ml, and were cultured under shaking and continuous irradiation with a light intensity of 100 $\mu E/m^2/sec$ (main culture). The strain with overexpression of GSH1 (22-2) was used as a control.

The results are shown in FIG. **6**. FIG. **6** illustrates the transition of the amount of starch, where the vertical axis represents the amounts of starch per culture calculated as glucose level (mg glucose/dL culture) and the horizontal axis represents culture time in the main culture process.

FIG. **6** demonstrates that the modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2/amiATG8) exhibited an increase in the amount of starch per culture after 72 hours from the initiation of the main culture, as compared to that of the strain with overexpression of GSH1 (22-2). Such results indicate that suppressing the expression of the ATG8 gene further increases the amount of accumulated starch in the strain with overexpression of GSH1.

Example 8

Influences on the Cell Proliferation in the Strain with Overexpression of GSH1 by ATG8 Silencing

The modified strain with overexpression of GSH1 and suppressed expression of ATG8 produced in Example 1 (22-2/ATG8amiRNA) were cultured and analyzed to observe the cell proliferation and the amount of starch. In detail, the cells were cultured in a TAP agar medium, and a small amount of the cells was then removed and transferred to a TAP liquid medium with a plastic inoculation loop. The cells were cultured under shaking and continuous irradiation with a light intensity of 10 $\mu E/m^2/sec$ (preliminary culture). When the cells during the preliminary culture reached almost the stationary phase, the culture was centrifuged to collect the cells. The cells were then diluted in afresh TAP liquid medium at a cell density of 1.0×10^4 cells/ml, and were cultured under shaking and continuous irradiation with a light intensity of 100 $\mu E/m^2/sec$ (main culture). The strain with overexpression of GSH1 (22-2) was used as a control.

The results are shown in FIG. **7**. FIG. **7**(*a*) illustrates the transition of the cell count, and FIG. **7**(*b*) illustrates the transition of the microparticle count. In the graph (a), the vertical axis represents the cell counts per culture (mL culture), and the horizontal axis represents the culture time in the main culture process. In the graph (b), the vertical axis

represents the microparticle counts per culture (mL culture), and the horizontal axis represents the culture time in the main culture process.

FIG. **7**(*a*) indicates that the cell proliferation was promoted in the modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2amiATG8) as compared to that of the control strain with overexpression of GSH1 (22-2). FIG. **7**(*b*) indicates that the modified strain with overexpression of GSH1 and suppressed expression of ATG8 (22-2amiATG8) had a higher microparticle count after 120 hours from the initiation of culture as compared to that of the strain with overexpression of GSH1 (22-2). The microparticles are presumed to be starch granules leaking from dead cells into the medium. Accordingly, the results suggest that extracellular release of starch was promoted.

Example 9

Influences on the Production of Oils and Fats in the Strain with Overexpression of GSH1 by Suppression of ATG8 Expression

The modified strains with overexpression of GSH1 and suppressed expression of ATG8 (22-2/CrMEX1ox, 22-2/AtMEX1ox, and 22-2/ATG8amiRNA) produced in Example 1 were cultured and analyzed to determine the amount of oils and fats. The strain with overexpression of GSH1 (22-2) was used as a control. Each strain was cultured under the same conditions as in Example 6, except that the light intensity in the continuous irradiation in the main culture was 170 $\mu E/m^2/sec$. A part of each culture was daily collected in a glass tube and was cryopreserved until the analysis.

Oils and fats were collected from the cells by the following procedure. The gaseous phase of the glass tube was purged with nitrogen, and 20 μg of pentadecanoic acid was added as internal control for determination of oils and fats. A suitable amount of a methanol-hexane mixture (1:1) was added, and then the resulting mixture was stirred and left to stand. The solution was then centrifuged to be separated into aqueous and organic solvent phases. The organic solvent fraction was transferred to a different glass tube, and was dried under vacuum. The product was dissolved in a suitable amount of hexane, and was mixed with a suitable amount of 2.5% methanolic sulfuric acid solution. The gaseous phase of the glass tube was purged with nitrogen gas, and the glass tube was heated at 80° C. for one hour. After the glass tube was cooled to a room temperature, 1 ml of saturated aqueous sodium carbonate solution was added, and the mixture was stirred. The solution was then centrifuged, and the organic solvent phase was collected and dried under vacuum. The product was dissolved in 200 μL of hexane, and the solution was injected in a gas chromatograph/mass spectrometer (Clarus SQ8; available from PerkinElmer, Inc.) and was analyzed to determine fatty acid methyl esters, using a column Elite-225 available from PerkinElmer, Inc. (length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 μm). The oven was heated at 3° C./minute to 200° C. and then was maintained at 200° C. for 6.5 minutes.

Commercially available fatty acid methyl esters were used to generate a calibration curve. The sum of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) was calculated as the amount of fatty acids. FIGS. **8**(*a*) and **8**(*b*) illustrate the measured amount of fatty acids and cell count, respectively. The vertical axis represents amounts of fatty acids (pg) or cell count ($\times 10^6$ cells) per culture (mL culture), and the horizontal axis represents the culture time (days) after the initiation of the main culture. The results for 22-2/CrMEX1ox clone 1 and 22-2/AtMEX1ox clone 7 are shown as representative.

FIG. **8** demonstrates that the modified strains with overexpression of GSH1 and suppressed expression of ATG8 accumulated more fatty acids and reached to the maximum level in a shorter culture time, as compared to the strain with overexpression of GSH1 (22-2). Such results indicate that suppression of ATG8 expression increases an amount of oils and fats.

INDUSTRIAL APPLICABILITY

The present invention provides algal biomass production with lower costs and higher efficiency than traditional methods. Biomass is a promising raw material for biofuels, and therefore the invention is applicable to a wide variety of industries including the energy industry.

SEQUENCE LISTING FREE TEXT

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SEQ ID NO: 2: a base sequence of a polynucleotide encoding the MEX1 protein derived from *Arabidopsis thaliana*

SEQ ID NO: 3: an amino acid sequence of an MEX1 protein derived from *Chlamydomonas reinhardtii*

SEQ ID NO: 4: a base sequence of a polynucleotide encoding the MEX1 protein derived from *Chlamydomonas reinhardtii*

SEQ ID NO: 5: a base sequence of ATG8-amiRNA

SEQ ID NO: 6: an amino acid sequence of γ-glutamylcysteine synthetase derived from *Chlamydomonas reinhardtii*

SEQ ID NO: 7: a base sequence of a polynucleotide encoding γ-glutamylcysteine synthetase derived from *Chlamydomonas reinhardtii*

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SEQ ID NO: 18: a base sequence of a polynucleotide encoding the MEX1 protein derived from *Arabidopsis thaliana*

SEQ ID NO: 19: an amino acid sequence of an MEX1 protein derived from *Chlamydomonas reinhardtii*

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Ile Gly Gly Ala Val Ser Cys Ser Gly Val Asn Gly Leu Thr Arg Trp
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Asn Ser Ile Val Ser Thr Arg Arg Leu Val Pro Val Arg Ser Ile Asn
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Ala Gln Leu Thr Met Ala Glu Ala Met Pro Ile Gln Tyr Phe Val Ala
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Thr Ser Ala Val Val Thr Ile Gly Leu Ile Val Asn Cys Leu Tyr Tyr
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Phe Gly Lys Leu Ser Lys Thr Val Trp Gln Leu Trp Glu Asp Val Ile
225                 230                 235                 240

Thr Ile Gly Gly Leu Ser Val Leu Pro Gln Ile Met Trp Ser Thr Phe
                245                 250                 255

Val Pro Leu Val Pro Asn Ser Ile Leu Pro Gly Thr Thr Ala Phe Gly
            260                 265                 270

Ile Ala Val Ala Ala Ile Ile Met Ala Arg Thr Gly Lys Leu Ser Glu
        275                 280                 285

Lys Gly Val Arg Phe Val Gly Ser Leu Ser Gly Trp Thr Ala Thr Leu
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Met Phe Met Trp Met Pro Val Ser Gln Met Trp Thr Asn Phe Leu Asn
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145                 150                 155                 160

Gln Thr Gly Phe Leu Gly Val Gly Phe Asp Pro Lys Trp Ala Ile Ser
                165                 170                 175

Asp Val Pro Met Met Pro Lys Gly Arg Tyr Lys Leu Met Lys Ser Tyr
            180                 185                 190

Met Pro Thr Val Gly Ser Met Gly Leu Asp Met Met Phe Arg Thr Cys
        195                 200                 205

Thr Val Gln Val Asn Leu Asp Phe Glu Ser Glu Gln Asp Met Val Glu
    210                 215                 220

Lys Phe Arg Ile Gly Leu Ala Leu Gln Pro Ile Ala Asn Ala Leu Phe
225                 230                 235                 240

Ala Ser Ser Pro Phe Lys Glu Gly Lys Pro Thr Gly Tyr Leu Ser Thr
                245                 250                 255

Arg Gly His Val Trp Thr Asp Val Asp Ala Ser Arg Thr Gly Asn Leu
            260                 265                 270

Pro Phe Val Phe Glu Lys Asp Met Cys Phe Glu Ser Tyr Val Asp Tyr
        275                 280                 285

Ala Met Ala Val Pro Met Tyr Phe Val Tyr Arg Asn Gly Gln Tyr Ile
    290                 295                 300

Asn Ala Leu Gly Met Ser Trp Lys Asp Phe Met Ala Gly Lys Leu Pro
305                 310                 315                 320

Ala Leu Pro Gly Glu Tyr Pro Thr Ile Ala Asp Trp Ala Asn His Leu
                325                 330                 335

Thr Thr Ile Phe Pro Glu Val Arg Leu Lys Lys Phe Leu Glu Met Arg
            340                 345                 350

Gly Ala Asp Gly Gly Pro Trp Arg Met Leu Cys Ala Leu Pro Ala Leu
        355                 360                 365

Trp Val Gly Leu Ile Tyr Asp Pro Glu Ala Gln Arg Gln Ala Leu Ala
    370                 375                 380

Leu Ile Glu Asp Trp Thr Pro Ala Glu Arg Asp Tyr Leu Arg Thr Glu
385                 390                 395                 400

Val Thr Arg Phe Gly Leu Arg Thr Pro Phe Arg Ala Gly Thr Val Gln
                405                 410                 415

Asp Val Ala Lys Gln Val Val Ser Ile Ala His Gly Gly Leu Glu Arg
            420                 425                 430

Arg Gly Tyr Asp Glu Thr Ser Phe Leu Lys Arg Leu Glu Val Ile Ala
        435                 440                 445

Glu Thr Gly Leu Thr Gln Ala Asp His Leu Leu Glu Leu Tyr Glu Thr
    450                 455                 460

Lys Trp Gln Arg Ser Val Asp Pro Leu Tyr Lys Glu Phe Met Tyr
465                 470                 475


<210> SEQ ID NO 7
<211> LENGTH: 1440
<212> TYPE: DNA
<213> ORGANISM: Chlamydomonas reinhardtii

<400> SEQUENCE: 7
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cggggtgtgc caagcccacg cttgagccct gtccacgcga acgcgcctgc ggttgcggag 120

cgtcgcacag agcccctctt aaagcaggag ctggtggatt acctgaaatc ggggtgtagg 180

cctcgcagcg cgtggcgaat cggcaccgag cacgagaagc tgggtttcaa cctggcagac 240

aacagccgca tgaactacga ccagattgca caggtgctac gcaagctgga ggcccggttt 300

ggttgggagc ccatcatgga ggagggccgt atcatcggcg tgcagttgga tggtcagagt 360

gtgacgctgg agcccggcgg ccagtttgaa ctgagcgggg cgcccgtgga gaccattcac 420

aagacgtgtg cggaggtgaa cagccacctc taccaggtca aggccatctg cgaggagctt 480

cagacaggat tcctgggcgt gggctttgac cccaagtggg ccatcagcga cgttcccatg 540

atgcccaagg gccgctacaa gctgatgaag tcgtacatgc ccacggtggg ctccatgggc 600

ctggacatga tgttccgcac atgcaccgtg caggtgaacc tggactttga gagcgagcag 660

gacatggtgg agaagttccg catcggcctg gcgctgcagc ccatcgccaa cgcgctcttc 720

gccagctcgc cattcaagga gggcaagccc accgggtacc tgagcacccg cggtcacgtg 780

tggacggacg tggacgcctc gcgcaccggc aacctgccgt tcgtgttcga gaaggacatg 840

tgcttcgaga gctacgtgga ctacgccatg gcggtgccca tgtacttcgt gtaccgcaac 900

gggcagtaca tcaacgcgct gggcatgagc tggaaggact tcatggccgg caagctgccc 960

gcgctgccgg gcgaataccc caccatcgcc gactgggcca accacctgac caccatcttc 1020

cccgaggtgc ggctcaagaa gttcctggag atgcgcggcg cggacggcgg cccctggcgc 1080

atgctgtgcg cgctgccggc gctgtgggtg gggctcatat acgatccgga ggcgcagcgc 1140

caggccctgg cgctgattga ggattggacg cccgcggagc gcgactacct gcgcaccgag 1200

gtgacccgct tcggcctgcg cacgcccttc cgcgccggca ccgtgcagga cgtggccaag 1260

caggtggtgt ccatcgcgca cggcggcctg gagcggcgag gctacgacga gacgtccttc 1320

ctcaagcgcc tggaggtcat cgcggagact ggcctcacac aggccgacca cctgcttgag 1380

ctgtacgaga ccaagtggca gcgctcggtg gacccgctgt acaaggagtt catgtactga 1440

<210> SEQ ID NO 8
<211> LENGTH: 1830
<212> TYPE: DNA
<213> ORGANISM: Chlamydomonas reinhardtii

<400> SEQUENCE: 8

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ttgcaacgcc cgcattgtgt cgacgaaggc ttttggctcc tctgtcgctg tctcaagcag 120

catctaaccc tgcgtcgccg tttccatttg caggagattc gaggtaccga gatctttgca 180

accggcaccg gcaaggagtt tttcggggat ggaagggcga cggacaacag gcacgcgaac 240

gctggtgact gtcagtagcc ggaaaaagcc cgtgcccccg ttgcatgcgg gagcaacgag 300

cggtccggca agcgacagcc agcgatggaa tgcgctcacg gacaagctgg tggctgcatc 360

cagcattccg ttctctattt tggtgcttcc gcaagtcgtc cagaatgcca tcaacatggc 420

tggcggccac cccacggccc tgtccattat cagctgggag ggctacctct ctgccatgtt 480

cggcaacaca ttaatgtgca gccatttcgc ggcgtcaggc gagcgcagcg ccgtaaacgt 540

gcagcttgtg ggaatcctca acaacttcct cattctcaca caggtggctc ttgccggctt 600

catgccgctc gcggtgtttt tggcggcggc ggccttcacg gcctttgcca cttttatgaa 660

cctggcacgc gtggcggcgc tggcgggcgc agcgcagccg gcggacgaga agtggggcag 720

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ctggcagatg tggcagctgg gcagcgggct ggtggggctg gcggtggtgc cgcaggtgct 780

ctacaacacc gtgtccccgg cggcatccac gctgctgccc ttcatttgca cgcttgggct 840

gcttggagcg gtgctagggc tacggctgtc gtccaagggc ggtagcgacg cggcaacgct 900

ggtgcggcag ctgccgggct ggggcgccac gctgctgttt gcgctgtcgc cgctaccgca 960

gctggtccgc aacctgcttg agccacagag cctggagggc ctgagcgtgg gcaccatgct 1020

gctggcgctg ctgggcaacg cgctcatggt gccgcgggcg ctgttcgtgc gcgacgtggt 1080

gtggctcagc ggcacctgct gggcctgcgt agcgggctgg ggtcagctgt tcagcatgtt 1140

ccgcagcgcg tcggcggcca cagggcggcg cttcctggac ccctggctgt tcttctccat 1200

caccggggcg ctgctgctgt acactggcta cgtggtggtg cagcaccgga gagccacagc 1260

ggcggcagca agcccgcggc ccgcataaac agctgctgct cgtgatgctt aggtggacca 1320

tgttcgggac cgctggcgga gcttggcgtg gcttttgttg tgcggcgtgg ctgcaacgct 1380

ttgtcgttga acggcacatg cggtctgcgg attgcgtcgg cgggtagggc gcggagggaa 1440

ggtggtcggg tagaggctga ttccgcggtt ggatgaaggg accagtcggc ggaatcaggg 1500

cggcgcgcct gcatccaggc aagtgcaggc cggcagccag catatattca tgacatgtcg 1560

tactgctata atgtgctaga ttcaaagcca gcagctgact tggttgcaac cggcggtatt 1620

gctggtatcg tgcataaggc tcggaggcag gctactgcgc atgacactgc ctactgatac 1680

acagatagcg catggcttgc atgctatgaa aagggccagt ctgccactgc aggtacaatc 1740

gggctgtagg cggcgtaagt gagctcgggg gtgatggtgc gcaggcacat acctgcaaac 1800

aaacgacacc acagtaacaa gtgcggccgc 1830

<210> SEQ ID NO 9
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 9

ttttggtacc gagatctttg caaccggcac cggcaaggag tttttcg 47

<210> SEQ ID NO 10
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 10

ttttgcggcc gcacttgtta ctgtggtgtc gtttgtttgc agg 43

<210> SEQ ID NO 11
<211> LENGTH: 1442
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana

<400> SEQUENCE: 11

atggccaggt gagtcgacga gcaagcccgg cggatcaggc agcgtgcttg cagatttgac 60

ttgcaacgcc cgcattgtgt cgacgaaggc ttttggctcc tctgtcgctg tctcaagcag 120

catctaaccc tgcgtcgccg tttccatttg caggagattc gaggtaccgg aaggtaaagc 180

catcgcgacg tctctcggtg gtgatcgtgt attgatattc ccgtgttctc ctcgctcttc 240

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cttcgttttt acatcccggc tctctagcct gcctctaaag cgtgcgtcta tcggtggtgc 300

tgtctcttgt tccggcgtca atggcttgac tcggtggaat tccattgttt cgactcgccg 360

actcgttcct gttcgttcaa ttaactcgga atcggactcg gactccgact tccctcacga 420

gaatcagcag ggaaatccag gtttggggaa atttaaggaa taccaagaat gggactcatg 480

gacggccaag ttctccggtg gagcaaacat tccgtttctc atgctccaat tgcctcagat 540

catcctcaat acccagaatc ttttggcggg aaacaatacc gctctttcgg ctgtcccatg 600

gctgggaatg ttgactggtt tgttaggaaa cctttcgttg ctttcttatt ttgctaagaa 660

gagagaaaaa gaagcagctg tggtgcaaac actgggagtg gtctctactc acattgtgct 720

tgcacagctc acaatggctg aagcaatgcc tattcagtat tttgttgcta cttcagctgt 780

tgtcaccatc ggtctcattg tgaactgttt gtactatttc ggtaagctta gcaaaactgt 840

gtggcaactg tgggaagacg ttatcactat tggtggactc tccgttcttc ctcaaatcat 900

gtggtcaaca tttgtccctc ttgtaccaaa cagtatcttg cctgggacaa ctgcttttgg 960

tattgctgtg gcagctataa tcatggctcg aactgggaaa ctttcagaga aaggtgttag 1020

gtttgtaggg tctttatctg gatggacagc aactcttatg ttcatgtgga tgccagtttc 1080

ccaaatgtgg acaaattttc taaacccgga caacataaaa ggcttatcgt caatcacaat 1140

gttgctctcg atgatgggaa acgggcttat gatccctcga gcactattta tccgtgattt 1200

gatgtggctc actggttcgc tatgggcaac tctcttttat ggatatggaa atattctttg 1260

cttatacctg gtaaattgca ccagccagtc attcttcgtg gcagctacaa ttggtttgat 1320

ctcatggata ggactggctt tgtggagaga tgcagtggct tatggtcaca actcgccgtt 1380

tagatctttg aaagaacttg tttttggacc gtaatgaatg aatgtacacg ccatgcggcc 1440

gc 1442

<210> SEQ ID NO 12
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 12

cgaggtaccg gaaggtaaag ccatcgcgac gtctc 35

<210> SEQ ID NO 13
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 13

atggcggccg catggcgtgt acattcattc attacggtcc 40

<210> SEQ ID NO 14
<211> LENGTH: 98
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Template for ATG8-amiRNA

<400> SEQUENCE: 14

ctagagaagg caccaggtac ttcatctttc tcgctgatcg gcaccatggg ggtggtggtg 60

atcagcgcta aagaagaagt acctggtgcc ttcgctag 98

<210> SEQ ID NO 15
<211> LENGTH: 94
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleic acid sequence of a single chain constituting template for ATG8-amiRNA

<400> SEQUENCE: 15

ctagagaagg caccaggtac ttcatctttc tcgctgatcg gcaccatggg ggtggtggtg 60

atcagcgcta aagaagaagt acctggtgcc ttcg 94

<210> SEQ ID NO 16
<211> LENGTH: 94
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleic acid sequence of a single chain constituting template for ATG8-amiRNA

<400> SEQUENCE: 16

ctagcgaagg caccaggtac ttcttcttta gcgctgatca ccaccacccc catggtgccg 60

atcagcgaga aagatgaagt acctggtgcc ttct 94

<210> SEQ ID NO 17
<211> LENGTH: 415
<212> TYPE: PRT
<213> ORGANISM: Arabidopsis thaliana

<400> SEQUENCE: 17

Met Glu Gly Lys Ala Ile Ala Thr Ser Leu Gly Gly Asp Arg Val Leu
1 5 10 15

Ile Phe Pro Cys Ser Pro Arg Ser Ser Phe Val Phe Thr Ser Arg Leu
20 25 30

Ser Ser Leu Pro Leu Lys Arg Ala Ser Ile Gly Gly Ala Val Ser Cys
35 40 45

Ser Gly Val Asn Gly Leu Thr Arg Trp Asn Ser Ile Val Ser Thr Arg
50 55 60

Arg Leu Val Pro Val Arg Ser Ile Asn Ser Glu Ser Asp Ser Asp Ser
65 70 75 80

Asp Phe Pro His Glu Asn Gln Gln Gly Asn Pro Gly Leu Gly Lys Phe
85 90 95

Lys Glu Tyr Gln Glu Trp Asp Ser Trp Thr Ala Lys Phe Ser Gly Gly
100 105 110

Ala Asn Ile Pro Phe Leu Met Leu Gln Leu Pro Gln Ile Ile Leu Asn
115 120 125

Thr Gln Asn Leu Leu Ala Gly Asn Asn Thr Ala Leu Ser Ala Val Pro
130 135 140

Trp Leu Gly Met Leu Thr Gly Leu Leu Gly Asn Leu Ser Leu Leu Ser
145 150 155 160

Tyr Phe Ala Lys Lys Arg Glu Lys Glu Ala Ala Val Val Gln Thr Leu
165 170 175

Gly Val Val Ser Thr His Ile Val Leu Ala Gln Leu Thr Met Ala Glu
180 185 190

Ala Met Pro Ile Gln Tyr Phe Val Ala Thr Ser Ala Val Val Thr Ile
195 200 205

Gly Leu Ile Val Asn Cys Leu Tyr Tyr Phe Gly Lys Leu Ser Lys Thr

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    210                 215                 220

Val Trp Gln Leu Trp Glu Asp Val Ile Thr Ile Gly Gly Leu Ser Val
225                 230                 235                 240

Leu Pro Gln Ile Met Trp Ser Thr Phe Val Pro Leu Val Pro Asn Ser
                245                 250                 255

Ile Leu Pro Gly Thr Thr Ala Phe Gly Ile Ala Val Ala Ala Ile Ile
            260                 265                 270

Met Ala Arg Thr Gly Lys Leu Ser Glu Lys Gly Val Arg Phe Val Gly
        275                 280                 285

Ser Leu Ser Gly Trp Thr Ala Thr Leu Met Phe Met Trp Met Pro Val
    290                 295                 300

Ser Gln Met Trp Thr Asn Phe Leu Asn Pro Asp Asn Ile Lys Gly Leu
305                 310                 315                 320

Ser Ser Ile Thr Met Leu Leu Ser Met Met Gly Asn Gly Leu Met Ile
                325                 330                 335

Pro Arg Ala Leu Phe Ile Arg Asp Leu Met Trp Leu Thr Gly Ser Leu
            340                 345                 350

Trp Ala Thr Leu Phe Tyr Gly Tyr Gly Asn Ile Leu Cys Leu Tyr Leu
        355                 360                 365

Val Asn Cys Thr Ser Gln Ser Phe Phe Val Ala Ala Thr Ile Gly Leu
    370                 375                 380

Ile Ser Trp Ile Gly Leu Ala Leu Trp Arg Asp Ala Val Ala Tyr Gly
385                 390                 395                 400

His Asn Ser Pro Phe Arg Ser Leu Lys Glu Leu Val Phe Gly Pro
                405                 410                 415
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<210> SEQ ID NO 18
<211> LENGTH: 1248
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana

<400> SEQUENCE: 18

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atggaaggta aagccatcgc gacgtctctc ggtggtgatc gtgtattgat attcccgtgt     60

tctcctcgct cttccttcgt ttttacatcc cggctctcta gcctgcctct aaagcgtgcg    120

tctatcggtg gtgctgtctc ttgttccggc gtcaatggct tgactcggtg gaattccatt    180

gtttcgactc gccgactcgt tcctgttcgt tcaattaact cggaatcgga ctcggactcc    240

gacttccctc acgagaatca gcagggaaat ccaggtttgg ggaaatttaa ggaataccaa    300

gaatgggact catggacggc caagttctcc ggtggagcaa acattccgtt tctcatgctc    360

caattgcctc agatcatcct caatacccag aatcttttgg cgggaaacaa taccgctctt    420

tcggctgtcc catggctggg aatgttgact ggtttgttag gaaacctttc gttgctttct    480

tattttgcta agaagagaga aaaagaagca gctgtggtgc aaacactggg agtggtctct    540

actcacattg tgcttgcaca gctcacaatg gctgaagcaa tgcctattca gtattttgtt    600

gctacttcag ctgttgtcac catcggtctc attgtgaact gtttgtacta tttcggtaag    660

cttagcaaaa ctgtgtggca actgtgggaa gacgttatca ctattggtgg actctccgtt    720

cttcctcaaa tcatgtggtc aacatttgtc cctcttgtac caaacagtat cttgcctggg    780

acaactgctt ttggtattgc tgtggcagct ataatcatgg ctcgaactgg gaaactttca    840

gagaaaggtg ttaggtttgt agggtcttta tctggatgga cagcaactct tatgttcatg    900

tggatgccag tttcccaaat gtggacaaat tttctaaacc cggacaacat aaaaggctta    960

tcgtcaatca caatgttgct ctcgatgatg ggaaacgggc ttatgatccc tcgagcacta   1020
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tttatccgtg atttgatgtg gctcactggt tcgctatggg caactctctt ttatggatat 1080

ggaaatattc tttgcttata cctggtaaat tgcaccagcc agtcattctt cgtggcagct 1140

acaattggtt tgatctcatg gataggactg gctttgtgga gagatgcagt ggcttatggt 1200

cacaactcgc cgtttagatc tttgaaagaa cttgtttttg gaccgtaa 1248

<210> SEQ ID NO 19
<211> LENGTH: 371
<212> TYPE: PRT
<213> ORGANISM: Chlamydomonas reinhardtii

<400> SEQUENCE: 19

Met Leu Gln Pro Ala Pro Ala Arg Ser Phe Ser Gly Met Glu Gly Arg
1 5 10 15

Arg Thr Thr Gly Thr Arg Thr Leu Val Thr Val Ser Ser Arg Lys Lys
20 25 30

Pro Val Pro Pro Leu His Ala Gly Ala Thr Ser Gly Pro Ala Ser Asp
35 40 45

Ser Gln Arg Trp Asn Ala Leu Thr Asp Lys Leu Val Ala Ala Ser Ser
50 55 60

Ile Pro Phe Ser Ile Leu Val Leu Pro Gln Val Val Gln Asn Ala Ile
65 70 75 80

Asn Met Ala Gly Gly His Pro Thr Ala Leu Ser Ile Ile Ser Trp Glu
85 90 95

Gly Tyr Leu Ser Ala Met Phe Gly Asn Thr Leu Met Cys Ser His Phe
100 105 110

Ala Ala Ser Gly Glu Arg Ser Ala Val Asn Val Gln Leu Val Gly Ile
115 120 125

Leu Asn Asn Phe Leu Ile Leu Thr Gln Val Ala Leu Ala Gly Phe Met
130 135 140

Pro Leu Ala Val Phe Leu Ala Ala Ala Ala Phe Thr Ala Phe Ala Thr
145 150 155 160

Phe Met Asn Leu Ala Arg Val Ala Ala Leu Ala Gly Ala Ala Gln Pro
165 170 175

Ala Asp Glu Lys Trp Gly Ser Trp Gln Met Trp Gln Leu Gly Ser Gly
180 185 190

Leu Val Gly Leu Ala Val Val Pro Gln Val Leu Tyr Asn Thr Val Ser
195 200 205

Pro Ala Ala Ser Thr Leu Leu Pro Phe Ile Cys Thr Leu Gly Leu Leu
210 215 220

Gly Ala Val Leu Gly Leu Arg Leu Ser Ser Lys Gly Gly Ser Asp Ala
225 230 235 240

Ala Thr Leu Val Arg Gln Leu Pro Gly Trp Gly Ala Thr Leu Leu Phe
245 250 255

Ala Leu Ser Pro Leu Pro Gln Leu Val Arg Asn Leu Leu Glu Pro Gln
260 265 270

Ser Leu Glu Gly Leu Ser Val Gly Thr Met Leu Leu Ala Leu Leu Gly
275 280 285

Asn Ala Leu Met Val Pro Arg Ala Leu Phe Val Arg Asp Val Val Trp
290 295 300

Leu Ser Gly Thr Cys Trp Ala Cys Val Ala Gly Trp Gly Gln Leu Phe
305 310 315 320

Ser Met Phe Arg Ser Ala Ser Ala Ala Thr Gly Arg Arg Phe Leu Asp
325 330 335

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Pro Trp Leu Phe Phe Ser Ile Thr Gly Ala Leu Leu Leu Tyr Thr Gly
            340                 345                 350

Tyr Val Val Val Gln His Arg Arg Ala Thr Ala Ala Ala Ala Ser Pro
        355                 360                 365

Arg Pro Ala
    370


<210> SEQ ID NO 20
<211> LENGTH: 1116
<212> TYPE: DNA
<213> ORGANISM: Chlamydomonas reinhardtii

<400> SEQUENCE: 20

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acgcgaacgc tggtgactgt cagtagccgg aaaaagcccg tgcccccgtt gcatgcggga    120

gcaacgagcg gtccggcaag cgacagccag cgatggaatg cgctcacgga caagctggtg    180

gctgcatcca gcattccgtt ctctattttg gtgcttccgc aagtcgtcca gaatgccatc    240

aacatggctg gcggccaccc cacggccctg tccattatca gctgggaggg ctacctctct    300

gccatgttcg gcaacacatt aatgtgcagc catttcgcgg cgtcaggcga gcgcagcgcc    360

gtaaacgtgc agcttgtggg aatcctcaac aacttcctca ttctcacaca ggtggctctt    420

gccggcttca tgccgctcgc ggtgtttttg gcggcggcgg ccttcacggc ctttgccact    480

tttatgaacc tggcacgcgt ggcggcgctg gcgggcgcag cgcagccggc ggacgagaag    540

tggggcagct ggcagatgtg gcagctgggc agcgggctgg tggggctggc ggtggtgccg    600

caggtgctct acaacaccgt gtccccggcg gcatccacgc tgctgccctt catttgcacg    660

cttgggctgc ttggagcggt gctagggcta cggctgtcgt ccaagggcgg tagcgacgcg    720

gcaacgctgg tgcggcagct gccgggctgg ggcgccacgc tgctgtttgc gctgtcgccg    780

ctaccgcagc tggtccgcaa cctgcttgag ccacagagcc tggagggcct gagcgtgggc    840

accatgctgc tggcgctgct gggcaacgcg ctcatggtgc cgcgggcgct gttcgtgcgc    900

gacgtggtgt ggctcagcgg cacctgctgg gcctgcgtag cgggctgggg tcagctgttc    960

agcatgttcc gcagcgcgtc ggcggccaca gggcggcgct tcctggaccc ctggctgttc   1020

ttctccatca ccggggcgct gctgctgtac actggctacg tggtggtgca gcaccggaga   1080

gccacagcgg cggcagcaag cccgcggccc gcataa                              1116
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What is claimed is:

1. A method of biomass production, comprising:

irradiating with light a modified alga, thereby photoirradiating the modified alga, wherein the modified alga has suppressed intracellular expression of ATG8 as compared to that of a reference strain, wherein the reference strain is an alga otherwise identical to the modified alga but which lacks suppression of intracellular ATG8 expression, and wherein the intracellular ATG8 expression in the modified alga is 0.9 times or less as compared to that of the reference strain cultured under the same conditions; and

recovering photosynthate by separating starch granules produced by the modified alga from the modified alga or a lysate thereof.

2. The method according to claim **1**, wherein the modified alga has an increased chloroplastic glutathione concentration as compared to that of the reference strain.

3. The method according to claim **2**, wherein the photoirradiation step is carried out on the modified alga in a culture medium having an inorganic nitrogen content of 0.001% to 0.1% by weight.

4. The method according to claim **3**, wherein the method comprises no cell lysis step to disrupt algal cells.

* * * * *