Title: DOUBLY SECURE TRANSGENIC ALGAE OR CYANOBACTERIA STRAINS TO PREVENT THEIR ESTABLISHMENT AND SPREAD IN NATURAL ECOSYSTEMS

Abstract: A dual method for preventing the establishment of cultivated transgenic algae and cyanobacteria in natural ecosystems should they be released from enclosed cultivation. Algae or cyanobacteria strains engineered by this dual method and to their uses for biotechnological applications.
DOUBLY SECURE TRANSGENIC ALGAE OR CYANOBACTERIA STRAINS TO PREVENT THEIR ESTABLISHMENT AND SPREAD IN NATURAL ECOSYSTEMS.

Field of the invention

The present invention relates to a dual method for preventing the establishment of cultivated transgenic algae and cyanobacteria in natural ecosystems should they be released from enclosed cultivation, to algae or cyanobacteria strains engineered by this method and to their uses for biotechnological applications thereof.

Background of the invention

Algae and cyanobacteria have recently attracted much interest as biofactories for production of foods, bioactive compounds and biofuels. Since algae and cyanobacteria need sunlight, carbon-dioxide and water for growth, they can be cultivated in open or enclosed water bodies. These systems are vulnerable to being contaminated by other algal species and cyanobacteria. Similarly, the cultivated algae may escape outside the cultivation system. This may become a serious concern when the cultivated cells are transgenically modified.

The release of organisms containing introgressed genetically engineered genetic traits may have negative environmental impacts and be of regulatory concern, and thus it is imperative that algae and cyanobacteria containing transgenic traits do not establish and compete with indigenous species outside of their place of cultivation, or transfer their transgenes to indigenous organisms, or receive genes from indigenous organisms that might render the transgenic organisms more fit to survive in natural ecosystems. While the major type of introgression from transgenic crops is sexual interspecific genetic gene flow, and in some cases sexual gene flow to related species, in the case of algae and cyanobacteria, it is mainly that they themselves will establish and propagate asexually, as sexual exchanges are quite rare with most algal and cyanobacterial species. Still, cyanobacteria can be subject to horizontal gene flow through phages and possibly by conjugation.

Horizontal gene flow is more rare in eukaryotic organisms included algae, but conjugation-like processes have been confirmed by protoplast fusion (Sivan and Arad, 1998). What can
occur in the laboratory at high frequency intra-specifically, can happen at much lower frequencies in nature, posing a finite risk, possibly even between related species. Additionally, there is evidence that some algae, e.g. have had extensive genomic combinations of organism types or contain plasmids containing DNA originating from apparent horizontal gene flow.

A “transgenic mitigation” (TM) concept has been proposed for higher plants in US 7,612,255, WO2004/46362 and in Gressel, J. et al (Gressel 1999) and extended to transgenic algae and cyanobacteria US2009/0215179, US2010/0081177 and US2011/0045593. In this concept, transgenic algae or cyanobacteria are provided bearing at least one genetically engineered, commercially desirable genetic trait that is considered at risk of establishing in natural ecosystems but tandemly linked to, and co-expressing at least one mitigation transgene that is desirable or neutral to the cultivated transgenic algae or cyanobacteria but rendering the transgenic algae or cyanobacteria incapable of establishing by itself or in introgressed offspring in natural ecosystems. This concept allows obtaining a cultivated algae or cyanobacteria capable of mitigating the effects of release of said genetically engineered, commercially desirable genetic trait of the algae or cyanobacteria in natural ecosystems, the concept being based on the fact that the sequence encoding the desirable genetic trait and the sequence of the mitigating gene remain genetically linked in the transgenic algae or cyanobacteria because of the introduction of the sequences in tandem.

Several drawbacks of this system may emerge. Even if segregation from each other between desirable genetic trait sequence and mitigating gene sequence is exceedingly rare and far below the natural mutation rate, it is still possible to lose the mitigation trait, especially if sexual or asexual recombination is possible in a defined algae species and in the case of a spill in a breeding-conducive ecosystem. Even if the mitigation system described earlier can be seen as functionally dominant because the antisense or RNAi would still probably suppress the expression of the mitigation gene if said gene was re-appearing consequently to a rare conjugational event, one can avoid an eventual silencing of said antisense construct in some organisms, allowing them to survive, establish and multiply in natural ecosystems.

Also, it is known that in a case of non-targeted genomic events the risk to obtain a transgene expression over time that is unstable, or environmentally dependent, is high. With the current diatom transformation technology, transformed DNA randomly integrates into the genome,
which results in different expression levels for different transformants using the same DNA construct. This is particularly true in the case where the trait that is considered is silenced by an antisense or RNAi approach. Gene silencing in *P. tricornutum* using anti-sense or inverted-repeat containing RNAs have been reported in De Riso et al, (De Riso, Raniello et al. 2009) where endogenous genes expression have been modulated.

This kind of approach where the trait that is considered is not physically suppressed in the genome of the algae but only knocked-down, is not reliable enough to be applied to particular genes the silencing of which is able to render a transgenic algae or cyanobacteria incapable of establishing by itself or in introgressed offspring in natural ecosystems.

There is clearly a recognized need for safer anti-establishment mechanisms of algae and cyanobacteria released in natural ecosystems that also precludes establishment of rare cases where the transgenes interspecifically introgress into other algae or cyanobacteria.

Meganucleases have emerged as a powerful genome engineering tool to perform targeted genomic manipulations ranging from targeted insertions in chosen loci (knock-in), considered or not as "safe harbors" for gene addition (i.e. a loci allowing safe expression of a transgene), to targeted gene knock-out, allele swap, substitutions, marker excisions, deletions, inside algae genomes.

Meganucleases, also referred to as homing endonucleases, were the first endonucleases used to induce double-strand breaks and recombination in living cells (Rouet, Smih et al. 1994 ; Rouet, Smih et al. 1994 ; Choulika, Perrin et al. 1995 ; Puchta, Dujon et al. 1996 ). However, their use has long been limited by their narrow specificity. Although several hundred natural meganucleases had been identified over the past years (Belfort and Roberts 1997; Belfort and Roberts 1997; Chevalier and Stoddard 2001; Stoddard 2005; Stoddard 2005), this diversity was still largely insufficient to address genome complexity, and the probability of finding a meganuclease cleavage site within a gene of interest is still extremely low. These findings highlighted the need for artificial endonucleases with tailored specificities, cleaving chosen sequences with the same selectivity as natural endonucleases.

Meganucleases have emerged as scaffolds of choice for deriving genome engineering tools cutting a desired target sequence (Paques and Duchateau 2007). Combinatorial assembly processes allowing to engineer meganucleases with modified specificities has been described
by (Arnould, Chames et al. 2006; Smith, Grizot et al. 2006; Arnould, Perez et al. 2007; Grizot, Smith et al. 2009). Briefly, these processes rely on the identifications of locally engineered variants with a substrate specificity that differs from the substrate specificity of the wild-type meganuclease by only a few nucleotides. Up to four sets of mutations identified in such proteins can then be assembled in new proteins in order to generate new meganucleases with entirely redesigned binding interface.

These processes require two steps, wherein different sets of mutations are first assembled into homodimeric variants cleaving palindromic targets. Two homodimers can then be co-expressed in order to generate heterodimeric meganucleases cleaving the chosen non palindromic target. The first step of this process remains the most challenging one, and one cannot know in advance whether a meganuclease cleaving a given locus could be obtained with absolute certainty. Indeed, not all sequences are equally likely to be cleaved by engineered meganucleases, and in certain cases, meganuclease engineering could prove difficult (Galetto, Duchateau et al. 2009).

The knock-out of a genetic trait by an endonuclease such as a meganuclease is functionally recessive if the gene encoding said genetic trait re-appears consequently to a rare conjugational event that can still happen during the life cycle of an organism such as an alga or cyanobacteria.

The present invention successfully and synergistically addresses the shortcomings of the known limitations of both unneeded gene suppression with mitigation technologies, and gene excision by meganucleases by conceiving and providing a dual mechanism for avoiding the establishment of a transgenic algae or cyanobacteria and their progeny from establishing by self-propagation or by the effects of introgression of a genetically engineered trait of an alga or cyanobacterium to competing organisms.

Summary of the invention

In a first aspect, the present invention concerns a dual method to prevent algae or cyanobacteria to establish and spread in natural ecosystems by performing a) a gene knock-
out of at least one targeted gene, said targeted gene being not essential in the cultivated transgenic algae or cyanobacteria and being able to render said transgenic algae or cyanobacteria incapable of establishing by itself or in introgressed offspring in natural ecosystems when suppressed and b) by introducing into said same algae or cyanobacteria genome a blocking sequence which presence or expression can sufficiently suppress the function of a target gene being not essential in the cultivated transgenic algae or cyanobacteria and being able to render said transgenic algae or cyanobacterium unfit to establish outside of cultivation. This method may be used to prevent establishment or spread of recombinant algae or cyanobacteria in general and can be applied to negatively modulating or controlling the establishment, growth or survival of recombinant algae or cyanobacteria under other conditions such as those found in vivo (e.g., in or on plants, animals or microbial systems), in vitro (e.g., in liquid, semisolid, aerosol, or solid substrates), under industrial culture conditions, in biofilms, in or on buildings or artifacts, within artificial biological systems, or in disrupted natural ecosystems.

In a second aspect, the present invention relates to endonucleases, functional mutants, variants and/or derivatives thereof used to implement the previous method and to prevent algae or cyanobacteria from establishing and spreading in natural ecosystems.

In a third aspect, the present invention concerns a genomically engineered algae or cyanobacteria to prevent their establishment and spread in natural ecosystems.

The above objects highlight certain aspects of the invention. Additional objects, aspects and embodiments of the invention are found in the following detailed description of the invention.

**Brief description of the figures**

In addition to the preceding features, the invention further comprises other features that will emerge from the description that follows, which refers to examples illustrating endonuclease variants and their uses according to the invention, as well as to the appended drawings. A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following Figures in conjunction with the detailed description below.
- Figure 1: Illustrates different meganucleases-induced types of recombination events leading to stable and precise genomic modifications. 

A. Targeted integration (insertion of a transgene) through gene conversion; this approach can also be used for simultaneous gene insertion and gene knock-out by homologous recombination. 

B. Knock-out of a gene through NHEJ initiated by a unique double-stranded break. 

C. Knock-out of a gene initiated by two double-stranded breaks (gene excision), the presence of a knock-out matrix being not mandatory.

- Figure 2: Modular structure of homing endonucleases and the combinatorial approach for custom meganucleases design. 

A. Tridimensional structure of the I-CreI homing endonuclease bound to its DNA target. The catalytic core is surrounded by two folds forming a saddle-shaped interaction interface above the DNA major groove. 

B. A combinatorial process for meganuclease engineering: Four separable DNA binding subdomains (boxed) could be identified in the I-CreI scaffold, a homodimeric meganuclease that binds and cleaves a palindromic target. Each subdomain can be engineered specifically (boxed), resulting in novel meganucleases cleaving locally altered palindromic targets. Two different subdomains can be combined within a “half meganuclease”, a homodimeric meganuclease binding a palindromic target. Two such “half meganucleases” can be co-expressed to form a heterodimeric custom meganuclease that will cleave a novel non-palindromic target. Additional steps of engineering (by random or targeted mutagenesis and screening) are often required at this stage to optimize the activity of meganucleases, resulting in a refined meganuclease. In the final version, the two refined monomers can be connected by a linker in a single-chain meganuclease, as described in Grizot et al. (Grizot, Smith et al. 2009).

- Figure 3: Schematic showing meganucleases targeting carbonic anhydrase gene from *Phaeodactylum tricornutum*.

- Figure 4: Sequences and locations of meganuclease target sites in non-coding regions of the *Phaeodactylum tricornutum* genome.

**Detailed Description of the Invention**

Unless specifically defined herein below, all technical and scientific terms used herein have the same meaning as commonly understood by a skilled artisan in the fields of gene therapy, biochemistry, genetics, and molecular biology. Such techniques are explained fully in the

All methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, with suitable methods and materials being described herein. All publications, patent applications, issued patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. Further, the materials, methods, and examples are illustrative only and are not intended to be limiting, unless otherwise specified.

A first aspect of the present invention concerns a dual method to prevent transgenic algae or cyanobacteria from establishing and spreading in natural ecosystems, said method comprising the steps of:

a) Performing into said alga or cyanobacterium genome the knock-out of at least one target gene being not essential in the cultivated transgenic alga or cyanobacterium and being able to render said transgenic alga or cyanobacterium incapable of establishing by itself in natural ecosystems when suppressed; and
b) Introducing into same alga or cyanobacterium from step a) a blocking sequence which presence or expression can sufficiently suppress the function of a target gene being not essential in the cultivated transgenic alga or cyanobacterium and being able to render said transgenic alga or cyanobacterium or its introgressed or conjugated offspring incapable of establishing by itself in natural ecosystems.

In an embodiment of the method of the present invention, said target genes from step a) and step b), respectively, are different. In another embodiment of the method of the present invention, said target genes from step a) and step b), respectively, are identical.

In a preferred embodiment, the knock-out step a) of the method of the present invention is performed by using one endonuclease, a functional mutant, a variant or a derivative of such endonuclease (as illustrated in Figure 1B). In another preferred embodiment, said knock-out step a) of the method of the present invention is performed by using more than one endonuclease, i.e. two endonucleases as a non-limiting example, functional mutants, variants or derivatives of such endonucleases (as illustrated in Figure 1C).

As used herein, the term "endonuclease" refers to any wild-type or variant enzyme capable of catalyzing the hydrolysis (cleavage) of bonds between nucleic acids within a DNA or RNA molecule, preferably a DNA molecule. Endonucleases can be classified as rare-cutting endonucleases when having typically a polynucleotide recognition sequence greater than 12 base pairs (bp) in length, more preferably of 14-45 bp. Rare-cutting endonucleases significantly increase HR by inducing DNA double-strand breaks (DSBs) at a defined locus (Rouet, Smih et al. 1994; Rouet, Smih et al. 1994; Choulika, Perrin et al. 1995; Pingoud and Silva 2007).

Rare-cutting endonucleases according to the invention can for example be a homing endonuclease (Paques and Duchateau 2007), a chimeric Zinc-Finger nuclease (ZFN) resulting from the fusion of engineered zinc-finger domains with the catalytic domain of a restriction enzyme such as FokI (Porteus and Carroll 2005) or a chemical endonuclease (Arimondo, Thomas et al. 2006); (Eisenschmidt, Lanio et al. 2005 ; Simon, Carnata et al. 2008); (Cannata, Brunet et al. 2008 ). In chemical endonucleases, a chemical or peptidic cleaver is conjugated either to a polymer of nucleic acids or to another DNA recognizing a specific target sequence, thereby targeting the cleavage activity to a specific sequence. Chemical
endonucleases also encompass synthetic nucleases like conjugates of orthophenanthroline, a DNA cleaving molecule, and triplex-forming oligonucleotides (TFOs), known to bind specific DNA sequences (Kalish and Glazer 2005). Such chemical endonucleases are comprised in the term “endonuclease” according to the present invention.

Rare-cutting endonucleases can also be for example TALENs, a new class of chimeric nucleases using a FokI catalytic domain and a DNA binding domain derived from Transcription Activator Like Effector (TALE), a family of proteins used in the infection process by plant pathogens of the Xanthomonas genus (Boch, Scholze et al. 2009; Moscou and Bogdanove 2009; Christian, Cermak et al. 2010; Li, Huang et al. 2011). The functional layout of a FokI-based TALE-nuclease (TALEN) is essentially that of a ZFN, with the Zinc-finger DNA binding domain being replaced by the TALE domain. As such, DNA cleavage by a TALEN requires two DNA recognition regions flanking an unspecific central region. Rare-cutting endonucleases encompassed in the present invention can also be derived from TALENs.


In a preferred embodiment, the homing endonuclease according to the invention is a LAGLIDAGD endonuclease such as I-Sce I, I-Crel I, I-Ceu I, I-Msol I, and I-Dmol I.

In a most preferred embodiment, said LAGLIDAGD endonuclease is I-Crel I. To be active, I-Crel I needs to dimerize. Wild-type I-Crel I is a homodimeric homing endonuclease that is
capable of cleaving a 22 to 24 bp double-stranded target sequence. The sequence of a wild-type monomer of I-CreI includes the sequence shown as SEQ ID NO: 1 (which corresponds to the I-CreI sequence of pdb accession number 1g9y).

Different groups have also used a semi-rational approach to locally alter the specificity of the I-CreI (Seligman, Stephens et al. 1997; Sussman, Chadsey et al. 2004); International PCT Applications WO 2006/097784, WO 2006/097853, WO 2007/060495 and WO 2007/049156 (Cellectis); (Arnould, Chames et al. 2006; Rosen, Morrison et al. 2006; Smith, Grizot et al. 2006), I-SceI (Doyon, Pattanayak et al. 2006), PI-SceI (Gimble, Moure et al. 2003) and I-MsoI (Ashworth, Havranek et al. 2006).

In addition, hundreds of I-CreI derivatives with locally altered specificity were engineered by combining the semi-rational approach and High Throughput Screening. The method for producing mecanunase variants and the assays based on cleavage-induced recombination in mammal or yeast cells, which are used for screening variants with altered specificity are described in the International PCT Application WO 2004/067736; (Epinat, Arnould et al. 2003; Chames, Epinat et al. 2005; Arnould, Chames et al. 2006). These assays result in a functional LacZ reporter gene which can be monitored by standard methods. The assembly of four sets of mutations into heterodimeric endonucleases cleaving a model target sequence or a sequence from different genes has been described in the following Cellectis International patent applications: XPC gene (WO2007/093918), RAG gene (WO2008/010093), HPRT gene (WO2008/059382), beta-2 microglobulin gene (WO2008/102274), Rosa26 gene (WO2008/152523), Human hemoglobin beta gene (WO2009/13622) and Human interleukin-2 receptor gamma chain gene (WO2009019614). In the present application, I-CreI variants may be homodimers (mecanunase comprising two identical monomers) or heterodimers (mecanunase comprising two non-identical monomers; WO 2006/097854 and WO 2007/034262). It is understood that the scope of the present invention also encompasses the I-CreI variants per se, including heterodimers, obligate heterodimers, hybrid mecanunases composed of two monomers from different mecanunases, single chain mecanunases composed of two monomers from identical mecanunase, as described in applications WO03/078619 and WO2009095793, as non limiting examples, able to cleave one of the sequence targets in algae genome.
In the present patent application, the I-CreI variants can comprise an additional alanine after the first methionine of the wild type I-CreI sequence, and three additional amino acid residues at the C-terminal extremity, as shown in SEQ ID NO: 2. These three additional amino acid residues consist of two additional alanine residues and one aspartic acid residue after the final proline of the wild type I-CreI sequence. These additional residues do not affect the properties of the enzyme. For the sake of clarity, these additional residues do not affect the numbering of the residues in I-CreI or variants thereof. More specifically, the numbering used herein exclusively refers to the position of residues in the wild type I-CreI enzyme of SEQ ID NO: 1. For instance, the second residue of wild-type I-CreI is in fact the third residue of a variant which comprises an additional alanine after the first methionine.

The invention encompasses both wild-type and variant endonucleases. In a preferred embodiment, the endonuclease according to the invention is a "variant" endonuclease, i.e. an endonuclease that does not naturally exist in nature and that is obtained by genetic engineering or by random mutagenesis. The variant endonuclease according to the invention can for example be obtained by substitution of at least one residue in the amino acid sequence of a wild-type endonuclease with a different amino acid. Said substitution(s) can for example be introduced by site-directed mutagenesis and/or by random mutagenesis. In the frame of the present invention, such variant endonucleases remain functional, i.e. they retain the capacity of recognizing and specifically cleaving a target sequence.

The variant endonuclease according to the invention cleaves a target sequence that is different from the target sequence of the corresponding wild-type endonuclease. For example, the target sequence of a variant I-CreI endonuclease is different from the sequence of SEQ ID NO: 3 (C1221). Methods for obtaining such variant endonucleases with novel specificities are described in International PCT applications WO 2006/097784 and WO 2006/097853, Arnould et al. (J. Mol. Biol., 2006, 355, 443-458), Arnould et al. (Arnould, Perez et al. 2007)), Smith et al. (Smith, Grizot et al. 2006) and Chames et al. (Chames, Epinat et al. 2005).

By a blocking sequence as introduced in step b) of the present invention is intended a sequence which presence or expression can sufficiently suppress or silence the function of a target gene being not essential in the cultivated transgenic alga or cyanobacterium and being able to render said transgenic alga or cyanobacterium unfit to establish outside cultivation. By blocking sequence is also intended a sequence (or interfering sequence) silencing per se target
genes or respective products of said genes according to the invention or by the blocking agent/product (or interfering agent/product) encoded by said blocking sequence. As a non-limiting example, said blocking sequence can be one or more sequences selected from the group consisting of siRNAs, shRNAs, miRNA or cDNAs. As another non-limiting example, said blocking sequence is an interfering sequence being able to silence per se target gene according to the present invention (RNA interference well-known in the art). As another non-limiting example, said blocking sequence can encode for a protein inhibitor i.e. the interfering agent/product in this case, this protein inhibitor being able to interact and directly or indirectly inhibits a target protein/enzyme, product of the target gene according to the present invention.

A detailed in silico analysis referred in De Riso et al (De Riso, Raniello et al. 2009) of the diatoms genome for known components of the RNAi pathway indicates that molecular players involved in RNA silencing in other eukaryotes are only poorly conserved in diatoms and that distantly related proteins may fulfill their functions in these particular organisms. As shown at least in mammalian cells, the enzyme Dicer cleaves long dsRNAs into short-interfering RNAs (siRNAs) of approximately 21-23 nucleotides. One of the two siRNA strands is then incorporated into an RNA-induced silencing complex (RISC). RISC compares these “guide RNAs” to RNAs in the cell and efficiently cleaves target RNAs containing sequences that are perfectly, or nearly perfectly complementary to the guide RNA. “iRNA construct” also includes nucleic acid preparation designed to achieve an RNA interference effect, such as expression vectors able of giving rise to transcripts which form dsRNAs or hairpin RNA in cells, and or transcripts which can produce siRNAs in vivo.

A “short interfering RNA” or “siRNA” comprises an RNA duplex (double-stranded region) and can further comprises one or two single-stranded overhangs, 3’ or 5’ overhangs. Each molecule of the duplex can comprise between 17 and 29 nucleotides, including 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, and 29 nucleotides. siRNAs can additionally be chemically modified.

“MicroRNAs” or “miRNAs” are endogenously encoded RNAs that are about 22-nucleotide-long, that post-transcriptionally regulate target genes and are generally expressed in a highly tissue-specific or developmental-stage-specific fashion. At least more than 200 distinct miRNAs have been identified in plants and animals. These small regulatory RNAs are believed to serve important biological functions by two predominant modes of action: (1) by repressing the translation of target mRNAs, and (2) through RNA interference, that means
cleavage and degradation of mRNAs. In this latter case, miRNAs function analogously to siRNAs. miRNAs are first transcribed as part as a long, largely single-stranded primary transcript (pri-miRNA) (Lee, Jeon et al. 2002). This pri-miRNA transcript is generally and possibly invariably, synthesized by RNA polymerase II and therefore is polyadenylated and may be spliced. It contains an about 80-nucleotides long hairpin structure that encodes the mature about 22-nucleotides miRNA part of one arm of the stem. In animal cells, this primary transcript is cleaved by a nuclear RNaseIII-type enzyme called Drosha (Lee, Ahn et al. 2003) to liberate a hairpin mRNA precursor, or pre-miRNA of about-65 nucleotides long. This premiRNA is then exported to the cytoplasm by exportin-5 and the GTP-bound form of the Ran cofactor (Yi, Qin et al. 2003). Once in the cytoplasm, the pre-miRNA is further processed by Dicer, another RNaseIII enzyme to produce a duplex of about-22 nucleotides base pairs long that is structurally identical to a siRNA duplex (Hutvagner, McLachlan et al. 2001). The binding of protein components of the RISC, or RISC cofactors, to the duplex results in incorporation of the mature, single-stranded miRNA into a RISC or RISC-like protein complex, while the other strand of the duplex is degraded (Bartel 2004). Thus, one can design and express artificial miRNAs based on the features of existing miRNA genes. The miR-30 (microRNA 30) architecture can be used to express miRNAs (or siRNAs) from RNA polymerase II promoter-based expression plasmids (Zeng, Cai et al. 2005). In some instances the precursor miRNA molecules may include more than one stem-loop structure. The multiple stem-loop structures may be linked to one another through a linker, such as, for example, a nucleic acid linker, a miRNA flanking sequence, other molecules, or some combination thereof.

A “short hairpin RNA (shRNA)” refers to a segment of RNA that is complementary to a portion of a target gene (complementary to one or more transcripts of a target gene), and has a stem-loop (hairpin) structure, and which can be used to silence gene expression. A “stem-loop structure” refers to a nucleic acid having a secondary structure that includes a region of nucleotides which are known or predicted to form a double strand (stem portion) that is linked on one side by a region of predominantly single-stranded nucleotides (loop portion). The terms “hairpin” is also used herein to refer to stem-loop structures.

Artificial Interfering RNA or anti-sense constructs can be performed to suppress or silence one or several targeted genes in the genome of algae and cyanobacteria, based on the
sequence of the considered targeted gene or a homologous form of this gene, depending on
the physiological need for the gene product in cultivation. These constructs could be included
in a vector, such a plasmid, before being inserted into the targeted genome at a site of interest
and transcribed to provide their silencing effects.

In a preferred embodiment, the blocking sequence introduced in step b) of the method of the
present invention, which presence or expression can sufficiently suppress the function of a
target gene being not essential in the cultivated transgenic alga or cyanobacterium and being
able to render said transgenic alga or cyanobacterium unfit to establish outside of cultivation,
can be tandemly linked to a transgene encoding a trait of commercial interest such that both
said blocking sequence and said trait of commercial interest will be genetically linked in said
organism, precluding easy genetic segregation from each other and the transfer of said trait of
commercial interest only to indigenous organisms.

Traits of commercial interest according to the present invention are those that increase directly
or indirectly the potential commercial value of alga or cyanobacterium in various
biotechnological applications such as, in non-limiting examples, those related to : - Secretion
of hydrocarbons (without limitation, lipids, isoprenoids, polyunsaturated aldehydes as source
of alkanes or alkenes, production of polymers such as alginates), - Fatty acid composition
(lipid branching), - Lipid accumulation (biofuel production), - Lipids and antibacterial
therapeutic applications, - Photosynthesis (additional pigments to enlarge useful light
wavelengths), - Pigment production (carotenoids and phycobiliproteins as non-limiting
examples), - Herbicide resistance, - Mercury volatilization, - Frustule composition and
organization (nanostructured materials and devices).

In a preferred embodiment, the blocking sequence introduction step of the method of the
present invention, i.e. step b), is a targeted event into the genome of said alga or
cyanobacterium to introduce at a genomic site of interest said blocking sequence which
presence or expression can sufficiently suppress the function of a target gene to render the
organism unfit to establish outside of cultivation. In another preferred embodiment, step b) of
the method of the present invention is performed by using one endonuclease, a functional
mutant, a variant or a derivative of such endonuclease, wherein said blocking sequence
presence or expression can sufficiently suppress the function of a target gene, said target gene
being not essential in the cultivated transgenic alga or cyanobacterium and being able to
render said transgenic alga or cyanobacterium unfit to establish outside of cultivation. In another preferred embodiment, step b) of the method of the present invention, is performed by using more than one endonuclease, i.e. two endonucleases as a non-limiting example, functional mutants, variants or derivatives of such endonucleases. When step b) of the method of the present invention is a targeted event, i.e. when at least one endonuclease is used to introduce said blocking sequence into alga or cyanobacterium, said “blocking sequence introduction step” can be qualified as a “blocking sequence insertion step”.

In another embodiment, the blocking sequence according to step b) of the present invention is introduced at the genomic site of step a) target gene, i.e. the knock-out step a) is performed by the insertion of blocking sequence according to step b). In other words, step a) target gene is knocked-out by the insertion of the blocking sequence of step b) in its coding region. In this embodiment, the insertion of step b) blocking sequence can be performed by the same unique endonuclease variant. In this embodiment, the insertion of step b) blocking sequence can also be performed by using more than one endonuclease. In this embodiment, the insertion of step b) blocking sequence can be performed by using two different endonuclease variants.

In another embodiment, the blocking sequence according to step b) of the present invention is introduced at a genomic site distinct of the genomic site of step a) target gene. In this embodiment, steps a) and b) are performed by using, respectively, two different endonuclease variants, i.e, step a) is performed by using a first endonuclease variant and step b) is performed by using a second endonuclease variant. In another more preferred embodiment, steps a) and steps b) can be performed by using more than two different endonuclease variants. In this embodiment, as non-limiting example, the knock-out of step a) can be performed by using two different endonucleases variants and the insertion of blocking sequence according to step b) can be performed by using two different endonucleases variants, distinct of the previous ones.

The chosen locus (or targeted site of interest or target sequence) to insert the blocking sequence or said blocking sequence tandemly linked with a trait of commercial interest according to step b) of this first aspect of the invention allows stable expression of said blocking sequence over time. In a preferred embodiment, said target sequence to insert the blocking sequence is not environment dependent. In another preferred embodiment, the target sequence according to the invention is only present once within the genome of said algae or
cyanobacteria. Ideally, insertion into said chosen locus should have no impact on the expression of other genes. Preferably, said chosen locus to insert the blocking sequence is a "safe harbor" locus, i.e. a locus allowing safe and stable expression of the blocking sequence. This locus can be an intragenic (within an intron) or an intergenic locus i.e the chosen locus can be located inside or outside a gene within a defined genome. In a preferred aspect of this embodiment, the locus for targeted insertion is chosen close to a gene that is essential for survival of the targeted algae or cyanobacteria, said blocking sequence inserted at this locus becoming genetically linked to this essential gene and the probability of their independent segregation from each other becoming extremely low. In another preferred aspect of this embodiment, the locus for targeted insertion is chosen inside a gene that is essential for survival of the targeted algae or cyanobacteria. In this preferred aspect of this embodiment, said gene essential for the survival of the targeted algae or cyanobacteria is a housekeeping gene. In a non-limiting list, housekeeping genes that are comprised in the scope of the present invention are those required for the maintenance of basal cellular function, like as non-limiting examples, transcription factors, translation factors (tRNA synthetases, RNA binding proteins), ribosomal proteins, RNA polymerases, processing proteins, Heat Shock Proteins, histones, cell cycle genes, metabolism genes (carbohydrate metabolism, citric acid cycle, lipid metabolism, amino acid metabolism, nucleotide synthesis, structural genes (cytoskeleton, organelle synthesis), genes from chloroplast origin involved in photosynthesis.

In another embodiment, as mentioned earlier, said blocking sequence can also be inserted at the locus of the gene chosen for targeted knock-out.

In addition, in another specific embodiment, insertion of said blocking sequence into said locus preferably does not substantially modify the phenotype of said algae or cyanobacteria (except for the phenotype due to expression of the genetic element). By "phenotype" is meant an observable trait in considered organisms. The phenotype includes e.g. the viability, the cellular proliferation and/or the growth rate.

According to the present invention, "Algae" or "algae cells" or "cells", refer to different species of algae, including without limitation one or more algae selected from the genera *Amphora, Anabaena, Ankistrodesmus, Botryococcus, Chaetoceros, Chlorella, Chlorococcum, Cyclotella, Cylindrotheca, Dunaliella, Emiliana, Euglena, Haematococcus, Isochrysis, Monochrysis, Monoraphidium, Nannochloris, Nannochloropsis, Navicula, Nephrochloris,*
Nephroselmis, Nitzschia, Nodularia, Nostoc, Ochromonas, Oocystis, Oscillatoria, Pavlova, Phaeodactylum, Playmonas, Pleurochrysis, Porphyra, Pseudoanabaena, Pyramimonas, Stichococcus, Synechococcus, Synechocystis, Tetraselmis, Thalassiosira, and Trichodesmium (see Table 1).

According to further features in preferred embodiments of the invention described below, the cultivated algae or cyanobacteria is one of the following Synechococcus PCC7002, Phaeodactylum tricornutum, Nannochloropsis sp CS-246, Nannochloropsis oculata, Nannochloropsis salina, Pavlova lutheri CS-182, Synechococcus PCC7942, Synechocystis PCC6803, Chlamydomonas reinhardtii, Chlorella vulgaris, Chlorella spp., Isochrysis sp. CS-177, Tetraselmis chuii CS-26 Tetraselmis suecica CS-187, Nannochloris spp. and Athrospira plantesis.

<table>
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<tr>
<th>Genus</th>
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<th>Order</th>
<th>Phylum</th>
<th>Sub-Kingdom</th>
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Table 1: Phylogeny of some of the eukaryotic algae used [Phylogeny according to: http://www.algaebase.org/browse/taxonomy/; Note: Many genes that in higher plants and Chlorophyta are encoded in the nucleus are encoded on the chloroplast genome (plastome) in the Chromobiota red lineage algae (Grzebyk, et al., 2003)].

By “gene knock-out”, “knock-out of one targeted gene”, “targeted knock-out of a gene” or “knock-out” is intended the most powerful tool for permanently modifying the phenotypic
characteristics of a cell by suppressing a gene and/or a gene function. One of the subject-matter of the present invention is a method for making a knocked-out algae or knocked-out cyanobacterium for at least one targeted mitigation gene. In a preferred embodiment of the present invention, an endonuclease variant can be used, said endonuclease variant inducing a double-stranded cleavage at a site of interest inside said targeted mitigation gene. In this case, the subject matter of the present invention is a method for making a targeted knocked-out algae or cyanobacterium for at least one gene, by using an endonuclease variant, said endonuclease variant allowing it to induce the formation of a double-stranded cleavage at a site of interest inside said targeted gene. Preferably, said endonuclease variant has been engineered, as previously mentioned, to induce a double-stranded cleavage at a site of interest inside said targeted gene. Preferably, the endonuclease of the present invention is a meganuclease. The repair of double-stranded break (DSB) in eukaryotic cells can occur via the distinct mechanisms of homology directed repair (HDR) or nonhomologous end joining (NHEJ). Although HDR typically uses the sister chromatid of the damaged DNA as a template from which to perform perfect repair of the genetic lesion, NHEJ is an imperfect repair process that often results in changes to the DNA sequence at the site of the DSB. During NHEJ, the cleaved DNA is, for example, further processed by exonuclease activity, and more bases may be added or deleted in an irregular fashion before the two ends of the damaged DNA are rejoined, resulting in a non-conservative repair pathway leading to potential insertion/deletion events and knock-out of the target gene coding region.

In a particular embodiment, the knocked-out algae or knocked-out cyanobacterium is made by introducing into said organism, an endonuclease as defined above, so as to induce a double-stranded cleavage at a site of interest within the targeted gene comprising a DNA recognition and cleavage site for said endonuclease, and thereby generate genetically modified algae or cyanobacterium knocked-out for said targeted gene, said modified algae or cyanobacterium having repaired the double-strands break by NHEJ (figure 1B), and isolating said genetically modified knocked-out organism by any appropriate means.

In another particular embodiment the knocked-out algae or knocked-out cyanobacterium is made by introducing into said organism, 1) an endonuclease as defined above, so as to induce a double-stranded cleavage at a site of interest within the targeted gene comprising a DNA recognition and cleavage site for said endonuclease, 2) a knock-out template to be introduced
flanked by sequences sharing homologies with the region surrounding the genomic DNA cleavage site and thereby generate genetically modified algae or cyanobacterium knocked-out for the gene located at this site of interest of the genome, said modified organism having repaired the double stranded break by HDR, and isolating said genetically modified knocked-out organism by any appropriate means.

Another subtype of knock-out strategy consists in the elimination of a large region within targeted gene by the action of two mecanucleases and optionally a knock-out matrix, for the deletion of large sequences (figure 1C only illustrates an option including KO matrix). Such an optional knock-out matrix can be built using sequences of flanking homology outside and / or within the targeted gene open reading frame and deleted for the coding exon regions.

By "targeting DNA construct/minimal repair matrix/repair matrix/template (knock-out template or knock-out matrix)" it is intended to mean a DNA construct comprising first and second portions which are homologous to regions 5’ and 3’ of the DNA target in situ, at a site of interest in algae genome. The DNA construct also comprises a third portion positioned between the first and second portion which can comprise some homology with the corresponding DNA sequence in situ (in the cases of allele/promoter swap as non-limiting examples) or alternatively can comprise no homology with the regions 5’ and 3’ of the DNA target in situ (insertion of a selectable marker). Following cleavage of the DNA target, a homologous recombination event is stimulated between the genome containing the targeted gene or part of the targeted gene and the repair matrix, wherein the genomic sequence containing the DNA target is replaced by the third portion of the repair matrix and a variable part of the first and second portions of the repair matrix.

Preferably, homologous sequences of at least 50 bp, preferably more than 100 bp and more preferably more than 200 bp are used. Therefore, the targeting DNA construct is preferably from 200 bp to 6000 bp, more preferably from 1000 bp to 2000 bp. Indeed, shared DNA homologies are located in regions flanking upstream and downstream the site of the break and the DNA sequence to be introduced should be located between the two arms. The targeting construct comprises advantageously a positive selection marker between the two homology arms and eventually a negative selection marker upstream of the first homology arm or
downstream of the second homology arm. The marker(s) allow(s) the selection of algae having inserted the sequence of interest by homologous recombination at the target site.

According to the present invention, said at least one target gene, to perform knock-out of a gene being not essential in the cultivated transgenic alga or cyanobacterium and being able to render said transgenic alga or cyanobacterium incapable of establishing by itself in natural ecosystems when suppressed, may encode for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia or cell wall formation as non-exhaustive examples. According to the present invention, the targeted gene being not essential in the cultivated transgenic alga or cyanobacteria and that prevents transgenic algae or cyanobacteria to establish and spread in natural ecosystems may be one or more of the following:

- A targeted gene participating in the encoding of cilia or flagella (or similar motility organ) formation or action such that the transgenic algae or cyanobacteria cannot optimally position themselves based on environmental stimuli. Such movement is required to compete in natural ecosystems, but is unnecessary and utilizes energy in commercial cultivation;

- A targeted gene participating in the inhibition or suppression of the formation of an intracellular CO$_2$ pool that will not interfere with cultivation of algae or cyanobacteria in the presence of high levels of carbon dioxide but will preclude their ability to live in natural ecosystems, where the ambient carbon dioxide concentration is too low to allow them to photosynthesize effectively. Said targeted gene can be a gene participating to the function of the carbon concentrating mechanism such as carbonic anhydrase gene as a non limiting example;

- A targeted gene participating in the encoding of enzymes that catalyze essential metabolic pathways in nature but not essential in cultivation conditions of algae or cyanobacteria such as nitrate reductase as a non-limiting example. Nitrate reductase knock-out should make an algae strain or cyanobacteria strictly dependent on an abundant source of reduced nitrogen such as urea, ammonia, etc…

- Any other gene participating in the encoding of a totally non essential gene to the algae or cyanobacteria when cultivated commercially, but able to render the algae or...
cyanobacteria unfit to compete in natural ecosystems, overcoming any benefit that may derive from another commercially desirable trait that can be engineered into the same algae or cyanobacteria.

In a particular embodiment, the scope of the present invention encompasses the realization of more than one targeted gene knock-out into algae or cyanobacteria by using at least one specific endonuclease variant for each targeted gene, said each targeted gene being not essential in the cultivated transgenic or cyanobacteria and being able to render said transgenic algae or cyanobacteria incapable of establishing by itself or in introgressed offspring in natural ecosystems when silenced. The scope of the present invention encompasses the performance of two, three, four, five, up to ten gene knocks-out into algae or cyanobacteria, said genes being part, as non limiting examples, of classes of genes mentioned above or any other gene participating in the encoding of a non essential gene to the algae or cyanobacteria when cultivated commercially, but able to render the algae or cyanobacteria unfit to compete in natural ecosystems, overcoming any benefit that may derive from another commercially desirable trait that can be engineered into the same algae or cyanobacteria.

In a particular embodiment, said knock out from step a) according to the present invention is performed with an endonuclease whose target sequence in the targeted genome of said alga or cyanobacterium is selected from the group consisting of the SEQ ID NO: 4 to SEQ ID NO: 9.

One other subject matter of the method of the present invention is to perform, into the genome of said alga or cynaobacterium that has been knocked-out according to the present invention, the insertion of at least one blocking sequence or one blocking sequence tandemly linked with a trait of commercial interest, said blocking sequence presence or expression being able to sufficiently suppress the function of a target gene being not essential in the cultivated alga or cyanobacterium and being able to render said transgenic alga or cyanobacterium unfit to establish outside of cultivation. In a preferred embodiment, at least one endonuclease variant can be used to insert said at least one blocking sequence or said blocking sequence tandemly linked with a trait of commercial interest. In another preferred embodiment, two different endonuclease variants can be used to insert said at least one blocking sequence or said blocking sequence tandemly linked with a trait of commercial interest. When insertion of said blocking sequence is performed by using at least one endonuclease, said targeted event can be qualified as a “knock-in”.
In a preferred embodiment, said blocking sequence presence or expression is able to sufficiently suppress the function of a gene distinct from the gene that has been knocked out according to step a) of the method of the present invention; in another preferred embodiment, said blocking sequence presence or expression is able to sufficiently suppress the function of the gene that has been knocked-out according to step a) of the method of the present invention, both embodiments resulting in transgenic alga or cyanobacterium synergistically displaying higher degrees of biosafety against the risk of establishment outside specialized cultivation conditions.

In these embodiments the knocked-in algae or cyanobacteria is made by introducing into said organisms, 1) an endonuclease as defined above, so as to induce a double stranded cleavage at a site of interest for sequence insertion of the genome comprising a DNA recognition and cleavage site of said endonuclease, 2) a knock-in template to be introduced flanked by sequences sharing homologies with the region surrounding the genomic DNA cleavage site and thereby generate genetically modified algae or cyanobacteria at this site of interest of their genomes, said modified organisms having repaired the double-strands break by HDR, and isolating said genetically modified organisms by any appropriate means.

By “sequence insertion”, it is intended to mean the introduction into a target genome of an exogenous nucleotidic sequence, i.e. a “transgene”.

In this aspect of the invention, the knock-in template to be introduced is the transgene of interest, flanked by sequences sharing homologies with the region surrounding the genomic DNA cleavage site and thereby generate genetically modified algae or cyanobacteria at this site of interest of the genome.

By “targeting DNA construct/minimal repair matrix/repair matrix/template (knock-in template or knock-in matrix)” it is intended to mean a DNA construct comprising first and second portions which are homologous to regions 5’ and 3’ of the DNA target in situ, at a site of interest in algae genome. The DNA construct also comprises a third portion positioned between the first and second portion which can comprise some homology with the corresponding DNA sequence in situ (in the cases of allele/promoter swap as non-limiting examples) or alternatively can comprise no homology with the regions 5’ and 3’ of the DNA target in situ (insertion of a selectable marker). Following cleavage of the DNA target, a homologous recombination event is stimulated between the genome containing the targeted
gene or part of the targeted gene and the repair matrix, wherein the genomic sequence containing the DNA target is replaced by the third portion of the repair matrix and a variable part of the first and second portions of the repair matrix.

Preferably, homologous sequences of at least 50 bp, preferably more than 100 bp and more preferably more than 200 bp are used. Therefore, the targeting DNA construct is preferably from 200 bp to 6000 bp, more preferably from 1000 bp to 2000 bp. Indeed, shared DNA homologies are located in regions flanking upstream and downstream the site of the break and the DNA sequence to be introduced should be located between the two arms. The targeting construct comprises advantageously a positive selection marker between the two homology arms and eventually a negative selection marker upstream of the first homology arm or downstream of the second homology arm. The marker(s) allow(s) the selection of algae having inserted the sequence of interest by homologous recombination at the target site.

In this aspect of the invention, the transgene of interest, introduced by the knock-in template, or its encoding product, is able to silence the gene targeted for knock-out in step a). This aspect provided in the method of the present invention adds a supplementary degree of biosafety and synergistically reduces the risk of establishment outside specialized cultivation.

According to the present invention, said at least one gene, target of a blocking sequence according to the present invention, may encode for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia, RUBISCO, carotene content, photosystem II antennae contents or cell wall formation as non-exhaustive examples.

According to the present invention, the targeted gene being not essential in the cultivated transgenic algae or cyanobacteria and that render said transgenic algae or cyanobacteria unfit to establish outside from cultivation may be one or more of the following:

- A targeted gene participating in the encoding of cilia or flagella (or similar motility organ) formation or action such that the transgenic algae or cyanobacteria cannot optimally position themselves based on environmental stimuli. Such movement is required to compete in natural ecosystems, but is unnecessary and utilizes energy in commercial cultivation;

- A targeted gene participating in the inhibition or suppression of the formation of an intracellular CO₂ pool that will not interfere with cultivation of algae or cyanobacteria
in the presence of high levels of carbon dioxide but will preclude their ability to live in natural ecosystems, where the ambient carbon dioxide concentration is too low to allow them to photosynthesize effectively. Said targeted gene can be a gene participating to the function of the carbon concentrating mechanism such as carbonic anhydrase gene as a non limiting example;

- A targeted gene participating in the encoding of enzymes that catalyze essential metabolic pathways in nature but not essential in cultivation conditions of algae or cyanobacteria such as nitrate reductase as a non-limiting example. Nitrate reductase knock-out should make an algae strain or cyanobacteria strictly dependent on an abundant source of reduced nitrogen such as urea, ammonia, etc...

- Any other gene participating in the encoding of a totally non essential gene to the algae or cyanobacteria when cultivated commercially, but able to render the algae or cyanobacteria unfit to compete in natural ecosystems, overcoming any benefit that may derive from another commercially desirable trait that can be engineered into the same algae or cyanobacteria.

In a particular embodiment, said blocking sequence suppresses the function of a gene encoding for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia, or cell wall formation.

In another particular embodiment, said blocking sequence partially suppresses the function of a gene encoding for a trait selected from the group consisting of RUBISCO, carotene content or photosystem II antennae contents.

In a preferred embodiment, said blocking sequence is introduced (i.e. knocked-in) by using an endonuclease whose target sequence in the targeted genome of said alga or cyanobacterium is selected from the group consisting of the SEQ ID NO: 10 to SEQ ID NO: 12.

In another particular embodiment, the scope of the present invention also encompasses the realization of more than one targeted gene knock-in into algae or cyanobacteria by using at least one specific endonuclease variant for each targeted gene, said each targeted gene being not essential in the cultivated transgenic or cyanobacteria and being able to render said transgenic algae or cyanobacteria unfit to establish outside from cultivation. The scope of the present invention encompasses the performance of two, three, four, five, up to ten gene
knocks-in into algae or cyanobacteria, said genes being part, as non limiting examples, of classes of genes mentioned above or any other gene participating in the encoding of a non essential gene to the algae or cyanobacteria when cultivated commercially, but able to render the algae or cyanobacteria unfit to compete in natural ecosystems, overcoming any benefit that may derive from another commercially desirable trait that can be engineered into the same algae or cyanobacteria.

The method of the present invention is a safer anti-establishment mechanism as it proposes both a way to physically suppress a mitigation gene by a targeted knock-out, reducing the probability of losing this mitigation phenotype compared to a mitigation trait conferred by a classical non targeted approach and also a targeted knock-in of a transgene to silence product of the gene previously targeted for knock-out, the targeted aspect of said knock-in allowing to insert said transgene in a “safe” site of interest of the considered genome giving this insertion robustness, stability of expression over time and environment expression independency, compared to classical non-targeted approaches.

In a second aspect, the present invention concerns the use of endonuclease variants as previously described to be used in the previous method to prevent algae or cyanobacteria to establish and spread in natural ecosystems. The endonuclease variants in the scope of the present invention are engineered to create a double-stranded break at a site of interest into the genome of algae or cyanobacteria. Preferably, the endonuclease variants in the scope of the present invention are used to create a double-stranded break within a targeted gene into the genome of algae or cyanobacteria to knock-out said targeted gene into said organisms, wherein said targeted gene is not essential in the cultivated transgenic or cyanobacteria and being able to render said transgenic algae or cyanobacteria incapable of establishing by itself or in introgressed offspring in natural ecosystems when silenced. Preferably, the endonuclease variants in the scope of the present invention are used to target genes encoding for a trait selected from the group consisting of nitrate reductase, RUBISCO, carbonic anhydrase, photosystem II antennae, carotene content, starch content, inuline inhibition, flagella, cilia, cell wall, as previously described. Preferably, the endonuclease variants in the scope of the present invention are engineered as previously described to be used to target a suitable sequence inside target genes encoding traits selected from the previous list.
In this second aspect, the present invention also concerns endonuclease variants as previously described to be used in the previous method to prevent algae or cyanobacteria to establish and spread in natural ecosystems, by creating a double-stranded break variant to insert at least a transgene at a targeted site of interest into an algae genome, said at least one transgene product being able to silence the gene (or its product) targeted for knock-out and conferring a supplementary degree of biosafety by synergistically reducing the risk of establishment outside specialized cultivation. Preferably, said insertion consecutively to the double-stranded break created by the endonuclease variants in the scope of the present invention does not modify the expression of genes located in the vicinity of the target sequence. More preferably, said insertion of a transgene is close to a gene essential for the survival of said algae or cyanobacteria. More preferably, said insertion is close to a housekeeping gene. It is understood that multiple transgenes can be inserted at a targeted site of interest into an algae or cyanobacteria genome.

In another preferred embodiment, endonuclease variants provided in the present invention target sequences selected from the group consisting of the SEQ ID NO 4 to 9 from the genome of \textit{Phaeodactylum tricornutum} for carbonic anhydrase gene knock-out as a non limiting example and target sequences selected from the group consisting of the SEQ ID NO 10 to 12 from the genome of \textit{Phaeodactylum tricornutum} to insert transgenes at chosen locus of said genome.

In another embodiment, the same endonuclease variant can be engineered to perform the knock-out of the targeted gene to prevent algae or cyanobacteria to establish and spread in natural ecosystems and to perform the insertion of the transgene the products of which are able to silence the gene (or its product) targeted for knock-out. In a preferred embodiment, different variants can be engineered to perform the knock-out of the targeted gene to prevent algae or cyanobacteria to establish and spread in natural ecosystems and to perform the insertion of the transgene the products of which are able to silence the gene (or its product) targeted for knock-out.

Another subject-matter of the present invention is also a recombinant vector for the expression of an endonuclease, a functional mutant, a variant or a derivative as mentioned above. The recombinant vector comprises at least one polynucleotide fragment encoding an endonuclease, a functional mutant, a variant or a derivative as mentioned above. In another embodiment,
said vector can comprise two different polynucleotide fragments, each encoding one of the monomers of a heterodimeric variant.

By "vector" is intended a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. A vector which can be used in the present invention includes, but is not limited to, a viral vector, a plasmid, an RNA vector or a linear or circular DNA or RNA molecule which may consists of a chromosomal, non chromosomal, semi-synthetic or synthetic nucleic acids. Preferred vectors are those capable of autonomous replication (episomal vector) and/or expression of nucleic acids to which they are linked (expression vectors). Large numbers of suitable vectors are known to those skilled in the art and commercially available. Some useful vectors include, for example without limitation, pGEM13z, pGEMT and pGEMTEasy (Promega, Madison, WI); pSTBluel (EMD Chemicals Inc. San Diego, CA); and pcDNA3.1, pCR4-TOPO, pCR-TOPO-II, pCRBlunt-II-TOPO (Invitrogen, Carlsbad, CA).

Vectors can comprise selectable markers, for example: neomycin phosphotransferase, histidinol dehydrogenase, dihydrofolate reductase, hygromycin phosphotransferase, herpes simplex virus thymidine kinase, adenosine deaminase, Glutamine Synthetase, and hypoxanthine-guanine phosphoribosyl transferase for eukaryotic cell culture; TRP1, URA3 and LEU2 for S. cerevisiae; tetracycline, rifampicin or ampicillin resistance in E. coli. Preferably said vectors are expression vectors, wherein the sequence(s) encoding the variant/single-chain meganuclease of the invention is placed under control of appropriate transcriptional and translational control elements to permit production or synthesis of said variant. Therefore, said polynucleotide is comprised in an expression cassette. More particularly, the vector comprises a replication origin, a promoter operatively linked to said polynucleotide, a ribosome-binding site, an RNA-splicing site (when genomic DNA is used), a polyadenylation site and a transcription termination site. It also can comprise an enhancer. Selection of the promoter will depend upon the cell in which the polypeptide is expressed. Preferably, when said variant is a heterodimer, the two polynucleotides encoding each of the monomers are included in one vector which is able to drive the expression of both polynucleotides, simultaneously. Suitable promoters include tissue specific and/or inducible promoters. Examples of inducible promoters are: eukaryotic metallothionine promoter which is induced by increased levels of heavy metals, prokaryotic lacZ promoter which is induced in
response to isopropyl-β-D-thiogalacto-pyranoside (IPTG) and eukaryotic heat shock promoter which is induced by increased temperature.

In some embodiments, the vector for the expression of the endonucleases according to the invention can be operably linked to an algal-specific promoter. In some embodiments, the algal-specific promoter is an inducible promoter. In some embodiments, the algal-specific promoter is a constitutive promoter or a light-induced promoter such as the RUBISCO rbcS promoter (see patent application 20100081177 from Jonathan Gressel). Promoters that can be used include, for example without limitation, a NIT1 promoter, an AMT1 promoter, an AMT2 promoter, an AMT4 promoter, an RHI promoter, a cauliflower mosaic virus 35S promoter, a tobacco mosaic virus promoter, a simian virus 40 promoter, a ubiquitin promoter, a PBCV-I VP54 promoter, or functional fragments thereof, or any other suitable promoter sequence known to those skilled in the art. In cyanobacteria, the light-responsive suf promoters, involved in *Synechocystis* sp. PCC 6803 Fe-S cluster biogenesis, have also been well-characterized (Seki, Nakano et al. 2006).

In another most preferred embodiment according to the present invention the vector is a shuttle vector, which can propagate both in *E. coli* (the construct containing an appropriate selectable marker and origin of replication) and be compatible for propagation or integration in the genome of the selected algae.

According to another advantageous embodiment of said vector, it includes a targeting construct comprising sequences sharing homologies with the region surrounding the targeted genomic DNA cleavage site in algae as defined above.

For instance, said sequence sharing homologies with the regions surrounding the genomic DNA cleavage site of the variant is a fragment of the targeted genomic DNA. Alternatively, the vector coding for an endonuclease variant/single-chain meganuclease and the vector comprising the targeting construct are different vectors.

Endonucleases provided in the present invention can be delivered in various formats: DNA, messenger RNA, or as a protein.

A variety of different methods are known for the introduction of DNA into host cell nuclei or chloroplasts. In various embodiments, the vectors can be introduced into algae nuclei by, for example without limitation, electroporation, microporation particle inflow gun bombardment,
or magnetophoresis. The latter is a nucleic acid introduction technology using the processes of magnetophoresis and nanotechnology fabrication of micro-sized linear magnets (Kuehnle et al., U.S. Patent No. 6,706,394; 2004; Kuehnle et al., U.S. Patent No. 5,516,670; 1996) that proved amenable to effective chloroplast engineering in freshwater Chlamydomonas, improving plastid transformation efficiency by two orders of magnitude over the state-of-the-art of biolistics (Champagne et al., Magnetophoresis for pathway engineering in green cells. Metabolic engineering V: Genome to Product, Engineering Conferences International Lake Tahoe CA, Abstracts pp 76; 2004). Polyethylene glycol treatment of protoplasts is another technique that can be used to transform cells (Maliga 2004). In various embodiments, the transformation methods can be coupled with one or more methods for visualization or quantification of nucleic acid introduction to one or more algae.

Direct microinjection of purified endonucleases of the present invention in algae can be considered. Also, appropriate mixtures commercially available for protein transfection can be used to introduce endonucleases in algae according to the present invention. More broadly, any means known in the art to allow delivery inside cells or subcellular compartments of agents/chemicals and molecules (proteins) can be used to introduce endonucleases in algae according to the present invention including liposomal delivery means, polymeric carriers, chemical carriers, lipoplexes, polyplexes, dendrimers, nanoparticles, emulsion, natural endocytosis or phagocytose pathway as non-limiting examples.

In a third aspect, the present invention concerns a genomically engineered algae or cyanobacterium to prevent its establishment and spread in natural ecosystems, said organism comprising:

a) at least one genomic knock-out of one target gene, said at least one target gene being not essential in the cultivated transgenic alga or cyanobacterium and being able to render said transgenic alga or cyanobacterium incapable of establishing by itself or in introgressed offspring in natural ecosystems when suppressed; and

b) the introduction of at least one blocking sequence which presence or expression can sufficiently suppress the function of a target gene being not essential in the cultivated transgenic alga or cyanobacterium and being able to render said transgenic alga or cyanobacterium unfit to establish outside of cultivation.
In an embodiment of this aspect of the invention, said genomically engineered alga or cyanobacterium comprises a target gene from step b) which is different from target gene from step a). In another embodiment of this aspect of the invention, said genomically engineered alga or cyanobacterium comprises a target gene from step b) which is the same as said target gene from step a).

In another embodiment of this aspect of the invention, said blocking sequence construct in genomically engineered alga or cyanobacterium is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest are genetically linked in said same alga or cyanobacterium, precluding easy genetic segregation from each other.

In a preferred embodiment of this aspect of the invention, said introduction of blocking sequence in genomically engineered alga or cyanobacterium is a targeted event performed at a genomic site of interest of said alga or cyanobacterium. In another aspect of this preferred embodiment, said blocking sequence that is introduced by a targeted event is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest will be genetically linked in said same alga or cyanobacterium, precluding easy genetic segregation from each other. In another preferred embodiment, said genomic site of interest for introduction of said blocking sequence is the site for the target gene knock-out of step a) of this aspect of the invention.

In another preferred embodiment, said genomic site of interest for introduction of said blocking sequence allows stable expression of said introduced blocking sequence.

In another preferred embodiment, said genomic site of interest for introduction of said blocking sequence does not modify the expression of genes located in the vicinity of said targeted site of interest.

In another preferred embodiment, said genomic site of interest for introduction of said blocking sequence interest is close to a gene essential for the survival of said alga or cyanobacterium. In another preferred embodiment, said genomic site of interest for introduction of said blocking sequence interest is inside a gene essential for the survival of said alga or cyanobacterium. In another preferred embodiment, said gene essential for the survival of said alga or cyanobacterium is a housekeeping gene.
In another preferred embodiment, said genomically engineered alga is derived from the genera selected from the group consisting of: *Amphora, Anabaena, Anikstrodesmus, Botryococcus, Chaetoceros, Chlamydomonas, Chlorella, Chlorococcum, Cyclotella, Cylindrotheca, Dunaliella, Emiliana, Euglena, Haematococcus, Isochrysis, Monochrysis, Monoraphidium, Nannochloris, Nannochloropsis, Navicula, Nephrochloris, Nephroselmis, Nitzschia, Nodularia, Nostoc, Ochromonas, Oocystis, Oscillatoria, Pavlova, Phaeodactylum, Playmonas, Pleurochrysis, Porphyra, Pseudoanabaena, Pyramimonas, Stichococcus, Synechococcus, Synechocystis, Tetraselmis, Thalassiosira, and Trichodesmium.*

In another preferred embodiment, said genomically engineered cyanobacteria is derived from the group consisting of *Synechococcus* PCC7002, *Synechococcus* PCC7942, *Synechocystis* PCC6803 and *Arthrospira plantesis*.

In another embodiment, said at least one target gene to perform knock-out from step a) of this aspect of the invention encodes for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia, or cell wall formation.

In another embodiment, said at least one target gene for blocking sequence from step b) of this aspect of the invention encodes for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia, RUBISCO, carotene content, photosystem II antennae contents or cell wall formation. In a preferred embodiment, said blocking sequence from step b) of this aspect of the invention suppresses the function of a gene encoding for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia or cell wall formation. In another preferred embodiment, said blocking sequence from step b) of this aspect of the invention partially suppresses the function of a gene encoding for a trait selected from the group consisting of RUBISCO, carotene content or photosystem II antennae contents.

In this aspect of the present invention, said genomically engineered algae or cyanobacteria displays a double degree of biosafety by reducing the risk of establishment outside specialized cultivation conditions.

A first degree of biosafety is provided in the genome engineered algae or cyanobacteria of the present invention by the targeted knock-out of a gene that is not essential in the culture.
conditions of cultivated transgenic algae or cyanobacteria but render said modified algae or cyanobacteria incapable of establishing by itself or in introgressed offspring in natural ecosystems when silenced.

A second degree of biosafety is provided in the genome engineered algae or cyanobacteria of the present invention by the insertion of a transgene at a targeted site of interest within said organism genome, said transgene product being able to silence product of the knock-out targeted gene in case knock-out gene is lost by sexual or asexual recombination.

In other words, genome engineered algae or cyanobacteria of the present invention contain a safer anti-establishment mechanism, targeted approaches of mitigation concept giving more robustness, stability of expression over time and environment expression independency, compared to classical non-targeted approaches.

**Other definitions:**

By “gene” is meant the basic unit of heredity, consisting of a segment of DNA arranged in a linear manner along a chromosome, which codes for a specific protein or segment of protein. A gene typically includes a promoter, a 5' untranslated region, one or more coding sequences (exons), optionally introns, a 3' untranslated region. The gene may further comprise a terminator, enhancers and/or silencers.

By mitigation gene is intended a gene that when silenced by mutation or knock-out, will render considered algae or cyanobacteria species incapable to propagate in the open sea or in natural ecosystems.

By “nearest genes” is meant the two genes that are located the closest to the target sequence, centromeric and telomeric to the target sequence respectively.

As used herein, the term “locus” is the specific physical location of a DNA sequence (e.g. of a gene) on a chromosome. As used in this specification, the term “locus” usually refers to the specific physical location of an endonuclease’s target sequence on a chromosome. Such a locus, which comprises a target sequence that is recognized and cleaved by an endonuclease according to the invention, is referred to as “locus according to the invention” or “chosen locus” or “genomic site of interest”.

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As used herein, the expression “genomic site of interest” or more broadly any mention of genome in the present application, encompasses nuclear genetic material but also a portion of genetic material that can exist independently to the main body of genetic material such as plasmids, episomes, virus, transposons or in organelles such as mitochondria or chloroplasts as non-limiting examples, at which a double stranded break (cleavage) can be induced by the endonuclease.

As used herein, the term “transgene” refers to a sequence inserted at a site of interest in an algae or cyanobacteria genome. Preferably, it refers to a sequence encoding a polypeptide. The polypeptide encoded by the transgene can be either not expressed, or expressed but not biologically active, in the algae or algae cells in which the transgene is inserted. The transgene can encode a polypeptide useful for increasing the usability and the commercial value of algae. Also, the transgene can be a sequence inserted at a site of interest in an algal or cyanobacterial genome for producing an interfering RNA. Also, the transgene can be a sequence inserted at a site of interest of previously mentioned organisms, encoding for more than one polypeptide or producing more than one interfering RNA. From these definitions, it is understood that under the names “product of a transgene” or “transgene encoded product” or equivalent expressions, are encompassed a polypeptide or a RNA molecule. In the present application, a transgene can also be qualified as a blocking sequence when only composed by such a blocking sequence according to the present invention; it can also be composed of a blocking sequence tandemly linked to a trait of commercial interest. Blocking sequence and trait of commercial interest according to the present invention, respectively, can be both independent transgenes within the genome of alga or cyanobacteria according to the present invention.

As used herein, the expressions "gene of interest", "nucleotide sequence of interest", "nucleic acid of interest" or “sequence of interest" refer to any nucleotide or nucleic acid sequence that encodes a protein or other molecule that is desirable for expression in an algal cell (e.g. for production of the protein or other biological molecule [e.g., an RNA product like interfering RNA as a non limiting example] in the target cell). The nucleotide sequence of interest is generally operatively linked to other sequences that are needed for its expression, e.g., a promoter. Further, the sequence itself may be regulatory in nature and thus of interest for expression in the target cell.
By "homologous" is intended a sequence with enough identity to another one to lead to homologous recombination between sequences, more particularly having at least 95 % identity, preferably 97 % identity and more preferably 99 %.

By "identity" is intended sequence identity between two nucleic acid molecules or polypeptides. Identity can be determined by comparing a position in each sequence that may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base, then the molecules are identical at that position. A degree of similarity or identity between nucleic acid or amino acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleic acid sequences. Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, or BLAST that are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default setting.

By “mutation” is intended the substitution, deletion, insertion of one or more nucleotides/amino acids in a polynucleotide (cDNA, gene) or a polypeptide sequence. Said mutation can affect the coding sequence of a gene or its regulatory sequence. It may also affect the structure of the genomic sequence or the structure/stability of the encoded mRNA.

By “functional variant” is intended a catalytically active variant of a protein, such variant can have additional properties compared to its parent protein. As a non-limiting example, a functional variant of a meganuclease can be able to cleave a DNA target sequence, said target possibly being a new target which is not cleaved by the parent meganuclease.

By “derived from” or “derivative(s)” it is intended to mean for instance a meganuclease variant which is created from a parent meganuclease and hence the peptide sequence of the meganuclease variant is related to (primary sequence level) but derived from (mutations) the peptide sequence of the parent meganuclease. In this definition, mutations encompass deletions or insertions of at least one amino acid residue; as non-limiting example, a truncated variant of an I-CreI meganuclease is considered as a scaffold derived from I-CreI meganuclease. More broadly, this definition applies for other endonucleases and rare-cutting endonucleases.
By "DNA target", "DNA target sequence", "target DNA sequence", "target sequence", "target-site", "target", "site", "site of interest", "recognition site", "polynucleotide recognition site", "recognition sequence", "homing recognition site", "homing site", "cleavage site" is intended a double-stranded palindromic, partially palindromic (pseudo-palindromic) or non-palindromic polynucleotide sequence that is recognized and can be cleaved by, as non-limiting examples, a LAGLIDADG homing endonuclease such as I-CreI, or a variant, or a single-chain meganuclease derived from I-CreI. These terms refer to a specific DNA location, preferably a genomic location, but also a portion of genetic material that can exist independently to the main body of genetic material such as plasmids, episomes, virus, transposons or in organelles such as mitochondria or chloroplasts as non-limiting examples, at which a double stranded break (cleavage) can be induced by the endonuclease, rare-cutting endonuclease, or meganuclease as non-limiting examples. For the LAGLIDADG subfamily of rare-cutting endonucleases, the DNA target is defined by the 5' to 3' sequence of one strand of the double-stranded polynucleotide, as indicate above for C1221 (SEQ ID NO: 2). Cleavage of the DNA target can occur at the nucleotides at positions +2 and -2, respectively for the sense and the antisense strand. Unless otherwise indicated, the position at which cleavage of the DNA target by an I-CreI-derived variant can occur corresponds to the cleavage site on the sense strand of the DNA target.

By a "TALE-nuclease" (TALEN) is intended a fusion protein consisting of a DNA-binding domain derived from a Transcription Activator Like Effector (TALE) and one FokI catalytic domain, that need to dimerize to form an active entity able to cleave a DNA target sequence.

The above written description of the invention provides a manner and process of making and using it such that any person skilled in this art is enabled to make and use the same, this enablement being provided in particular for the subject matter of the appended claims, which make up a part of the original description.

As used above, the phrases "selected from the group consisting of," "chosen from," and the like include mixtures of the specified materials.

Where a numerical limit or range is stated herein, the endpoints are included. Also, all values and subranges within a numerical limit or range are specifically included as if explicitly written out.
The above description is presented to enable a person skilled in the art to make and use the invention, and is provided in the context of a particular application and its requirements. Various modifications to the preferred embodiments will be readily apparent to those skilled in the art, and the generic principles defined herein may be applied to other embodiments and applications without departing from the spirit and scope of the invention. Thus, this invention is not intended to be limited to the embodiments shown, but is to be accorded the widest scope consistent with the principles and features disclosed herein.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples, which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

**Examples:**

Algae are able to acclimate to variable limiting availability of CO₂ by using carbon-concentrating mechanisms, to enhance CO₂ assimilation, when inorganic carbon is limited. One of the important molecular components of this system is carbonic anhydrase. Algal spills are a major environmental problem, and therefore it is of general interest to identify mitigation factors that render the algal species incapable to propagate and unfit to compete in natural ecosystems.

Carbonic anhydrase (the *Phaeodactylum tricornutum* sequence accession number, AF414191) is a well-conserved protein within all algal and photosynthetic organisms, and its inactivation can lead to strains incapable of growing in environments with ambient CO₂ concentration. Thus, when there is a knock-out of gene function, this will render the organism inviable in its natural marine environment, but viable under culture conditions of high carbon dioxide. Two types of meganucleases are envisioned by the inventors, giving a double insurance that carbonic anhydrase is inactivated. First type of meganucleases can inactivate the carbonic anhydrase open reading frame, using for example the NHEJ process to generate a knock-out, and the second type of meganucleases can perform a knock-in of an RNAi silencing construct, nearby an essential housekeeping gene, that if lost during sexual breeding, will render the algal species inviable.

Therefore, the first main aspect of this invention concerns the engineering of meganucleases within the carbonic anhydrase gene to create a knock-out of gene function. The second aspect
of this invention uses meganucleases for knock-in of silencing constructs against carbonic anhydrase (RNAi) that will allow additional assurance of the knock-out of carbonic anhydrase gene function in the wild.

**Example 1: Engineering meganucleases to knock-out the carbonic anhydrase gene (a mitigation factor) in *Phaeodactylum tricornutum***

A) **Knock-Out of the Carbonic Anhydrase gene (using meganucleases targeting the carbonic anhydrase gene without a repair matrix).**

One strategy envisioned by the inventors to knock-out the carbonic anhydrase (CA) gene, involves mutating the coding sequence by non homologous end joining (NHEJ), using a CA meganuclease targeting a sequence within the open reading frame (see Figure 3, and Table 2). In this case, the CA meganuclease will create a double-strand break, and in absence of homology or a repair matrix, the double-stranded ends can either rejoin perfectly, or rejoin imperfectly, using micro-homologies near the break site. Imperfect end-joining, can give rise to small deletions or insertions within the open reading frame, and therefore generate loss of gene function. The loss of carbonic anhydrase activity is easily measured by cultivating the transgenically modified strain in either high CO₂ conditions (>5% CO₂), where it should survive, or in ambient CO₂ conditions as in natural ecosystems, where it should not survive. As an example, we see that several meganucleases can be generated targeting the exons of the carbonic anhydrase sequence from *P. tricornutum*. Using degenerate primers, homologous to this sequence, one can easily identify, clone, and sequence carbonic anhydrase homologs in other algal species, and identify meganucleases to do the same type of knock-out approach.

<table>
<thead>
<tr>
<th>Targets</th>
<th>Location</th>
<th>Sequences</th>
<th>SEQ ID NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1</td>
<td>303-326</td>
<td>TT-GAC-AC-CCT-GGGA-ACA-GT-CCA-CT</td>
<td>4</td>
</tr>
<tr>
<td>CA2</td>
<td>363-386</td>
<td>GT-GCT-CC-TCC-GAAC-ATG-AT-CAT-GG</td>
<td>5</td>
</tr>
<tr>
<td>CA3</td>
<td>462-485</td>
<td>CT-GCC-AT-CCA-GTTC-GGA-AT-TAA-CG</td>
<td>6</td>
</tr>
<tr>
<td>CA4</td>
<td>489-512</td>
<td>TC-AAG-AT-CCC-GCAC-GTT-AT-CGT-CT</td>
<td>7</td>
</tr>
<tr>
<td>CA5</td>
<td>793-816</td>
<td>CT-TCG-AC-CCG-AAGA-CTG-AT-GCC-AT</td>
<td>8</td>
</tr>
<tr>
<td>CA6</td>
<td>906-929</td>
<td>CC-TAA-AC-TTG-GAAA-ATG-AT-TCT-GT</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 2 : Sequences and locations of targeted sites in the carbonic anhydrase gene from *Phaeodactylum tricornutum* (Gene Bank accession number AF414191).
B) Knock-Out of the Carbonic Anhydrase gene (using meganucleases and a Knock-Out repair matrix).

A second strategy to knock-out gene function, takes advantage of a knock-out repair matrix. This consists of disrupting a large region within the carbonic anhydrase gene, using one or two CA meganucleases (for example CA1 and CA5) (Figure 3, Table 2), and a knock-out repair matrix to generate a large deletion. This repair matrix is designed using sequences of flanking homology (typically 500 -1000 bp are used) outside and/or just within the carbonic anhydrase open reading frame and deleted for the coding exon regions. One can also design the repair knock-out construct, with for example, a marker resistance gene such as the phytoene desaturase gene (pds), (Frommolt, Werner et al. 2008; Junchao et al., 2008, J. of Phycology, Volume 44, issue 3, June 2008, pages 684–690), embedded between the same flanking homologous sequences for targeted gene replacement.

Example 2: Engineering meganucleases to target different sites in the Phaeodactylum tricornutum genome for targeted knock-in of RNAi silencing vectors.

Example 1 provides an illustration of how to knock-out carbonic anhydrase gene function, for example using meganucleases to generate mutagenic events by NHEJ, within the gene. Although these knock-out events are stable, algae might be able to sexually reproduce at low frequencies. Therefore, mating of one strain mutated for carbonic anhydrase with a wild type strain, proficient in this gene, could re-establish carbonic anhydrase function, and thus viability in ambient CO₂ environments. To avoid this, we can insert anti-sense and or RNAi constructs against the carbonic anhydrase gene. Different silencing cassettes have been described, using various promoters with different strengths. To generate an RNAi carbonic anhydrase gene, a 240bp fragment within the sequence of carbonic anhydrase can be PCR amplified or chemically synthesized in both directions, separated by an intron from the CA gene, producing an RNA containing double-stranded stem and loop (need figure). The RNAi fragment is then cloned downstream of the Phaeodactylum tricornutum (Pt) fcpA promoter and upstream to fcpA terminator (as described in Lioudmila et al, 2000). Alternative the RNAi stem and loop fragment can be fused to the Ble resistance gene and the cassette is cloned downstream to the Pt psbA promoter.rbcS2 promoter.
We have identified three meganucleases in the *P. tricornutum* genome that target within non-coding regions, within close proximity to essential metabolic genes involved in nitrogen, thiamine or starch metabolism. The meganuclease target site sequences and locations within the genome are described in Table 3 and Figure 4. A silencing construct can then be inserted in one of these locations, using a repair matrix with homology to the target site. Because the target sites are located near these essential metabolic genes, this type of targeted insertion will insure retention of the RNAi construct in the genome.

Construction of the transgenic algal strain, containing the meganuclease targeted RNAi knock-in construct, as well the meganuclease knock-out construct for carbonic anhydrase, will provide double security, in order that such an algal strain cannot propagate in the natural environment.

<table>
<thead>
<tr>
<th>Chromosome / Location</th>
<th>Target site for Meganuclease</th>
<th>SEQ ID NO</th>
<th>Gene 5' to target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chr 9 (736640-736663)</td>
<td>ACGAAAATCAGTACTCCATTTGTG</td>
<td>10</td>
<td>*Ferredoxin-nitrite reductase (Nitrogen metabolism)/ 1703 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*Ubiquitin protein ligase/ 681 bp</td>
</tr>
<tr>
<td>Chr 16 (162706-162729)</td>
<td>GCAAACTCTGTATACATAGACAG</td>
<td>11</td>
<td>*Thiamine pyrophosphokinase (Thiamine metabolism)/ 4885 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*Unknown gene/ 1494 bp</td>
</tr>
<tr>
<td>Chr 20 (467336-467359)</td>
<td>CCA4ACGTGTACATAGATATCCA</td>
<td>12</td>
<td>*Unknown gene/ 442 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*Glucan 1,4-alpha glucosidase (Starch and sucrose metabolism)/ 996 bp</td>
</tr>
</tbody>
</table>

Table 3: Sequences and locations of meganuclease target sites in non-coding regions of the *Phaeodactylum tricornutum* genome. The descriptions of nearby coding regions are also indicated. Distances between the target site and closest neighboring genes are also shown. Sequences and annotations were downloaded from this public webpage: http://genome.jgi-psf.org/Phatr2/Phatr2.download.ftp.html.

Example 3: Transformation of algae and cyanobacteria by different protocols

Transformation of *Chlamydomonas*

Chlamydomonas cell wall deficient (CW15) strain was transformed with the plasmid (1±5mg) by the glass bead vortexing method (Kindle, 1990). The transformation mixture was then transferred to 50 ml of non-selective TAP growth medium for recovery. The cells were kept for at least 18 h at 25°C in the light. Cells were collected by centrifugation and plated at a density of 10^8 cells per 80 mm plate. *Chlamydomonas* transformants were selected on fresh TAP agar plates containing 5 μM Zeocin, for 7-10 days at 25°C.
Transformation of Marine Algae by Particle Bombardment.

Cultures of marine algae are grown in artificial sea water (ASW)+f/2 media until they reach a density of $10^6$ cells/ml. The cells are then centrifuged (2500g, 10min, room temp) and washed twice with fresh ASW media. After washing, the cells are re-suspended in an appropriate volume to reach a cell density of $10^8$ cells/ml. 0.5ml of this cell suspension in then spotted into the center of a Petri dish containing ASW+f/2+15mM HCO$_3$- (solidified by 1.5% Bacto-Agar). The Petri dishes are incubated for 24-48 hrs under standard growth conditions. 0.7 micron tungsten particles (M-10 tungsten powder, Bio-Rad), 0.6 micron gold particles (Bio-Rad) or tungsten powder comprised of particles smaller than 0.6 microns (FW06, Canada Fujian Jinxin Powder Metallurgy Co., Markham, ON, Canada) are prepared according to the manufacturer’s instructions and coated with linear DNA using CaCl$_2$ and spermidine. Particles are then placed onto macrocarriers and bombarded onto the cells using the Biolistic PDS-1000/He unit (BioRad), 1100psi, 1350psi or 1550psi rupture discs. This method was adopted, with changes, from Kroth (2007). After bombardment the cells are placed in the growth room for 24-48 hrs then transferred to a fresh Petri dish containing ASW+f/2+15mM HCO$_3$- and a selection agent under standard growth conditions. Colonies of transformed cells appear after 2-3 weeks. Conditions are modified for each organism according to its needs, based on modifications of standard protocols.

Transformation of Marine Algae by Electroporation

Cultures of *Nannochloropsis* are grown in ASW+f/2 media for a few days, until they reach a density of $10^6$ cells/ml. To form protoplasts, cells are centrifuged (2500g, 10 min, room temp) and washed twice with fresh ASW media. After washing, the cells are resuspended in fresh ASW containing 4% hemicellulase (Sigma) and 2% Driselase (Sigma) and incubated in the dark for 4 hrs. Following incubation protoplasts are washed twice (5 min centrifuge, 400g, room temp) with ASW containing 0.6M sorbitol and 0.6M mannitol (Sigma). Protoplasts are resuspended in an appropriate volume to reach a density of $10^8$ protoplasts/ml. 100μl of protoplasts are incubated with 10μg of linear DNA in a 0.1cm electroporation cuvette (BioRad) on ice for 5 minutes. The protoplasts are then pulsed using the BTX ECM830 (Harvard Apparatus, Holliston, MA, USA) electroporator. A series of pulse conditions are applied, ranging between 1000-1400 volts, 6-10 pulses, 10-20ms each pulse. Samples are then placed immediately on ice for 10 minutes. Protoplasts are transferred to fresh liquid ASW+f/2
media and placed under standard growth conditions for 24 hrs. The treated protoplasts are then transferred to a fresh Petri dish containing ASW+f/2+15mM HCO3- and a selection agent and placed under standard growth conditions. Colonies of transformed cells appear after 2-3 weeks. Conditions are modified for each organism according to its needs, based on modifications of standard protocols.

Transformation of Marine Algae by Microporation

A fresh algal culture is grown to mid exponential phase in ASW+f/2 media. A 10ml sample of the culture is harvested, washed twice with Dulbecco's phosphate buffered saline (DPBS, Gibco, Invitrogen, Carlsbad, CA, USA) and resuspended in 250μl of buffer R (supplied by Digital Bio, NanoEnTek Inc., Seoul, Korea, the producer of the microporation apparatus and kit). After adding 8μg linear DNA to every 100μl cells, the cells are pulsed. A variety of pulses is usually needed, depending on the type of cells, ranging from 700 to 1700 volts, 10-40ms pulse length; each sample is pulsed 1-5 times. Immediately after pulsing the cells are transferred to 200μl fresh growth media (without selection). After incubating for 24-120 hours in low light at 25°C, the cells are plated onto selective solid media and incubated under normal growth conditions until single colonies appear.

Agrobacterium-Mediated Transformation of Marine Algae

Cultures of marine algae are grown in ASW+f/2+HCO3- media for a few days, until they reach a density of 10^6 cells/ml. Approximately 106 algae cells are plated on solid ASW+f/2 media in Petri dishes and incubated under normal growth conditions until a lawn of cells is observed. Agrobacterium (A600=0.5) bearing the appropriate plasmid (pCAMBIA1301 containing the gene of interest, see (Kathiresan, Manivannan et al. 2009)) is grown overnight in liquid LB medium then harvested by centrifugation at 3000g for 10 min. The pellet is resuspended in ¼ASW+f/2 medium. A 200 μl aliquot of the bacterial culture is then plated on a lawn of marine algae and the plates are incubated under normal growth conditions. After 48h the cells are harvested and washed with ASW+f/2 containing 200 μg/ml augmentin to kill the Agrobacterium. The algae cells are recovered by centrifugation, washed, and then transferred to a fresh Petri dish containing ASW+f/2+15mM HCO3- and a selection agent under standard growth conditions. Colonies of transformed cells appear after 2-3 weeks. Conditions are modified for each organism according to its needs, based on modifications of standard protocols.
Transformation of Cyanobacteria

For transformation of *Synechococcus* PCC7002, cells are cultured in 100 ml of BG11+ Turk Island Salts liquid medium (http://www.crbip.pasteur.fr/fiches/fishemedium.jsp?id=648) at 28°C under white fluorescent light and subcultured at the mid-exponential phase of growth. To 1.0 ml of cell suspension containing 2x10^7 cells, which are cultured at the mid-exponential phase of growth, 0.5 or 1.0 μg of donor DNA (in 10 mM Tris/1 mM EDTA, pH 30 8.0) is added, and the mixture is incubated in the dark at 26°C overnight. After incubation for a further 6 h in the light, the transformants are directly selected on BG11+ Turk Island.

Transformation of *Isochrysis* sp. cells by particle bombardment

Cultures of *Isochrysis* sp. are grown in artificial sea water (ASW)+/2 media until they reach a density of 106 cells/ml. The cells are then centrifuged (2500g, 10 min, room temp) and washed twice with fresh ASW media. After washing, the cells are resuspended in an appropriate volume to reach a cell density of 108 cells/ml. 0.5ml of this cell suspension is then spotted into the center of 55mm Petri dishes containing ASW+f/2+15mM HCO3- (solidified by 1.5% Bacto-Agar). The Petri dishes are incubated for 24 h under standard growth conditions. 0.7micron tungsten particles (M-10 tungsten powder, Bio-Rad) are prepared according to the manufacturer’s instructions and coated with linear DNA using CaCl2 and spermidine. Particles are then placed onto macrocarriers and bombarded onto the cells using the Biolistic PDS-1000/He unit (BioRad), 1100psi rupture discs. This method was adopted, with changes, from Kroth (2007). After bombardment the cells are placed in the growth room for 24 h then transferred to fresh 90mm Petri dishes containing ASW+f/2+15mM HCO3- and a selection agent under standard growth conditions. Colonies of transformed cells appear after 2-3 weeks.

Transformation of *Nannochloropsis* sp. cells by electroporation

Cultures of *Nannochloropsis* are grown in ASW+f/2 media until they reach a density of 106 cells/ml. To form protoplasts, cells are centrifuged (2500g, 10 min, room temp) and washed twice with fresh ASW media. After washing, the cells are resuspended in fresh ASW containing 4% hemicellulase (Sigma) and 2% Driselase (Sigma) and incubated in the dark for 4 h. Following incubation protoplasts are washed twice (5 min centrifugation, 400g, room temp) with ASW containing 0.6M sorbitol and 0.6M mannitol (Sigma). Protoplasts are resuspended in an appropriate volume to reach a density of 108 protoplasts/ml. 100μl of
protoplasts are incubated with 10µg of linear DNA in a 0.1cm electroporation cuvette (BioRad) on ice for 5 minutes. The protoplasts are then pulsed using the BTX ECM 830 electroporator (Harvard Apparatus, Holliston, MA, USA). A series of pulse conditions are applied, ranging between 1000-1400 volts, 6-10 pulses, 10-20ms each pulse. Samples are then placed immediately on ice for 10 minutes. Protoplasts are transferred to fresh liquid ASW+f/2 media and placed under standard growth conditions for 24 h. The treated protoplasts are then transferred to a fresh Petri dish containing ASW+f/2+15mM HCO3- and a selection agent and placed under standard growth conditions. Colonies of transformed cells appear after 2-3 weeks.

Transformation of *Tetraselmis* sp. cells by Microporation

Cultures of *Tetraselmis* are grown to mid exponential phase in ASW+f/2 media. A 10ml sample of the culture is harvested, washed twice with Dulbecco’s phosphate buffered saline (DPBS, Gibco, Invitrogen, Carlsbad, CA, USA) and resuspended in 250µl of buffer R (supplied by Digital Bio, NanoEnTek Inc., Seoul, Korea, the producer of the microporation apparatus and kit). After adding 8µg linear DNA to every 100µl cells, the cells are pulsed. A variety of pulses is usually needed, depending on the type of cells, ranging from 700 to 1700 volts, 10-40ms pulse length; each sample is pulsed 1-5 times. Immediately after pulsing the cells are transferred to 200µl fresh growth media (without selection). After incubating for 24 hours in low light at 25oC, the cells are plated onto selective solid media and incubated under normal growth conditions until single colonies appear.

**Example 4: Physiological assessment of genetically modified algae**

To assess physiological properties of genetically modified algae compared with their relevant wild type strains we performed a set of procedures that enabled us to evaluate each strain. This includes measurement of growth rate, photosynthetic activity, respiration activity, tolerance to abiotic parameters, lipid content and protein content.

**Growth Rate**

Growth rates are measured using one or more of the following techniques:

- Direct cell count
- Optical density at a relevant wavelength (e.g. 750nm)
- Pigment/chlorophyll concentration (where this method is applicable)
• Dry weight

**Photosynthetic Activity**

One of the important parameters indicating the welfare of a photoautotrophic culture is its photosynthetic capability. Photosynthetic activity is monitored by measuring oxygen evolution and/or by variable fluorescence measurements. We also evaluate oxygen consumption in the dark in order to estimate net photosynthetic potential of the algal culture. As part of the photosynthetic evaluation we follow several abiotic parameters that potentially influence the physiological state of a culture.

- Light intensity tolerance (at a given cell density) is evaluated. P/I (photosynthesis vs. irradiance) curves are used to determine optimal light intensity per cell.
- Performance at different CO₂ concentrations.
- Temperature tolerance. Each culture is tested at its optimal temperature for growth. In addition, temperatures are raised gradually and culture activities (as described above) are measured.

**Growth conditions**

Cells of eukaryotic marine cultures (e.g. *Chlorella vulgaris*, *Phaeodactylum tricornutum*, *Isochrysis* sp., *Nannochloris* spp. and *Nanochloropsis* sp.) and transformants thereof are grown on artificial seawater medium (Goyet and Poisson, 1989) supplemented with f/2 (Guillard and Ryther, 1962). Marine cultures are grown at 18-22°C with a 16/8 h light/dark period. Fresh water cultures (e.g. *Chlamydomonas reinhardtii*) and mutants thereof are grown photoautotrophically on liquid medium, using mineral medium as described in (Harris, 1989), with the addition of 5mM NaHCO₃⁻, with continuous shaking and illumination at 22°C.

**Growth rate estimation**

Cells are harvested in the logarithmic growth phase and resuspended in fresh growth media. Cultures are brought to a cell density corresponding to ~3 µg/ml chlorophyll a. Light intensity is optimized for each culture and temperature is maintained at growth temperature 25 ±1°C. Where required, cells are concentrated by centrifugation (3000g, 5 min, room temperature) and resuspended in a fresh media. A time-series sampling procedure is followed where a
subsampling of each culture is collected and the number of cells per ml measured. Direct counting, optical density at different wavelengths, packed volume at stacked assay and chlorophyll concentrations are also measured.

**Oxygen evolution**

Measurements of O₂ concentrations are performed using a Clark type O₂ electrode (Pasco Scientific, Roseville, CA, USA). 20 ml of cell suspension corresponding to 15 μg chlorophyll/ml are placed in the O₂ electrode chamber, at relevant temperatures. Cells are exposed to various light intensities and net O₂ production is measured. Dark incubations are performed in air-tight vessels to follow light-independent O₂ consumption.

**Fluorescence measurements**

Electron transfer activity of photosystem II is measured by pulse modulated fluorescence (PAM) kinetics using PAM-101 (Walz, Effeltrich, Germany). Light intensity (measured at the surface of the chamber) of the modulated measuring beam (at 1.6 kHz frequency) is 0.1 μmol photons m⁻² s⁻¹. White actinic light is delivered at 50-1500 μmol photons m⁻² s⁻¹ as required in different experiments and is used to assess steady state fluorescence (Fs). Maximum fluorescence (Fm) is measured with saturating white light pulses of 4000 μmol photons m⁻² s⁻¹ for 1 s.

**Additional experiments**

- Light intensity tolerance (at a given cell density) is evaluated. P/I (photosynthesis vs. irradiance) curves are used to determine optimal light intensity per cell. 20 ml of cell suspension corresponding to 15 μg chlorophyll/ml are placed in the O₂ electrode chamber, at relevant temperature and various light intensities. Oxygen evolution rates are measured at different light intensities.

- Performance at different CO₂ levels (e.g. ambient; 1%; 5%). Growth rate estimations and photosynthetic activity (methodology described above) are evaluated when cultures are maintained at different CO₂.
List of references cited in the description:


Claims:

1. A dual method to prevent transgenic algae or cyanobacteria from establishing and spreading in natural ecosystems, said method comprising the steps of:
   a) performing into said alga or cyanobacterium genome the knock-out of at least one target gene being not essential in the cultivated transgenic alga or cyanobacterium and being able to render said transgenic alga or cyanobacterium incapable of establishing by itself in natural ecosystems when suppressed; and
   b) introducing into same alga or cyanobacterium from step a) a blocking sequence which presence or expression can sufficiently suppress the function of a target gene being not essential in the cultivated transgenic alga or cyanobacterium and being able to render said transgenic alga or cyanobacterium unfit to establish outside of cultivation.

2. The method of claim 1 wherein said target gene from step b) is different from said target gene from step a).

3. The method of claim 1 wherein said target gene from step b) is the same as said target gene from step a).

4. The method of claim 1 wherein said blocking sequence is coding for a RNAi or antisense construct.

5. The method of claim 1 wherein said knock-out of at least one said target gene is performed by the use of at least one endonuclease.

6. The method of claim 1 wherein said blocking sequence is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest will be genetically linked in said same alga or cyanobacterium, precluding easy genetic segregation from each other.

7. The method of claim 1 wherein the introduction of step b) is a targeted event into the genome of said alga or cyanobacterium to introduce at a genomic site of
interest said blocking sequence which presence or expression can sufficiently suppress the function of a target gene to render the organism unfit to establish outside of cultivation.

8. The method of claim 7 wherein said introduction is performed by using at least one endonuclease.

9. The method of claim 7 wherein steps a) and b) are performed by using, respectively, at least two different endonucleases.

10. The method of claim 8 wherein said endonuclease is a variant.

11. The method of claim 9 wherein said endonucleases are variants.

12. The method of claim 1 wherein the introduction of step b) is a targeted event into the genome of said alga or cyanobacterium to introduce at a genomic site of interest said blocking sequence which presence or expression can sufficiently suppress the function of a target gene to render the organism unfit to establish outside of cultivation and wherein said genomic site of interest to introduce said blocking sequence is the site of the target gene knock-out of step a).

13. The method of claim 12 performed by using the same endonuclease.

14. The method of claim 12 wherein said blocking sequence is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest will be genetically linked in said same alga or cyanobacterium, precluding easy genetic segregation from each other.

15. The method of claim 7 wherein said genomic site of interest allows stable expression of said introduced blocking sequence.

16. The method of claim 15 wherein said blocking sequence is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest will be genetically linked in said same alga or cyanobacterium, precluding easy segregation between them.
17. The method of claim 7, wherein said genomic site of interest to introduce said blocking sequence is inside a gene essential for the survival of said alga or cyanobacterium.

18. The method of claim 17, wherein said blocking sequence is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest will be genetically linked in said same alga or cyanobacterium, precluding easy segregation between them.

19. The method of claim 7, wherein said genomic site of interest to introduce said blocking sequence is close to a gene essential for the survival of said alga or cyanobacterium.

20. The method of claim 17, wherein said blocking sequence is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest will be genetically linked in said same alga or cyanobacterium, precluding easy segregation between them.

21. The method of claim 7, wherein said genomic site of interest to introduce said blocking sequence is close to a housekeeping gene of said alga or cyanobacterium.

22. The method of claim 21, wherein said blocking sequence is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest will be genetically linked in said same alga or cyanobacterium, precluding easy segregation between them.

23. The method of claim 7, wherein the introduction of said blocking sequence at a genomic site of interest does not modify the expression of genes located in the vicinity of said targeted site of interest.
24. The method of claim 23, wherein said blocking sequence is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest will be genetically linked in said same alga or cyanobacterium, precluding easy segregation between them.

25. The method according to claim 1, wherein said algae genome is selected from the group consisting of the following genera Amphora, Anabaena, Anistromonas, Botryococcus, Chaetoceros, Chlamydomonas, Chlorella, Chlorococcum, Cyclotella, Cylindrotheca, Dunaliella, Emiliana, Euglena, Haematococcus, Isochrysis, Monochrysis, Monoraphidium, Nannochloris, Nannochloropsis, Navicula, Nephrochloris, Nephrosemis, Nitzschia, Nodularia, Nostoc, Oocystis, Oscillatoria, Pavlova, Phaeodactylum, Playmna, Pleurochrysis, Porphyra, Pseudoanabaena, Pyramimonas, Stichococcus, Synechococcus, Synechocystis, Tetraselmis, Thalassiosira, and Trichodesmium.

26. The method according to claim 1, wherein said cyanobacterium genome is selected from the group consisting of Synechococcus PCC7002, Synechococcus PCC7942 and Synechosysist PCC6803 and Arthrospira plantesis.

27. The method of claim 1, wherein said at least one target gene to perform knock-out from step a) encodes for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia, or cell wall formation.

28. The method of claim 1, wherein said at least one target gene for blocking sequence from step b) encodes for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia, RUBISCO, carotene content, photosystem II antennae contents or cell wall formation.

29. The method of claim 1, wherein said blocking sequence suppresses the function of a gene encoding for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia, or cell wall formation.

30. The method of claim 1, wherein said blocking sequence partially suppresses the function of a gene encoding for a trait selected from the group consisting of RUBISCO, carotene content or photosystem II antennae contents.
31. The method of claim 1, wherein said knock-out from step a) is performed with an endonuclease whose target sequence in the targeted genome of said alga or cyanobacterium is selected from the group consisting of any one of SEQ ID NO: 4 to 9.

32. The method of claim 1, wherein said blocking sequence of step b) is introduced with an endonuclease whose target sequence in the targeted genome of said alga or cyanobacterium is selected from the group consisting of any one of SEQ ID NO: 10 to 12.

33. A genomically engineered alga or cyanobacterium comprising:

a) at least one genomic knock-out of one target gene, said at least one target gene being not essential in the cultivated transgenic alga or cyanobacterium and being able to render said transgenic alga or cyanobacterium incapable of establishing by itself or in introgressed offspring in natural ecosystems when suppressed; and

b) at least one introduced blocking sequence which presence or expression suppresses the function of a target gene being not essential in the cultivated transgenic alga or cyanobacterium and which is able to render said transgenic alga or cyanobacterium unfit to establish itself, grow or proliferate outside of cultivation.

34. The genomically engineered alga or cyanobacterium of claim 33 wherein said target gene from step b) is different from target gene from step a).

35. The genomically engineered alga or cyanobacterium of claim 33, wherein said target gene from step b) is the same as said target gene from step a).

36. The genomically engineered alga or cyanobacterium of claim 33, wherein said blocking sequence construct is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest will be genetically linked in said same alga or cyanobacterium, precluding easy genetic segregation from each other.
37. The genomically engineered alga or cyanobacterium of claim 33, wherein the introduction of step b) is a targeted event performed at a genomic site of interest of said alga or cyanobacterium.

38. The genomically engineered alga or cyanobacterium of claim 37, wherein said blocking sequence construct is tandemly linked to a transgene encoding a trait of commercial interest such that both said blocking sequence and said trait of commercial interest will be genetically linked in said same alga or cyanobacterium, precluding easy genetic segregation from each other.

39. The genomically engineered alga or cyanobacterium of claim 33, wherein the introduction of step b) is a targeted event performed at a genomic site of interest of said alga or cyanobacterium and wherein said genomic site of interest is the site for the target gene knock-out of step a).

40. The genomically engineered alga or cyanobacterium of claim 37, wherein said genomic site of interest allows stable expression of said introduced blocking sequence.

41. The genomically engineered alga or cyanobacterium of claim 37, wherein said genomic site of interest does not modify the expression of genes located in the vicinity of said targeted site of interest.

42. The genomically engineered alga or cyanobacterium of claim 37, wherein said genomic site of interest is inside a gene essential for the survival of said alga or cyanobacterium.

43. The genomically engineered alga or cyanobacterium of claim 37, wherein said genomic site of interest is close to a gene essential for the survival of said alga or cyanobacterium.

44. The genomically engineered alga or cyanobacterium of claim 37, wherein said genomic site of interest is close to a housekeeping gene of said alga or cyanobacterium.

45. The genomically engineered algae of claim 33, wherein said algae genome is selected from the group consisting of the following genera: Amphora, Anabaena,

46. The genomically engineered cyanobacteria of claim 33, wherein said cyanobacteria genome is selected from the group consisting of Synechococcus PCC7002, Synechococcus PCC7942, Synechosystis PCC6803 and Arthrospira plantesstis.

47. The genomically engineered algae or cyanobacteria of claim 33, wherein said at least one target gene to perform knock-out from step a) encodes for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia, or cell wall formation.

48. The genomically engineered algae or cyanobacteria of claim 33, wherein said at least one target gene for blocking sequence from step b) encodes for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia, RUBISCO, carotene content, photosystem II antennae contents or cell wall formation.

49. The genomically engineered algae or cyanobacteria of claim 33, wherein said blocking sequence suppresses the function of a gene encoding for a trait selected from the group consisting of nitrate reductase, carbonic anhydrase, starch content, flagella, cilia or cell wall formation.

50. The genomically engineered algae or cyanobacteria of claim 33, wherein said blocking sequence partially suppresses the function of a gene encoding for a trait selected from the group consisting of RUBISCO, carotene content or photosystem II antennae contents.
Figure 3

Hits within carbonic anhydrase CDS from P. tricornutum
<table>
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<tr>
<th>Chromosome / Location</th>
<th>Target site for Meganuclease</th>
<th>SEQ ID NO</th>
<th>Gene 5' to target</th>
<th>Gene 3' to target</th>
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<tr>
<td>Chr 9 (736640- 736663)</td>
<td>ACGAAACTACGTACTCCATTTTGG</td>
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<td>*Ferredoxin- nitrite reductase (Nitrogen metabolism)/ 1703 bp</td>
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<td>Chr 16 (162706- 162729)</td>
<td>GCAAACCTGTATCATGACAGCAAC</td>
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<td>*Thiamine pyrophosphokinase Thiamine metabolism /4865 bp</td>
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<td>*Unknown gene/ 442 bp</td>
<td>*Glucan 1,4-alpha glucosidase (Starch and sucrose metabolism)/ 996 bp</td>
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**Figure 4**
# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

**INV. C12N1/12 C12N15/09**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
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**X** Further documents are listed in the continuation of Box C.

**X** See patent family annex.

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<th>* Special categories of cited documents:</th>
<th><em>T</em> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</th>
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<tr>
<td><em>A</em> Document defining the general state of the art which is not considered to be of particular relevance</td>
<td><em>X</em> Document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td>
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<tr>
<td><em>E</em> Earlier application or patent but published on or after the international filing date</td>
<td><em>Y</em> Document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to person skilled in the art</td>
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<td><em>O</em> Document referring to an oral disclosure, use, exhibition or other means</td>
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<td><em>P</em> Document published prior to the international filing date but later than the priority date claimed</td>
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**Data of the actual completion of the international search**

6 July 2012

**Data of mailing of the international search report**

17/07/2012

**Name and mailing address of the ISA/**

European Patent Office, P. B. 5818 Patentlaan 2 NL-2280 HV Rijswijk

Tel. (+31-70) 340-2040, Fax (+31-70) 340-3016

Authorized officer:

Roscoe, Richard
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<td>D'HALLUIN KATHLEEN ET AL: &quot;Homologous recombination: a basis for targeted genome optimization in crop species such as maize&quot;, PLANT BIOTECHNOLOGY JOURNAL, BLACKWELL, OXFORD, GB, vol. 6, no. 1, 1 January 2008 (2008-01-01), pages 93-102, XP002501442, ISSN: 1467-7644, DOI: 10.1111/j.1467-7652.2007.00305.X the whole document</td>
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