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Genetic Engineering of Microalgae for Fuel Production

Scientific Note

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INTRODUCTION

The NREL Culture Collection contains a number of microalgae strains that have potential for growth in mass culture and that accumulate large quantities of lipids under conditions of nutrient stress. We plan to use recombinant DNA technologies to regulate lipid accumulation and growth characteristics of the algae in order to establish conditions for economically feasible liquid fuel production.

In order to use recombinant DNA technologies to manipulate algal biosynthetic pathways, we first need to develop genetic transformation systems for the algae of interest. In this article, we will review our progress towards the introduction of DNA into algal cells, monitoring of expression of foreign genes, and the development of methods to promote stable expression of exogenous DNA in the algae. Once the technology is available for algal transformation, genes that affect the quantity or quality of lipid accumulation in the algal cells will be substituted for the marker genes.

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We have identified two enzymes, acetyl-CoA carboxylase and nitrate reductase, that have the potential to affect photosynthate partitioning in the microalgae, and will describe our progress toward the isolation and characterization of these genes.

GENETIC TRANSFORMATION OF MICROALGAE

Transient Expression of Luciferase in *Chlorella ellipsoidea*

As with higher plants, the cell walls of microalgae present a significant barrier to the introduction of exogenous DNA into the cells. Plant cell walls can be removed enzymatically to form protoplasts that can then be induced to take up foreign DNA in the presence of polyethylene glycol (PEG) and/or calcium (*1*). We have successfully adapted plant protoplast transformation protocols for introduction of a foreign gene (the luciferase gene from firefly) into the green alga *Chlorella ellipsoidea*. This work has been published recently (*2*) and will be reviewed briefly here.

The firefly luciferase transient assay has been used to monitor DNA uptake and expression in a variety of cell types (*3,4*), including plants (*5*). In the presence of ATP, O_2, and Mg^{2+}, luciferase catalyzes the oxidation of luciferin, with the concurrent release of a photon of light. Luciferase can thus be detected in crude extracts from cells expressing this gene by monitoring light production in a scintillation counter or luminometer.

Protoplasts were produced from the green alga *C. ellipsoidea* by digestion of the cells with Cellulysin (Calbiochem, San Diego, CA), a crude preparation of cellulase (*6*). Protoplasts, defined as cells that could be disrupted when suspended in water and sonicated, were treated with a plasmid containing the luciferase gene under control of the CaMV 35S promoter (pDO432; generously provided by David Ow). Following PEG treatment and overnight incubation, luciferase activity could be detected in extracts of the protoplasts (*2*). Expression of luciferase was detectable only in cells treated with Cellulysin (protoplasts), and the addition of both carrier DNA and PEG was essential. A time-course of expression showed that luciferase was made rapidly, within about 7 h after addition of DNA, but that the activity disappeared over the course of a few days.

We have accomplished the first steps in the development of a transformation system for *C. ellipsoidea* in that protoplasts can be produced and induced to take up exogenously added DNA and to express a heterologous (i.e., a nonalgal) gene. This result is significant, since homologous genes were required to achieve transformation in another green alga,

Chlamydomonas reinhardtii (*7*) and doubts have been expressed as to whether algae can recognize heterologous genes. Further experiments will be directed towards increasing the level of expression, developing a selectable genetic marker, stabilizing the foreign DNA within the cell, and promoting recovery and proliferation of stable transformants.

Electroporation of *Cyclotella cryptica*

The transformation protocol for *C. ellipsoidea* described above may not be applicable to a number of algal species of interest to NREL. Development of procedures for the formation of protoplasts from microalgal cells is tedious, and the protocols can be difficult to reproduce because of the complexity of the algal cell walls and variation among species and strains. In addition, there are no reproducible methods available to obtain protoplasts from diatoms. Diatoms make up a significant proportion of the NREL Culture Collection, and several strains display considerable potential for mass culture and lipid production. The cell walls of these organisms consist of an organic component plus a silica frustule and have proven recalcitrant to enzymatic digestion.

Electroporation is a technique in which transient pores can be induced in cellular membranes by the application of an electric pulse (*8*). This technique has been used extensively to introduce molecules (DNA, protein) into animal cells and plant protoplasts. More recently, it has been shown that very short, high-voltage pulses will induce DNA uptake by walled cells (i.e., bacteria, yeast, plant suspension cells) without prior removal of the wall (*9–12*). We are currently exploring electroporation as a means to bypass protoplast production, and to introduce DNA directly into intact green algae and diatoms.

Using a modification of a protocol for transformation of yeast via electroporation (*12*), we attempted to introduce the luciferase gene into intact cells of the diatom *Cyclotella cryptica*. Upon exposure to increasingly high electric field strengths, cell viability decreased, with approx 50% survival at 2.0–3.0 kV/cm. For bacteria and yeast, the electroporation conditions that result in 40–60% cell death also result in maximal transformation efficiencies (*9,10*). As yet, we have been unable to detect expression of luciferase in electroporated *C. cryptica*, possibly owing to undetectably low levels of DNA uptake and expression. The fact that the cells die upon exposure to increasingly strong electric pulses is encouraging, however, since it indicates that electroporation is indeed perturbing the algal cell membrane, probably by inducing transient pores. We believe that electroporation is a promising method for introducing DNA into intact algal cells and should prove to be a useful technique for production of algal transformants in combination with the homologous selectable marker systems discussed below.

Development of Homologous Selectable Markers for Microalgal Genetic Transformation

Transient gene expression assays, such as the luciferase system described above, are useful for monitoring DNA entry into cells and short-term expression of a foreign gene. The next step in the development of a complete transformation system is to achieve expression of a selectable marker gene in transformed cells at levels sufficient to overcome selection and, thus, allow identification of rare stable transformants. For higher plants, selection is typically for resistance to an antibiotic, such as G418, induced by insertion of a bacterial antibiotic resistance gene (i.e., the neomycin phosphotransferase gene, NPTII) into the DNA of the transformed plant. Several factors suggest that this approach may not be successful for microalgal transformation. The single-celled green alga *Chlamydomonas reinhardtii* is the only microalga for which there exists an efficient transformation system. Initial attempts to transform this alga with heterologous genes were at best unreliable (*13,14*); only after the implementation of homologous systems were efficient and reproducible nuclear transformation protocols developed (*7,15*). *C. reinhardtii* DNA contains an unusually high proportion of the nucleoside bases guanosine and cytosine (>60% GC), which is reflected in an unusual codon bias (*16*). This preference for specific codons could result in inefficient expression of foreign DNA by the host cell. Transformation using homologous selectable marker systems, in which a lethal mutation in the host organism is complemented by introduction of a wild-type gene from the same organism, would overcome this obstacle.

As a preliminary step in the development of transformation systems for microalgae with potential for fuel production, we have analyzed the composition of total DNA isolated from several species of microalgae by high-performance liquid chromatography. A brief summary of these data is listed in Table 1; a manuscript describing the details of these experiments is in preparation (*17*). A number of the green algae exhibit an unusually high GC content, up to nearly 71%. As observed for *C. reinhardtii*, the elevated GC contents could indicate peculiar codon usages and may indicate the need to develop homologous selectable genetic markers for these algae as well. In addition, methylated bases were detected in many of the algae. DNA methylation can interfere with construction of genomic DNA libraries in standard bacterial hosts (*18,19*); therefore, we are using host strains insensitive to DNA methylation for production of algal genomic libraries.

As mentioned above, homologous selectable genetic marker systems utilize organisms containing a mutation in an essential gene that is lethal under particular growth conditions. The complementary wild-type gene is then isolated, cloned into a plasmid vector, and introduced into the mutant cells. Transformants can be isolated by their ability to grow under

Table 1
DNA Nucleoside Composition
of Several Microalgal Strains

Algal species	m^5dC	%GC
Chlamydomonas reinhardtii	0.16	61.6
Chlorella ellipsoidea	1.48	51.6
Cyclotella cryptica	1.95	43.2
Monoraphidium minutum	11.2	70.9
Navicula saprophila	0.20	46.2
Nitzschia pusilla	0.78	45.4
Phaeodactylum tricornutum	0.14	48.0
Stichococcus sp.	0.30	44.8
Tetaselmis suecica	3.32	57.5

selective conditions. Once the new gene is incorporated into the host DNA, it should be expressed and maintained in the cell, because it has the codon usage and transcriptional and translational regulatory signals of that specific organism.

We are currently working to develop two genes as potential selectable markers for algal transformation. The orotidine-5'-phosphate decarboxylase gene (OPD) codes for an essential enzyme in the pyrimidine biosynthesis pathway. OPD mutants grow only if provided with an alternate source of pyrimidines, such as uracil. There is also a positive selection for OPD mutants; OPD converts the drug 5-fluoroorotic acid (FOA) into a toxic compound, killing wild-type cells, whereas OPD mutants can grow on FOA-containing media. In addition, a relatively simple spectrophotometric assay can be used to measure OPD activity in cell extracts (*20*). The gene has been cloned and sequenced from a number of organisms (*21–24*), and has been used successfully in a number of transformation systems (*25–27*).

Preliminary screening has shown that several microalgal strains in the NREL collection are sensitive to FOA at concentrations between 0.5–1.0 mg/mL. Using the mutant selection method outlined above, we have screened UV-mutagenized cells of the green alga *Monoraphidium minutum* for FOA-resistance. Several colonies were isolated that showed both FOA resistance and a requirement for uracil; these isolates are putative OPD mutants. One of these mutants has been characterized more extensively. This isolate, "3180a-1," grows well on medium supplemented with 80 μg/mL uracil, either in liquid culture or on plates, but shows no growth in the absence of uracil. A spectrophotometric assay is being used to determine whether this mutant has lost its OPD activity (*20*).

The next step in the development of this system is to isolate the wild-type *M. minutum* OPD gene. We are exploring several cloning strategies.

Genomic and cDNA libraries are being constructed from *M. minutum* that can be screened with heterologous probes for the OPD gene. It may also be possible to clone the gene simply by complementation of the yeast or *E. coli* marker, though an initial attempt to do the latter has failed. As an alternative, the extensive homology between the known OPD genes can be used, taking into account the high GC content of *M. minutum* determined in the DNA analysis, to design primers for gene cloning using the polymerase chain reaction (PCR; *28,29*). Successful cloning of the OPD gene, in combination with the putative OPD mutants described above, should prove to be a suitable selectable marker system for use in transformation of *M. minutum.*

The nitrate reductase (NR) gene has also been used successfully as a transformation marker in a number of organisms, including fungi (*30*) and *C. reinhardtii* (*7*). Algal cell mutants lacking functional NR can be selected based on their resistance to chlorate. Cells having a functional NR protein will take up chlorate along with nitrate and reduce it to chlorite, which is toxic to the cells. The NR-minus mutants do not reduce the chlorate and so do not experience the cytotoxic effects of chlorite. Consequently, cells can be subjected to a positive selection regime by growing target organisms in the presence of chlorate and picking out resistant colonies. Using this protocol, we have isolated several putative NR-deficient mutants of *M. minutum* and of the diatom *C. cryptica.* These candidates grow in the presence of chlorate, and are unable to utilize nitrate on plates or in liquid culture. Experiments in progress to determine if the mutation is in the NR structural gene include assessment of the nitrogen nutritional requirements of the mutants, determination of partial and full NR activities, and detection of the NR protein by immunoblotting.

We are currently working to isolate wild-type NR genes from both *M. minutum* and *C. cryptica* using techniques similar to those described above for isolation of the OPD gene. Complementation of an NR-minus mutant with a functional NR gene will result in cells that can use nitrate as the sole nitrogen source, thus allowing selection for transformants.

GENES INVOLVED IN PHOTOSYNTHATE PARTITIONING

Our interest in the use of microalgae for the mass production of liquid oil fuels necessitates an increased research emphasis on algal lipid production and the means of increasing algal lipid content. Storage lipids accumulate when diatoms and other algae are grown under nutrient-deficient conditions (reviewed in *31*). Nitrogen deficiency induces lipid accumulation in several diverse algal groups that include members of the Chlorophyceae, Bacillariophyceae, and the Rhodophyceae. Because of the influence of nitrogen metabolism on photosynthate partitioning (*32*),

we are investigating the gene coding for nitrate reductase, an important enzyme in algal nitrogen assimilation. NR catalyzes the initial step in the conversion of nitrate to ammonia, and is the rate-limiting and regulated step in this pathway.

We have studied the biochemistry of lipid accumulation in silicon-deficient cultures of the diatom *Cyclotella cryptica,* a species that grows well in outdoor mass culture. Silicon deficiency causes an increase in the activity of acetyl-CoA carboxylase (ACC; *33,34*), which catalyzes one of the initial steps of fatty acid biosynthesis. The activity of this enzyme controls the rate of fatty acid biosynthesis and, consequently, of lipid accumulation in many other organisms (including mammals).

We have undertaken the isolation and characterization of the genes for NR and ACC, since these enzymes control the rate-limiting (and presumably regulated) steps in nitrogen assimilation and lipid biosynthesis. We will analyze these genes in an attempt to identify common genetic regulatory elements, information that will be valuable for future research directed towards the genetic engineering of cells in order to alter lipid production.

Cloning of the ACC and NR Genes from Microalgae

Genetic engineering of microalgae for increased lipid production will require detailed knowledge about the structure and regulation of the genes involved in this process. As mentioned above, construction of cDNA and genomic libraries from several algal species is in progress. These libraries will be screened for NR sequences using heterologous probes. Preliminary experiments in our lab showed that a cDNA probe for the NR gene from the green alga *Chlorella vulgaris* recognizes single-copy DNA sequences (putative NR genes) in DNA from *C. reinhardtii* and *M. minutum* on Southern blots. We are also attempting to clone the NR gene using the polymerase chain reaction. DNA sequences have been identified that are specific for the gene of interest, based on known conserved sequences of the gene from other organisms; the primers were designed to take into account the codon biases seen in the NR gene from *C. vulgaris* (*35*). These sequences are being used as primers to amplify NR-specific sequences from *M. minutum* and *C. cryptica* that, in turn, will be used to screen specific algal DNA libraries for full-length NR genes.

We are using two different approaches to identify sequences encoding ACC from the diatom *Cyclotella cryptica.* Polyclonal antibodies that react specifically with purified ACC from *C. cryptica* have been produced in rabbits. These antibodies are being used to screen *C. cryptica* cDNA expression libraries for the presence of ACC-encoding sequences. In addition, oligonucleotide probes specific for ACC are being designed based on partial amino acid sequences of the purified ACC protein. These probes will be used to amplify ACC sequences using PCR, and the amplified products will then be used to isolate ACC from genomic DNA libraries. Cloning of

the NR and ACC genes from microalgae will allow us to sequence the genes for analysis of their structural and regulatory regions. We will then be in a position to modify the genes in vitro and reintroduce them into cells in order to manipulate lipid accumulation within the cells.

SUMMARY

Significant progress has been made toward the successful genetic engineering of microalgal species with high potential for fuel production. Foreign DNA has been transferred into a green alga, *Chlorella ellipsoidea*, and has been successfully expressed in this heterologous system. In addition, electroporation has shown promise as a means of introducing DNA into intact algal cells. We have analyzed the composition of DNA from several algal species and demonstrated the presence of elevated GC contents in several green algal species. These results suggest that homologous selectable markers may be required for the development of stable transformation systems for the algae; the development of nitrate reductase and orotidine-5′-phosphate decarboxylase as homologous selectable markers for *Monoraphidium minutum* and for *Cyclotella cryptica* is in progress. We have constructed a cDNA expression library for *Cyclotella cryptica*, and are constructing cDNA and genomic libraries for several other algal species. The libraries will be screened with heterologous or homologous probes for nitrate reductase and acetyl-CoA carboxylase in order to clone these genes, which appear to influence lipid accumulation in the algae. This work represents important steps toward the genetic improvement of microalgae for fuel production.

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