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Homologous recombination in *Nannochloropsis*: A powerful tool in an industrially relevant alga

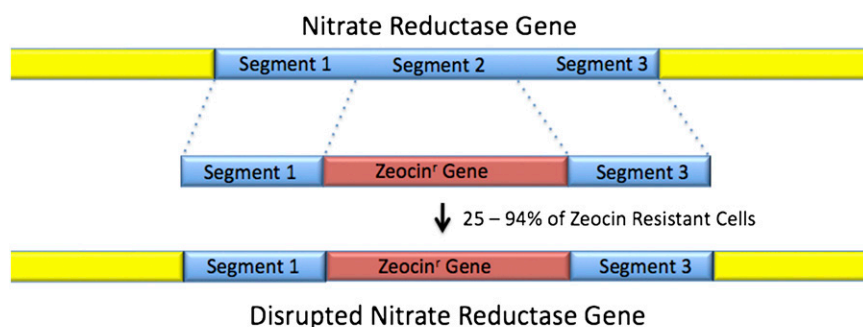
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The availability of facile methods for targeted gene knockout and gene replacement based on homologous recombination in bacteria and yeast systems has driven rapid progress in understanding many of the complex metabolic and regulatory networks in prokaryotic and eukaryotic cells. The lack of such tools in other organisms is a major impediment to progress both in fundamental research and in research that is directed toward practical economic and societal outcomes. For example, the current goal to replace fossil fuels with renewable energy sources produced by plants and algae is hampered in both the short term and the long term by our limited knowledge of their metabolic systems and how they can be modified to create organisms that produce more and better energy-rich molecules. Thus, the report in PNAS by Kilian et al. (1) of efficient and reliable genetic transformation of the commercially important alga, *Nannochloropsis*, via homologous recombination is a significant step forward.

Nannochloropsis species have been used for several decades to produce nutraceuticals and feed supplements (2–5). Several *Nannochloropsis* species have received attention more recently as oil-rich organisms with promise as a source of algal biofuels (6–9). Nonetheless, relatively little is known about the biology of this algal species, including the genes and enzymes involved in lipid biosynthesis. This situation is changing rapidly with the advent of important new tools, not the least of which is the ability to perform targeted gene knockouts and gene replacements using the methods of Kilian et al. (1). Coupled with the availability of genome sequences from at least a dozen independent genome sequencing and transcriptomics projects (www.ncbi.nlm.nih.gov/bioproject?term=nannochloropsis), a rapidly growing community of academic and industrial *Nannochloropsis* researchers (over 100 publications and patents thus far in 2011), and new DNA delivery methods (10), one or more *Nannochloropsis* species may emerge as significant new model alga systems that can build on progress made with *Chlamydomonas reinhardtii*, the most thoroughly studied alga to date (11).

To demonstrate gene replacement by homologous recombination in *Nannochloropsis* sp. (strain W2J3B), Kilian et al.



Nannochloropsis sp. (strain W2J3B)

Targeted Gene Knockout and Gene Replacement by Homologous Recombination
 High Genetic Transformation Rates Commercial-grade Organism
 Multiple Selectable Marker Genes High Lipid, EPA and DHA Contents
 Haploid for Ease of Mutant Selection Potential for Activation Tagging of Genes
 Multiple *Nannochloropsis* Genome Sequencing Projects

Fig. 1. Targeted gene replacement of the NR gene in *Nannochloropsis* sp. (strain W2J3B). Transformation of *Nannochloropsis* cells containing a WT copy of the NR gene with a DNA fragment containing the zeocin resistance gene flanked by ~1-kbp sections of the NR gene results in highly efficient replacement of the native NR gene by homologous recombination and the creation of zeocin resistant, NR-deficient (knockout) mutants. The molecular tools developed by Kilian et al. (1) and other factors (listed) make *Nannochloropsis* sp. (strain W2J3B) attractive both as a model algal system and as an organism for commercial production of algal biofuels and other high-value biomolecules.

(1) target the nitrate reductase (NR) and nitrite reductase (NiR) genes. WT cells can grow using ammonium (NH_4^+), nitrate (NO_3^-), or nitrite (NO_2^-) as a nitrogen source. Mutants lacking a functional NR gene grow in the presence of NH_4^+ and NO_2^- but not with NO_3^- as a nitrogen source. Mutants with a defective NiR gene can grow in the presence of NH_4^+ but not on media containing NO_2^- or NO_3^- . To disrupt the NR gene, the researchers produced a DNA construct containing a selectable marker gene (i.e., a zeocin resistance gene) flanked on either side by ~1-kbp fragments from the NR gene (Fig. 1). The DNA construct was introduced into WT cells by electroporation, and transformants were selected on a zeocin-containing medium. When replica-plated onto a medium containing NH_4^+ and onto another medium containing NO_3^- , it was found that between 25% and 94% of transformants lacked the ability to use NO_3^- . Subsequent analyses by PCR amplification of the NR gene region demonstrated that all cells unable to grow in the presence of NO_3^- contained a NR gene disrupted by the zeocin resistance gene.

Parallel experiments targeting the NiR gene confirmed high rates of cell transformation and gene disruption by homologous recombination.

In addition to demonstrating efficient homologous recombination in *Nannochloropsis* sp. (strain W2J3B), the publication by Kilian et al. (1) reports several other important findings and advances. For example, three different selectable markers (zeocin, hygromycin, and blastidicin resistance genes) were developed and used to demonstrate high rates of cotransformation with independent DNA constructs. The patterns of DNA insertion into the genome provided strong evidence that *Nannochloropsis* sp. (strain W2J3B) is a haploid organism. This finding implies that both dominant and recessive gene mutations caused by gene knockout or conventional mutagenesis, once created, will instantly exhibit any resulting

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phenotype. Although sexual reproduction and genetic analyses have not yet been demonstrated in *Nannochloropsis*, modern molecular tools make this issue much less of a disadvantage than in past times. An unexpected discovery to those who are aware that efficient transformation of *C. reinhardtii* requires removal of cell walls is the fact that *Nannochloropsis* sp. (strain W2J3B) can be genetically transformed at reasonably high rates without stripping its cell walls, albeit using exceptionally high electroporation voltages. An additional difference between *C. reinhardtii* and *Nannochloropsis* sp. (strain W2J3B) is that the latter apparently requires linearized DNA for efficient genetic transformation, whereas the former is readily transformed with either linear or circularized DNA constructs. Finally, Kilian et al. (1) engineer promoter and UTR regions from two unlinked genes encoding violaxanthin/chlorophyll *a*-binding proteins, VCP1 and VCP2, to allow expression of coding regions from various genes. The VCP2 gene construct may prove especially useful because it contains a single bidirectional promoter that allows simultaneous expression of two separate genes. This situation offers the potential to create a construct containing a selectable marker gene driven by one side of the VCP2

promoter region, while the other side remains available to drive the coding region of a gene next to which the construct integrates during an “activation tagging” protocol.

One or more *Nannochloropsis* species may emerge as significant new model alga systems.

Although various *Nannochloropsis* species have been used to produce a number of commercial biomolecules [e.g., docosahexaenoic acid (22:6n- Ω 3), eicosapentaenoic acid (20:5n- Ω 3) (2–5)] for a number of years, they, along with a few other “wild” algal species, are only now being subjected to detailed molecular and biochemical analyses. The present demonstration of gene knockout and gene replacement by homologous recombination in *Nannochloropsis* sp. (strain W2J3B) should stimulate efforts to find additional algae with this capacity, and the development of tools, such as TAL

effector nuclease (TALEN) technology (12, 13), that can achieve similar end points. Intense efforts are underway to improve the production of desirable oils and lipids in algae (6–9) and to develop economically viable facilities for large-scale algal production. Initial use of “omics” technologies already has resulted in laboratory breakthroughs, such as enhanced production of algal lipids without the need to impose nitrogen starvation on algal cultures (14). Nonetheless, it should be noted that development and modification of algae for commercial purposes is in its infancy and that just as modern agricultural crops emerged from primitive predecessors through long-term selection and, later, advanced breeding methods (e.g., modern hybrid corn derived from primordial teosinte), development of algae for commercially viable biofuel and specialty chemical production will depend on extensive expansion of our knowledge base and on concerted and well-reasoned “crop improvement” strategies.

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