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# Genetic Tool Development for *Nannochloropsis* species

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## Background

Algae are excellent candidates for providing a renewable source of liquid fuels with an overall reduction in carbon emissions compared to conventional petroleum fuels. However, the economic viability of algal biofuel production at industrial scales is limited by the low biomass productivities of natural algal species. As bioprospecting and classical breeding techniques have failed to produce an industrial algal strain, genetic modification of eukaryotic algal species is necessary. Strain development of algal species has lagged behind conventional industrial microbes due to the lack of available tools for genetic manipulation.

For growth in outdoor raceway ponds, *Nannochloropsis* species have shown superior performance compared to other strains under a wide range of biotic and abiotic conditions.[1] Furthermore, *Nannochloropsis* species also produce high levels of lipids under stress conditions (~40-50% lipids per cell dry weight). These natural properties make *Nannochloropsis* species excellent candidates for algal biofuel strain development. In this work, we present preliminary data on the development of genetic tools to enable the targeted genetic modification of *Nannochloropsis* species; these tools include novel transfection methods, expression tools, and assessment of CRISPR-Cas9 tools.

## Objectives

- To develop high efficiency transfection methods for *Nannochloropsis* species.
- To identify expression tools for stable transgene expression in *Nannochloropsis* species.
- To apply CRISPR-Cas9 editing tools for targeted nuclear modification in *Nannochloropsis* species.
- To genetically modify *Nannochloropsis* species for improved biomass productivity using the developed genetic tools.

## Results

### Expression Tools

- Developed plasmids for transfection of *Nannochloropsis* species (Table 1)
- Tested chloroplast targeting signal peptides fused to GFP for targeted expression of transgenes
  - Chalmy: MAVMMRTQAPAATRASSRVAVAA RPAARRAVVVRAEA
  - SBP: MKCSLLLALVAPTMAFIPMKPVVPMAK
  - LHC: MRTTACLLAGLGLTQAFLAPTLPSAR
- GFP integration confirmed by colony PCR (14/75 for Chlamy signal peptide and 26/75 for SBP signal peptide)

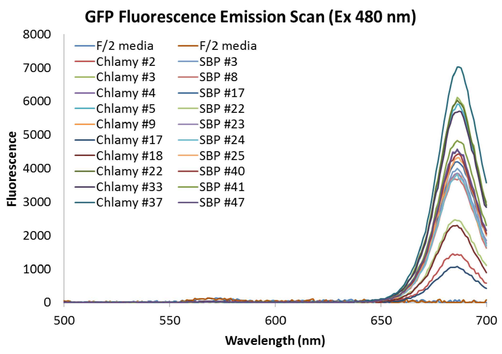


**Figure 1:** Fluorescence emission scans for wild type *N. oceanica* CCMP 526 and candidates modified with chloroplast-targeted GFP. The emission peak at 687 nm is chlorophyll fluorescence, while the GFP emission peak is expected at 510 nm.

**Table 1:** Plasmids for random genome integration in *Nannochloropsis* species with standard electroporation protocol (2200 V, 50 µF, 500 Ω).[2]

| Plasmid | Description | Transformation Efficiency (CFUs per $10^8$ cells) |
|---|---|---|
| pSP124s | Plasmid from the Chlamydomonas Resource Center containing the *ble* resistance gene with *RBCS2* promoter, intron 1, and 3' untranslated region. | 0 |
| pBleT | pSP124s modified by replacing *C. reinhardtii ble* operon with codon optimized *ble* driven by the β-tubulin 5' and 3' regions (500 bp) from *N. gaditana* CCMP 526 | 10 - 2000 |
| pBleV | pSP124s modified by replacing *C. reinhardtii ble* operon with codon optimized *ble* driven by the *VCP1* 5' and 3' regions (500 bp) from *N. gaditana* CCMP 526 | 10 - 2000 |
| pHygT | pBleT modified by replacing *ble* with codon optimized *hygR* | 30 - 2000 |
| pHygV | pBleV modified by replacing *ble* with codon optimized *hygR* | 30 - 2000 |

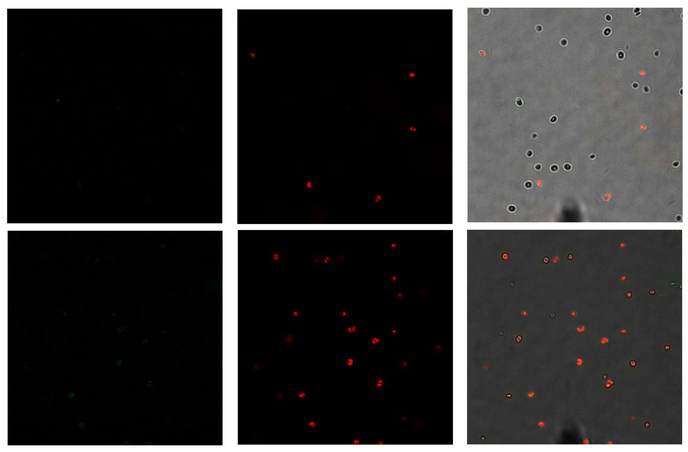

**Figure 2:** Confocal fluorescence microscopy images of wild type *N. oceanica* CCMP 526 (top row) and *N. oceanica* expressing GFP with the chloroplast signal peptide from *C. reinhardtii* (bottom row). Red is chlorophyll fluorescence; green is GFP fluorescence or autofluorescence; and grey is the brighfield overlay in the merged image.

### Cas9 Toxicity

**Table 2:** Results from transfection of *N. oceanica* CCMP 1779 with constructs containing Cas9-2A-GFP with native 5' and 3' fragments for expression.

| Plasmid | Number of Transfected Colonies | Colony PCR for GFP | Positive hits | Full Cas9 Integration |
|---|---|---|---|---|
| pBleT-Cas9 | 78 | 50 | 1 | 0 |
| pBleV-Cas9 | 1000's | 300 | 1 | 0 |

- Reduced number of transfected colonies with plasmids containing Cas9
- Nearly 400 transfected colonies were screened using colony PCR and no candidates contained the full *cas9.*
- β-tubulin and VCP1 promoters may be too strong for *cas9* expression if Cas9 is toxic to *N. oceanica*.

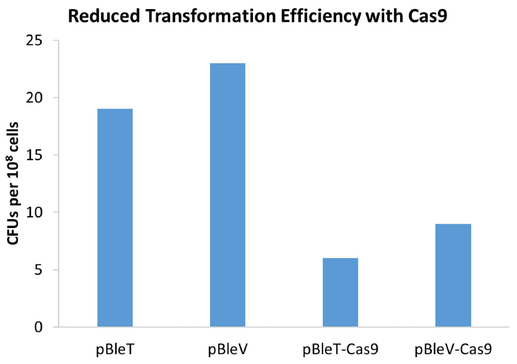


**Figure 3:** Transfection frequency reported as CFUs per $10^8$ cells using standard electroporation conditions[2] for plasmids containing the zeocin resistance gene with 500 bp promoter and terminator regions from β-tubulin and VCP1 (pBleT and pBleV) and modifications also containing Cas9-2A-GFP from pCMV-Cas9-GFP (Sigma-Aldrich) driven by the β-tubulin promoter from *N. gaditana* CCMP 526.

## Transfection Tools

We will explore 4 different transfection methods to optimize transfection efficiency in *Nannochloropsis* species:

- High voltage electroporation (conventional approach)
- Microparticle bombardment (conventional approach)
- Acoustic delivery (sonoporation)[3]
- Nanoparticle-mediated delivery

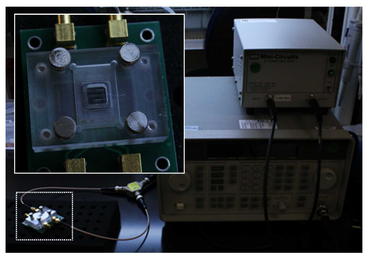

**Figure 4 (right):** Surface acoustic wave (SAW) device for acoustic DNA delivery (i.e., sonoporation).

### *Sonoporation*

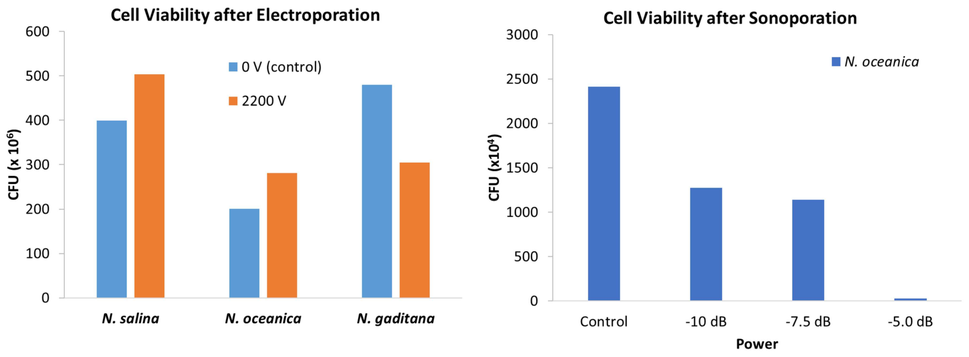


**Figure 5:** Cell viability of *Nannochloropsis* species after electroporation (left) and sonoporation (right). Cell viability was measured as CFUs on dilution plates.

- Electroporation has minimal effect on cell viability
- Sonoporation shows tunable control of cell viability
- Heat-induced damage of transducer element

### *Nanoparticle (NP) Mediated Delivery*

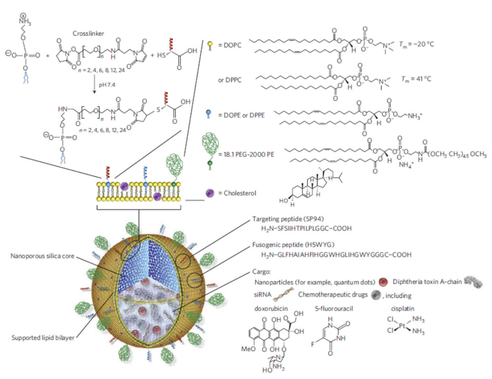


**Figure 6:** Protocell technology (a nanoporous silica nanoparticle (NP) surrounded by a lipid bilayer) allows for targeted delivery of cargo to mammalian cells by varying surface chemistries and biological constituents.[4]

- Presence of the octa-arginine peptide on the protocell surface enhances uptake in *N. salina* and *C. variabilis*

**Table 3:** Specifications for protocell variants tested for uptake in 3 strains of algae: *N. salina* CCMP 1776, *Chlorella variabilis* NC64A, and *Haematococcus pluvialis*.

| Sample ID | Avg. Diameter (nm) | Charge (mV) | Surface Coating |
|---|---|---|---|
| PF1.1 | 244 | +27 | CTAB |
| PF1.2 | 218 | -33 | Hydroxyl Silanes |
| PF2.1 | 312 | +4 | Amino Silanes, Octa-Arginine Peptide |
| PF2.2 | 279 | -8 | Zwitterionic Lipid, PEG, Cholesterol |
| PF2.3 | 309 | -2 | Zwitterionic Lipid, PEG, Cholesterol, Octa-Arginine Peptide |
| PF2.4 | 284 | -18 | Anionic Lipid (DOPS) |
| PF2.5 | 247 | +22 | Cationic Lipid (DOTAP) |

**Table 4:** Evidence of protocell uptake by algae observed by confocal fluorescence microscopy of protocell variants carrying DyLight 488.

| Sample ID | Conditions showing evidence of NP uptake | | |
|---|---|---|---|
| | *C. variabilis* | *H. pluvialis* | *N.salina* |
| PF 1.1 | No uptake observed | 1 mg/mL, 24h | No uptake observed |
| PF 1.2 | No uptake observed | 1 mg/mL, 24h | No uptake observed |
| PF 2.1 | 1 mg/mL, 21h | 1 mg/mL, 4, 21, and 24h | 100 µg/mL, 4h |
| PF 2.2 | No uptake observed | 1 mg/mL, 24h | No uptake observed |
| PF 2.3 | 100 µg/mL, 4h | No uptake observed | 100 µg/mL, 4h |
| PF 2.4 | No uptake observed | No uptake observed | No uptake observed |
| PF 2.5 | No uptake observed | No uptake observed | No uptake observed |

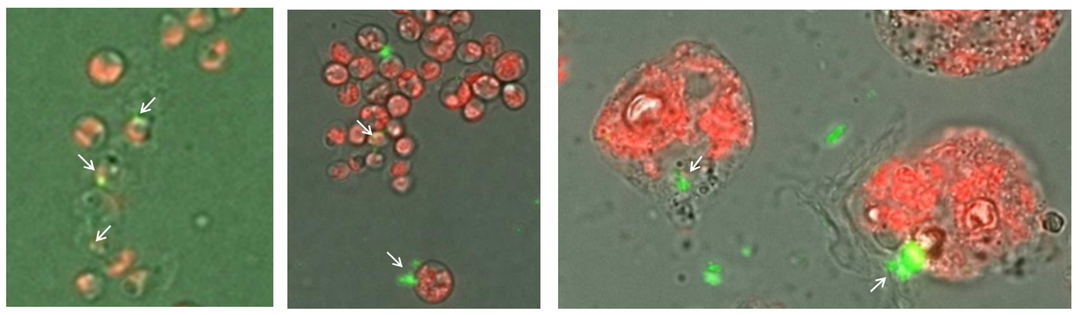


**Figure 7:** Merged confocal fluorescence microscopy images of protocell uptake in 3 algal strains. Red is chlorophyll fluorescence; green is DyLight 488; and grey is the brightfield image. *N. salina* was mixed with 100 µg/mL of PF 2.1 and imaged after 4 hrs of incubation. *C. variabilis* was mixed with 100 µg/mL of PF 2.3 and imaged after 4 hours of incubation. *H. pluvialis* was mixed with 1 mg/mL of PF 2.2 and imaged after 24 hrs of incubation. Incubation conditions = room temperature, 150 rpm, and 60 µmol $m^{-2}$ $s^{-1}$ of cool white fluorescent light.

## Conclusions

- Low expression of GFP in the chloroplast may be masked by chlorophyll autofluorescence.
- Cas9 expression may be toxic in *Nannochloropsis* species.
- Electroporation has a low efficiency of transformation for *Nannochloropsis* species.
- Sonoporation is able to lyse *Nannochloropsis* cells more efficiently than electroporation.
- Nanoparticle-mediated delivery shows some evidence of uptake with octa-arginine peptides on the protocell surface.

## References

1. Radakovits *et al.* 2012. Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropsis gaditana*. Nature Communications. 3:686.
2. Kilian *et al.* 2011. High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. PNAS. 108(52): 21265-21269.
3. Branch *et al.* "Microfluidic device for acoustic cell lysis." U.S. Patent No. 9,096,823. 4 Aug. 2015.
4. Ashley *et al.* 2011. The targeted delivery of multicomponent cargos to cancer cells by nanoporous particle-supported lipid bilayers. Nature Materials. 10: 389-397.

## Funding

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