Functional genomics of plant photosynthesis in the fast lane using Chlamydomonas reinhardtii

Rachel M. Dent, Miehie Han, and Krishna K. Niyogi

Oxynogenic photosynthesis by algae and plants supports much of life on Earth. Several model organisms are used to study this vital process, but the unicellular green alga Chlamydomonas reinhardtii offers significant advantages for the genetic dissection of photosynthesis. Recent experiments with Chlamydomonas have substantially advanced our understanding of several aspects of photosynthesis, including chloroplast biogenesis, structure-function relationships in photosynthetic complexes, and environmental regulation. Chlamydomonas is therefore the organism of choice for elucidating detailed functions of the hundreds of genes involved in plant photosynthesis.

With the genome sequence of the first photosynthetic eukaryote fully characterized, the path appears set for Arabidopsis to dominate the field of plant biology in the next decade. But Arabidopsis is not the ideal organism for all fields of plant research, and photosynthesis is one area where other models have major advantages (Table 1). Chlamydomonas reinhardtii (Fig. 1), for example, has been used as a model organism in photosynthesis research for >40 years, and the use of this unicellular green alga in biochemical, biophysical and genomic approaches, to the study of photosynthesis and photoprotection has been reviewed by several authors recently1-3. This review describes several examples of how molecular genetic studies of Chlamydomonas have provided new insights into photosynthesis. We will highlight the comparative merits of Chlamydomonas as a model photosynthetic organism and discuss how it can make future contributions to the functional genomics of photosynthesis.

Advantages of Chlamydomonas for studying photosynthesis

Chlamydomonas has several attributes that make it an excellent organism for basic genetic studies of plant photosynthesis (Table 1). Its photosynthetic apparatus is closely related to that of vascular plants, and it is also a eukaryote, with photosynthesis genes encoded by both the nuclear and chloroplast genomes. As a unicellular organism, Chlamydomonas has the advantages of a microbial lifestyle without the complications of multicellularity. Synchronous or asynchronous cultures of Chlamydomonas grow quickly with a doubling time of less than ten hours, and the cells behave homogeneously in terms of physiological and biochemical characteristics. Because Chlamydomonas is haploid and has a controlled sexual cycle with the possibility of tetrad analysis (Fig. 2), it is an excellent genetic model.
However, the most valuable characteristic that Chlamydomonas possesses with respect to the study of photosynthesis is its ability to grow heterotrophically using acetate as a sole carbon source. This allows the isolation of viable mutants that are unable to perform photosynthesis, and light-sensitive mutants can be maintained in complete darkness (Fig. 2). Plants such as Arabidopsis or maize with mutations that affect photosynthesis are often inviable or at least susceptible to light-induced photo-oxidation, which can complicate biochemical analyses. Unlike angiosperms, Chlamydomonas can synthesize chlorophyll and assemble a complete photosynthetic apparatus in the dark. It is therefore possible to perform detailed biochemical or biophysical studies of dark-grown nonphotosynthetic and/or light-sensitive mutants. Nonphotosynthetic mutants of other green algae, such as Chlordila and Scenedesmus, and the cyanobacterium Synechocystis PCC6803, can also be isolated and maintained in the dark, but genetic analysis is not possible with these organisms because of the lack of a known sexual cycle. Synechocystis has the advantage of a completely sequenced genome and photosynthetic reaction centers that are highly similar to those of plants, making it an especially useful organism for studying photosystem II and I (PSII and PSI). The limitations of Synechocystis as a plant model include differences in its light-harvesting system and oxygen-evolving complex, and, because the cytochrome b₆f complex is part of both the photosynthetic and respiratory electron transport chains in cyanobacteria, mutants severely affected in this complex cannot be recovered.

Chlamydomonas is the only known eukaryote in which the nuclear, chloroplast and mitochondrial genomes can all be transformed, and studies of photosynthesis have made extensive use of this. Chloroplast transformation is achieved using particle bombardment and occurs by homologous recombination, simplifying chloroplast gene knockouts and site-directed mutagenesis. Chloroplast transformation is not currently practical in Arabidopsis or maize; it is possible in tobacco, but the nuclear genetics are not as easy and rapid to perform in tobacco as they are in Chlamydomonas or Arabidopsis. Because Chlamydomonas contains a single chloroplast, the generation of homoplasmic strains is simplified compared with plants: a single plant mesophyll cell can contain hundreds of chloroplasts.

### Table 1. Comparison of model photosynthetic organisms

<table>
<thead>
<tr>
<th>Synechocystis PCC6803</th>
<th>Chlamydomonas</th>
<th>Arabidopsis</th>
<th>Maize</th>
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</thead>
<tbody>
<tr>
<td>Unicellular prokaryote</td>
<td>Unicellular eukaryote</td>
<td>Multicellular eukaryote</td>
<td>Multicellular eukaryote</td>
</tr>
<tr>
<td>Rapid growth rate (doubling time &lt;10 h)</td>
<td>Rapid growth rate (doubling time &lt;10 h) and life cycle (&lt;2 weeks)</td>
<td>Complete life cycle takes 6 weeks</td>
<td>Complete life cycle takes at least 4 months</td>
</tr>
<tr>
<td>Oxygenic photosynthesis; heterotrophic growth possible</td>
<td>Oxygenic photosynthesis; heterotrophic growth possible with normal assembly of photosynthetic machinery in the dark</td>
<td>Oxygenic photosynthesis; heterotrophic growth possible at seedling stage</td>
<td>Oxygenic photosynthesis; heterotrophic growth possible at seedling stage</td>
</tr>
<tr>
<td>Transformation by homologous recombination</td>
<td>Transformation of nuclear, mitochondrial and chloroplast genomes; homologous recombination in chloroplast and mitochondrial genomes</td>
<td>Transformation of nuclear genome; transformation of chloroplast genome has low success rate</td>
<td>Transformation of nuclear genome only</td>
</tr>
<tr>
<td>Segregation of wild-type copies of the circular genome required for expression of loss-of-function mutant phenotypes</td>
<td>Haploid, therefore immediate expression of nuclear mutant phenotype</td>
<td>Diploid (possible ancestral tetraploid), therefore recessive mutations not expressed in heterozygotes</td>
<td>Ancestral tetraploid, therefore recessive mutations not expressed in heterozygotes</td>
</tr>
<tr>
<td>Low level of functional overlap</td>
<td>Degree of functional overlap not known</td>
<td>High degree of functional overlap with many genes belonging to gene families</td>
<td>High degree of functional overlap</td>
</tr>
<tr>
<td>Replica plating for large-scale screening</td>
<td>Replica plating for large-scale screening</td>
<td>Replica plating for large-scale screening not possible</td>
<td>Field space required for large-scale screening</td>
</tr>
<tr>
<td>Tetrad analysis not applicable because no sexual reproduction</td>
<td>Tetrad analysis possible</td>
<td>Tetrad analysis possible, but only in specific genetic backgrounds (e.g. the qrt mutant)</td>
<td>Tetrad analysis not possible</td>
</tr>
<tr>
<td>Genome size = 3.5 Mbp</td>
<td>Genome size = 100–160 Mbp</td>
<td>Genome size = 120 Mbp</td>
<td>Genome size = 2500 Mbp</td>
</tr>
<tr>
<td>Genome sequenced</td>
<td>Limited sequence available, but sequencing projects have been initiated</td>
<td>Genome sequenced</td>
<td>Limited sequence available, but sequencing projects have been initiated</td>
</tr>
</tbody>
</table>
spectinomycin resistance has been the most widely used selectable marker for chloroplast transformation, but recently a new series of vectors have been developed for the targeted insertion and expression of foreign genes that are based on the bacterial gene aphA-6. As with Chlamydomonas chloroplasts, Synechocystis PCC6803 can also be transformed easily by homologous recombination. Site-directed mutagenesis has therefore been used extensively in the study of structure-function relationships in the reaction centers of this organism.

Transformation of the Chlamydomonas nuclear genome can be accomplished using either endogenous or foreign selectable marker genes. The expression of foreign genes in Chlamydomonas has been a longstanding problem, but this has been overcome by the use of strong endogenous promoters, such as that of RBCS2, and by the introduction of Chlamydomonas introns within the recombinant sequence. In contrast with chloroplast transformation, nuclear transformation results in random insertion of recombinant DNA. Insertional mutagenesis has often been used in forward genetic studies of photosynthetic processes. Highly efficient nuclear transformation has also been used to clone genes by the complementation of existing mutations. Recent successes in identifying, cloning and analyzing novel genes involved in photosynthesis using these techniques are apparent in the studies described below. Although these examples focus on the light reactions of photosynthesis, it should be noted that Chlamydomonas has also proved useful in studies of other aspects of photosynthesis, such as starch metabolism.

Biogenesis of the photosynthetic apparatus

The ability to transform both the nuclear and chloroplast genomes of Chlamydomonas has revolutionized the study of chloroplast biogenesis and the role of nuclear genes in chloroplast gene expression. Protein subunits of the photosynthetic complexes within the thylakoid membrane are encoded by both the nuclear and chloroplast genomes (Fig. 3). A complex crosstalk between the two separate genetic compartments is therefore required to ensure the correct subunit and photosystem stoichiometry. Whereas induction of nuclear gene expression is often controlled at the transcriptional level, mounting evidence suggests that chloroplast gene expression is primarily regulated by post-transcriptional events that are under the control of nucleus-encoded factors. Mutants with defects in these processes are generally nonphotosynthetic and light sensitive, and exhibit high chlorophyll fluorescence, enabling easy screening of mutant populations. Pulse-labeling experiments can also be performed routinely with Chlamydomonas to test whether a mutation affects RNA or protein synthesis or stability in vivo.

The NAC2 gene is a good example of a nuclear gene controlling chloroplast gene expression. It is involved specifically in the control of the half-life of the chloroplast psbD mRNA encoding the D2 reaction center polypeptide of PSI (Ref. 17). By chloroplast transformation and mutagenesis using chimeric psbD mRNAs, two cis-elements have been identified within the 5′UTR that are required for psbD RNA stabilization. There is also evidence to suggest a close connection between the processes of RNA stabilization, 5′-end maturation and translation initiation. Additional support for the interconnection of these processes has been provided by recent
cross-linking experiments showing that a 40 kDa protein interacts specifically with a U-rich translation element in a NAC2-dependent manner. The NAC2 gene was cloned by complementation of the psbD mutation with a cosmid library, and found to encode a hydrophilic polypeptide with nine tetratricopeptide-like repeats (TPRs) in its C-terminal domain. TPR motifs might coordinate the assembly of proteins into multistubunit complexes, and indeed the Nac2 protein was shown to be part of a high molecular weight complex associated with non-polysomal RNA. RNA binding by Nac2 has not been shown, therefore Nac2 might bind RNA via an additional unidentified subunit. Candidates for this role include three independent nuclear products that have been genetically identified as suppressors of psbd5'UTR mutations that destabilize psbd mRNA. This suppressor analysis of nonphotosynthetic mutants, a powerful approach for identifying interacting genes, is easily applied using Chlamydomonas. Although nonphotosynthetic mutants can be isolated using plant models such as Arabidopsis or maize, their mutants can only be propagated as heterozygotes, making suppressor analysis much more difficult.

The mechanism of control of psbd RNA stability is shared perhaps by other chloroplast mRNAs. The MBB1 gene, which encodes a Nac2 homolog, has recently been isolated and shown to be required for psbb mRNA accumulation. Mbb1 also functions as part of a high molecular weight complex, and although Mbb1 and Nac2 are different sizes, it is possible that these two specific complexes might recruit common factors via their TPR domains. This process might not be limited to Chlamydomonas because the maize Crp1 protein, which is needed for chloroplast RNA processing, is also part of a high molecular weight complex and contains pentatricopeptide repeats related to the TPR repeats.

**Structure-function analysis of photosystem I**

Nuclear and chloroplast transformation of Chlamydomonas can be coupled with sophisticated spectroscopic measurements in the study of structure-function relationships within photosynthetic complexes such as PSI. PSI functions as a light-driven oxido-reductase that transfers electrons from plastocyanin to cytochrome c₅₅₃ (in the thylakoid lumen) to ferredoxin (in the stroma), which in turn generates NADPH. The Chlamydomonas PSI consists of eight nucleus-encoded polypeptides (PsaA, PsaE, PsaF, PsaG, PsaH, PsaK, Psal, and PsaN) and at least six chloroplast-encoded polypeptides (PsaA, PsaB, PsaC, PsaJ, PsaL, and PsaM) (Fig. 3). PsaA and PsaB are related proteins that form the heterodimeric reaction center core of PSI. Structural studies of PSI and PsaI have revealed striking similarities in the arrangement of transmembrane helices that bind symmetrical cofactor branches.

To study the oxidizing side of PSI, insertional mutagenesis was used to generate a mutant in the nuclear psaF gene. Analysis of the mutant showed that PsaF is important for the docking of plastocyanin to PSI. The positively charged N-terminal domain of PsaF in Chlamydomonas and plants is not present in cyanobacteria, and it has been hypothesized that the evolution of this domain allowed the formation of a stable complex between plastocyanin and PSI, and thus fast electron transfer. Site-directed mutagenesis of PsaF has illustrated that a single amino acid (K23) might represent a specific recognition site for the interaction of plastocyanin with PSI (Ref. 27). The interaction of PsaF with plastocyanin might also involve the small hydrophobic subunit PsaJ. Although PsaJ could be cross-linked to PsaF, targeted mutation of the chloroplast psaJ gene does not affect the level of PsaF in isolated PSI particles. However, flash-absorption spectroscopy has revealed that only 30% of PSI particles isolated from the PsaJ-deficient strain oxidized plastocyanin with the same kinetics as the wild type. This suggests that PsaJ is involved in the maintenance of PsaF in the correct orientation to enable fast electron transfer from soluble donor proteins to the PSI reaction center.

On light-induced excitation of P700, the chlorophyll dimer in the PSI reaction center, an electron is first transferred to the primary acceptor A₀ and then rapidly to the secondary acceptor A₁ (a phylloquinone). From there, the electrons are transferred to the (4Fe–4S) cluster F₇₅₅ and finally to the terminal iron–sulfur acceptors F₅₅₃ and F₆₃₃, which are bound by the extrinsic polypeptide PsaC. Several site-directed
Fig. 3. Photosynthetic complexes of the thylakoid membrane. The composition of the complexes is drawn according to structural data where available, but position of subunits within the complexes is largely arbitrary. Nucleus-encoded subunits are shaded blue, chloroplast-encoded subunits are white. An arrow indicates the direction of movement of the rotor upon ATP synthesis. Based on Ref. 46.

Regulation of photosynthesis in response to light and nutrients

Light and nutrients are important environmental factors that affect photosynthesis in algae and plants. Photosynthetic light harvesting is regulated by changes in both light quantity and quality. In response to high light intensities that exceed a photosynthetic organism’s capacity for CO₂ fixation, nonphotochemical quenching (NPQ) mechanisms are induced that dissipate excess absorbed light energy harmlessly as heat. Chlamydomonas mutants that are defective in NPQ have been isolated by digital video imaging of chlorophyll fluorescence. The lack of the xanthophyll cycle in the npq1 mutant was the first genetic evidence for the importance of zeaxanthin synthesis in NPQ. Characterization of the lor1 mutant has revealed a possible role for lutein in NPQ (Ref. 32), and other mutants appear to identify specific proteins that are involved in the process.

In response to changes in light quality, a mechanism known as state transition regulates the relative excitation of PSII and PSI. When PSII is preferentially excited by illumination with red light, plastoquinone, the lipid-soluble electron carrier between PSII and PSI, becomes reduced. A protein kinase that phosphorylates the PSII light-harvesting complex (LHCII) proteins is then activated, resulting in a decrease in excitation energy reaching PSII (state 2). Far-red light excites PSI preferentially, resulting in oxidation of the plastoquinone pool, dephosphorylation of LHCII, and restoration of energy transfer from LHCII to PSII (state 1). A crucial role for the plastoquinol-binding site of the Chlamydomonas cytochrome b₅f complex in redox sensing and kinase activation has been demonstrated by site-directed mutagenesis. Nuclear mutants defective in state transition have been identified using chlorophyll fluorescence video imaging under conditions that induce either reduction or oxidation of the plastoquinone pool. The stt7 and stm1 mutants are blocked in state 1 and are deficient in LHCII phosphorylation. These mutants are still capable of photoautotrophic growth, although at a slower rate than the wild type, and further analysis should answer longstanding questions about the physiological role of state transitions.

Acclimation of the photosynthetic apparatus also occurs in response to a deficiency of various essential nutrients. In a screen to identify genes involved in the response to copper deficiency, crd1 mutants were isolated that failed to accumulate PSI, LHC1 and to a lesser extent LHCII under copper-deficient conditions. Because the mutants were produced and tagged by insertion mutagenesis, it was possible to
Insertional mutagenesis has been a valuable technique in the generation of banks of photosynthetic mutants. A complicating factor is that not all mutants are actually tagged by the DNA insertion. Genetic crosses have shown that ~50% of mutants are tagged, a frequency similar to that observed using comparable T-DNA tagging approaches in plants. Insertional mutagenesis in Chlamydomonas often results in the deletion of large fragments of genomic DNA (up to 20 kb). This makes identification of the gene responsible for a mutant phenotype more difficult, but does have the advantage that fewer transformants are needed to saturate the nuclear genome. As an alternative to insertional mutagenesis by transformation, the development of a transposon tagging system should provide a more precise mechanism for the insertion of foreign DNA into the genome. Several active transposons have already been identified in Chlamydomonas.

An additional tool that would aid in the isolation of photosynthetic genes is an autonomously replicating shuttle vector for cloning by complementation. Current methods require plasmid rescue to recover an integrated cosmid after complementation of the mutant phenotype. An alternative strategy involves transformation with indexed libraries that consist of pools of specific cosmids, with subsequent identification of an individual complementing cosmid done. It can therefore be seen that although there are technical challenges that remain to be met in Chlamydomonas research, progress is being made in finding solutions to each of these issues.

The ultimate goal of photosynthesis research is to identify all the genes that are involved in photosynthesis and to understand how their proteins function and interact with one another (Fig. 4). A central resource to this goal is a catalog of all the genes in Chlamydomonas, and progress towards achieving this aim is being made through a Chlamydomonas genome project. Current efforts include sequencing of full-length cDNAs that are expressed under various environmental conditions. These will be used to construct DNA microarrays to investigate global patterns of gene expression. However, the sequencing of cDNAs is unlikely to find all the genes, so complete DNA sequencing of the ~100–160 Mbp Chlamydomonas nuclear genome will be necessary.

In the meantime, cDNA sequencing from polymorphic strains of Chlamydomonas can be used to identify single nucleotide polymorphisms and DNA fragment length polymorphisms (insertions or deletions) that will enable construction of a detailed genetic map. The current map of 240 markers on 17 linkage groups is based on progeny from a cross between the standard laboratory strain of Chlamydomonas and a highly polymorphic field isolate from Minnesota, USA (strain S1D2). The markers used to generate the map include random cDNAs, random genomic fragments, and doned genes already in the database.

![Fig. 4. Functional genomics of photosynthesis using Chlamydomonas reinhardtii.](http://plants.trends.com)
The genome project has also initiated the development of a physical map linked to the genetic map of the nuclear genome. The physical map will consist of overlapping BAC clones that are anchored on the genetic map using the set of 240 unique markers as probes. To date, ~2000 different BACs have been aligned on the genetic map, representing ~25% of the genome. Work is currently being carried out to fill in gaps between BAC contigs, and an updated map will soon be placed in the Chlamydomonas database (C. Silflow and P. Lefebvre, pers. commun.).

These genetic and physical maps will facilitate the positional cloning of genes identified in classical forward genetics screens for nonphotosynthetic mutants. Use of mutagens such as EMS or UV light should enable the recovery of a variety of partial loss-of-function and gain-of-function mutations, in addition to complete null mutations. Because of the rapid life cycle of Chlamydomonas, map-based cloning can be completed much faster than the positional cloning of genes identified in classical reverse genetics approaches will also be necessary where, for example, members of gene families have overlapping function. To obtain mutations in specific genes, collections of random insertion mutants can be screened using PCR-based strategies similar to those used for Arabidopsis and maize. An additional reverse genetics approach, we are currently generating an EMS-induced Chlamydomonas mutant population that can be screened by denaturing HPLC for point mutations in any gene of interest. Because EMS usually causes C-to-T transition mutations, this approach is particularly suited to Chlamydomonas, given the GC-rich nature of its genome. The technique has the advantage that it can be used to find nonsense (null) alleles of a gene, as well as missense mutations that might have more subtle phenotypes.

Recently developed strong promoters should enable silencing of specific genes by antisense technology or RNA interference (RNAi). For example, the HSP70A–RBCS2 expression system has been used to silence the COP gene encoding the photoreceptor for phototaxis (Chlamydomonas; M. Fuhrmann and P. Hegemann, pers. commun.). After transformation of wild-type cells with an inverted repeat construct, COP gene expression was reduced by almost 90%. An advantage of this approach is the feasibility of silencing duplicated genes in a single experiment. The recent use of Chlamydomonas as a model system for the study of the mechanisms of post-transcriptional gene silencing.

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**Fig. 5** Comparison of map-based cloning in Chlamydomonas and Arabidopsis, showing the approximate minimum time to obtain a rough map position for a nonphotosynthetic mutation. A collection of markers for Chlamydomonas mapping is currently being developed. The Arabidopsis timeline assumes that a collection of M3 families (seeds from individual M2 plants) is available for screening, thereby enabling the identification of heterozygous siblings of mutants that are lethal as homozygotes.

<table>
<thead>
<tr>
<th>Chlamydomonas</th>
<th>Arabidopsis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutant picked</strong></td>
<td><strong>Mutant picked in M3 family</strong></td>
</tr>
<tr>
<td>Streak mutant to retest phenotype</td>
<td>Cross heterozygous sibling and get F1 seeds</td>
</tr>
<tr>
<td>Do cross and dissect tetrads</td>
<td>Grow F1 and get F2 seeds</td>
</tr>
<tr>
<td>Grow up tetrads</td>
<td>Grow F2, score phenotype and extract DNA</td>
</tr>
<tr>
<td>Restreak colonies, score phenotype and extract DNA</td>
<td>Score PCR-based markers</td>
</tr>
<tr>
<td><strong>Total = 6 weeks to map position</strong></td>
<td><strong>Total = 16 weeks to map position</strong></td>
</tr>
<tr>
<td>Confirmation of gene by complementation takes a further 2 weeks</td>
<td>Confirmation of gene by complementation takes a further 6 weeks</td>
</tr>
</tbody>
</table>

**Time (weeks)**

- 0
- 2
- 4
- 6
- 8
- 10
- 12
- 14
- 16

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http://plants.trends.com
Using a combination of these genetic approaches, it should thus be possible to obtain mutations in every Chlamydomonas gene involved in photosynthesis. Many photosynthetic mutants have already been generated in Synechocystis, and a complete collection of knockout mutants will soon be available for Arabidopsis. These resources should enable the annotation of many new genes that are necessary for photosynthesis24. However, because of the inviability of nonphotosynthetic mutants of Arabidopsis, it will be practically impossible to perform the kinds of detailed biochemical and biophysical analyses (Fig. 4) that are routine with such mutants of Chlamydomonas and Synechocystis. Functional genomic studies of eukaryotic photosynthesis will therefore undoubtedly need to involve Chlamydomonas. The unique advantages of Chlamydomonas should help bring to light the specific functions of genes responsible for the synthesis, assembly, function, and regulation of the photosynthetic apparatus.

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