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Functional genomics of photosynthesis using *Chlamydomonas reinhardtii*

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Introduction

The study of photosynthesis is ideal for functional genomic analysis. Because of the number of components involved in the process and the requirement for co-ordination of two different genomes in algae and higher plants, photosynthesis research will benefit from a global approach more than many other areas of plant biology. The recent completion of the *Arabidopsis* genome sequence, and the *Synechocystis* PCC6803 sequence, have generated large volumes of data that will be invaluable in the isolation of new photosynthetic genes and the elucidation of their functions. Neither of these organisms, however, are ideal model systems for the study of photosynthesis. *Chlamydomonas reinhardtii*, on the other hand, offers several significant advantages, and is the organism of choice for functional genomic studies of photosynthesis (Dent *et al* 2001). *Chlamydomonas* has a photosynthetic apparatus closely related to higher plants, but its microbial lifestyle allows for large-scale replica plating. Because *Chlamydomonas* is haploid there is immediate expression of nuclear mutant phenotypes. Most importantly, however, *Chlamydomonas* has the ability to grow heterotrophically using acetate as a sole carbon source. This allows the isolation of viable non-photosynthetic and light-sensitive mutants.

We are therefore developing a project that aims eventually to isolate mutants for all genes involved in photosynthesis and photoprotection from *Chlamydomonas.* To achieve this aim we are taking both forward and reverse genetics approaches. We are in the process of generating a bank of insertional mutants, using the *ble* gene, for use in classical forward genetics. As a complementary strategy we are using EMS to create a population of point mutants for screening using denaturing HPLC for reverse genetics.

Procedures

Strain and growth conditions

Mutagenesis of *Chlamydomonas* was carried out using the strain $4A^+$ (CC125 background, Cw^+, kindly supplied by J.-D. Rochaix, University of Geneva). Mutants were maintained on TAP agar medium (Harris 1989) in the dark at 25°C. For screens, 5 µl cells in liquid TAP medium were spotted onto TAP agar plates or HS agar plates (Harris 1989) and maintained at different light intensities. For chemical treatments, TAP or HS agar plates contained Rose Bengal (Sigma, USA) or Metronidazole (Sigma, USA) at different concentrations. High CO_2 treatments were carried out in anaerobic BBL GasPak Pouches (Becton Dickinson, USA).

Strain storage

Cells were stored under liquid nitrogen as described by Crutchfield *et al* (1999). Zygospores were stored according to Harris (1989).

Insertional mutagenesis

Insertional mutagenesis of $4A^+$ cells was carried out as described by Lumbreras *et al* (1998) with some modifications. The bacterial *ble* gene was used as a dominant selectable marker. This gene confers resistance to phleomycin and its derivatives, such as zeocin. In an attempt to limit the problem of deletions of genomic DNA which have been encountered with *Chlamydomonas* insertional mutagenesis we have employed the method of restriction enzyme-mediated insertion (REMI), which has been used successfully in fungal systems (Kuspa and Loomis 1992, Sànchez et al 1998). In brief, *Chlamydomonas* $4A^+$ cells at log phase were treated with gamete autolysin to digest the cell walls. For REMI transformation, 1 µg linearised pSP124S plasmid was added to the cells in the presence of 10 U BamHI and 20% PEG and the cells vortexed with glass beads for 30s. After recovery in the dark overnight, the cells were plated onto zeocin-containing TAP medium and maintained in the dark until resistant clones were visible. These were picked and patched onto zeocin-containing media to confirm their antibiotic resistance, then subsequently maintained on TAP agar plates in the dark.

Genomic DNA extraction and isolation of flanking sequences

Genomic DNA was isolated from *Chlamydomonas* cells using DNAzol isolation reagent for plants (Life Technologies, USA) according to the manufacturer's instructions. The isolation of fragments of flanking genomic sequence from insertional mutants was achieved either by plasmid rescue or TAIL-PCR. For plasmid rescue genomic DNA was digested with MluI to isolate fragments on both sides of the insertion. The digested DNA was re-ligated and used to transform XL10-Gold Ultracompetent cells (Stratagene, USA). TAIL-PCR was carried out following the method described by Liu *et al* (1995). The sequence for the arbitrary degenerate primer was kindly supplied by H. Cerutti (University of Nebraska).

Results and Discussion

To select a strain in which to create both banks of mutants we tested a number of available "wild-type" strains for the ability to remain green and grow well under constant dark. We selected $4A^+$ as the most vigorous when cultured in the dark.

An initial population of ~5000 insertional mutants was generated as described above using strain $4A^+$. Molecular analysis by Southern hybridization of a small number of random mutants has revealed that 70% of lines have single insertion events. Genetic analysis has shown that approximately 60% of mutations are tagged by the transforming DNA. This is comparable with other studies utilizing insertional mutagenesis in *Chlamydomonas.*

Screens

The insertional mutant lines generated were subject to 6 different growth conditions for primary phenotypic screening:

1) Very low light (10 µmol photons m^{-2} s^{-1}) on TAP medium
2) Low light (~80 µmol photons m^{-2} s^{-1}) on TAP medium
3) HS medium containing no alternative carbon source, under low light
4) High light (500 µmol photons m^{-2} s^{-1}) on HS medium
5) 1.5 mM Metronidazole (generates superoxide in the chloroplast) on TAP medium
6) 2 µM Rose Bengal (generates singlet oxygen) on TAP medium.

After selection of mutants from these initial screens, the phenotypes were confirmed by an expanded second screen that also included:

7) Low light (~80 µmol photons m^{-2} s^{-1}) on HS medium under anaerobic/elevated CO_2 conditions
8) High light (500 µmol photons m^{-2} s^{-1}) on HS medium under anaerobic/elevated CO_2 conditions
9) 1.5 mM Metronidazole on TAP and HS medium
10) 2 µM and 1 µM Rose Bengal on TAP and HS medium
11) 6.5 mM Cu^{2+} under low light conditions on TAP medium
12) Chorophyll fluorescence measurement using video imaging.

After secondary screening, 80% of the mutants isolated in the primary screen re-scored with the same phenotype. The proportions of each major class of mutant isolated by the screens are presented in Table 1. From this group mutants with any phenotype screened are occurring at an approximate frequency of 6.7%. The classes of phenotype presented in Table 1 are not mutually exclusive. Therefore many mutants show a phenotype in more than one of the screens.

Table 1: Percentage of mutants in each class of primary screen

Mutant class	**Percentage**
Very light-sensitive (10 µmol photons m^{-2} s^{-1})	0.2%
Light-sensitive (~80 µmol photons m^{-2} s^{-1})	1.8%
High light-sensitive (~500 µmol photons m^{-2} s^{-1})	1.0%
Acetate-requiring	2.5%
Rose Bengal-sensitive	2.5%
Metronidazole-sensitive	2.3%

The results of the primary and secondary screens are being used to develop a database with a detailed description of the phenotype for each individual mutant.

Characterization of dim *mutants*

From the mutant classes described above, one class was selected for further analysis. This class of mutants is sensitive to low light intensities such that they grow at 10

µmol photons m^{-2} s^{-1}, but not at 80 µmol photons m^{-2} s^{-1}. The phenotype of representative mutants of this class is illustrated in Figure 1. These mutants will subsequently be referred to as *dim* mutants. In addition to the light-sensitive phenotype 90% of these mutants are also acetate-requiring.

For genetic analysis, *dim1* has been crossed to a wild-type strain similar in phenotype to $4A^+$, named $17D^-$. The results show that the phenotype of *dim1* is due to a single nuclear insertion and that the mutation is tagged (Table 2).

Table 2: Genetic analysis of *dim1* mutant

Recombinants/complete tetrad	0/4
Recombinants/total progeny	0/34
$ZeocinR^+$/light sensitive : wild-type progeny per tetrad	2:2

Isolation of flanking sequence from insertional mutants

Two methods for the isolation of flanking genomic DNA from photosynthetic mutants – plasmid rescue and TAIL-PCR – were evaluated using the *dim* mutants. The plasmid rescue procedure resulted in low success of plasmid recovery from mutant lines. Flanking sequence was recovered from approximately 20% of mutants tested. This may be explained by the fact that deletion of fragments of the transforming DNA had occurred in many of the insertional mutants, such that either the ampicillin resistance gene, the origin of replication or both were no longer present.

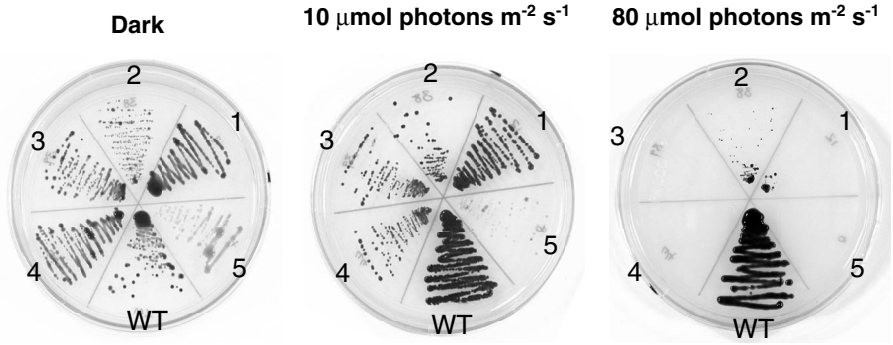

Figure 1: Phenotype of *dim* mutants compared to $4A^+$ (WT) grown on TAP agar plates in the dark, at 10 µmol photons m^{-2} s^{-1} and at 80 µmol photons m^{-2} s^{-1}. Mutant labeled 1 = *dim1*.

Plasmid rescue was, however, successfully used to isolate flanking genomic sequence from both sides of the insertion in *dim1*. In addition to sensitivity to low light intensity, *dim1* is also acetate-requiring but shows no detectable phenotype in any of the other screens. Sequencing of the flanking DNA showed that the *ble* gene had inserted 190 bp upstream of the start codon of the *rbcS2* gene

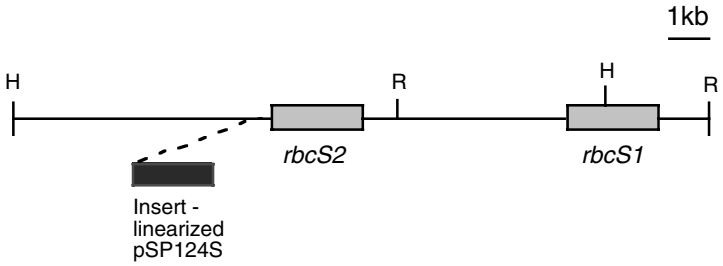

Figure 2: The *rbcS* locus in *Chlamydomonas reinhardtii* (from Khrebtukova and Spreitzer 1996) showing the position of insertion of the linearised pSP124S plasmid in *dim1*.

(Figure 2). The genomic sequence isolated from the other side of the insertion shows no significant homology to any Genbank sequences. It is therefore likely that the whole *rbcS* locus has been deleted in *dim1*. Unlike other Rubisco mutants that have been isolated (Khrebtukova and Spreitzer 1996), *dim1* has flagella and can cross to its opposite mating type easily, as illustrated by the genetic data presented in Table 2.

Due to the low success rate of plasmid rescue, TAIL-PCR was investigated as an alternative method for a high-throughput approach to the isolation of flanking sequence. As flanking sequence from *dim1* was already known, this mutant was used to confirm the specificity of amplification. Nested primers were designed for each end of the linearised transformation plasmid. Amplification of a single major DNA fragment was achieved on both sides of the insert (Figure 3). Sequencing of these fragments showed that the amplification was specific as these coincided with the flanking sequence isolated by plasmid rescue. TAIL-PCR is therefore being used to isolate flanking sequence from all the mutants isolated from the primary screening, using a 96-well format. Preliminary results show that fragments ranging from 100 bp to 1000 bp may be amplified using this technique. Amplification has a higher rate of success (approximately 75% of mutants tested) with flanking DNA at the *ble* gene end of the plasmid due to problems of deletion of the plasmid as described above. As plasmid rescue is no longer being pursued as a method to isolate flanking DNA we are investigating whether the problems of insert deletion may be solved by using a smaller fragment of recombinant DNA for transformation which only contains the *ble* gene. Thus selective pressure would ensure that only transformants containing the full sequence would be recovered after selection on zeocin. A second problem that has been encountered is a proportion of the mutants contain multiple concatamerised inserts at a single locus. Fragments amplified from these mutants usually only contain plasmid sequence. In several cases, however, additional fragments of flanking DNA are also amplified.

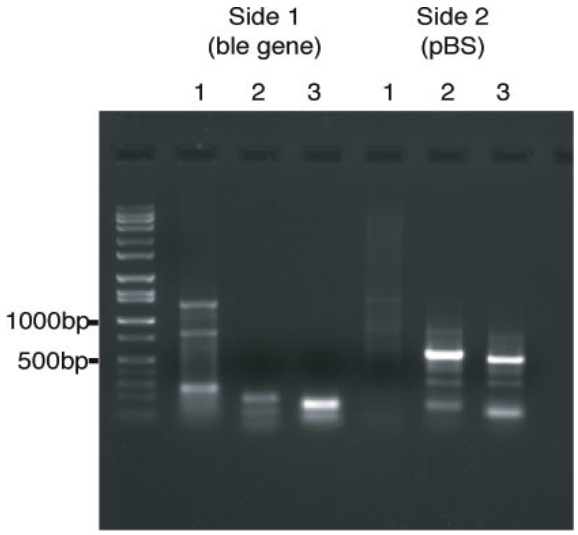

Figure 3: Fragments of flanking DNA generated by TAIL-PCR of *dim1* genomic DNA. The lanes are numbered to show the cycles of PCR carried out with nested primers and a arbitrary degenerate primer

Using this TAIL-PCR method, a preliminary group of 40 mutants have been investigated. Flanking sequence was recovered from the *ble* gene end of the insertion from 20. Many of these sequences show homology to *Chlamydomonas* ESTs in Genbank. We are currently optimising the high-throughput methods to increase the success rate.

Future work

The current goal of the project is to generate and screen insertional mutants until a bank of 5000 mutants with a phenotype related to photosynthesis, photoprotection and

oxidative stress tolerance is generated and stored. This should be sufficient to isolate most genes involved in these processes from the *Chlamydomonas* genome. Flanking sequence will be isolated from each of these mutants using TAIL-PCR to generate a database of insertion loci. Using the rapidly developing *Chlamydomonas* EST database, and hopefully in the near future the complete genome sequence, these short sequences will enable identification of the mutated gene.

In addition, a population of 10000 random insertional mutants will be maintained and stored as a resource for reverse genetics. These will be stored both as zygospores and as vegetative cells under liquid nitrogen. DNA will be isolated from these mutants, pooled and screened in a PCR-based strategy similar to those used for *Arabidopsis* and maize.

Due to the problems of storing large numbers of insertional mutants of *Chlamydomonas*, it will not be possible to saturate the genome using this method for the reverse genetics project. As a complementary approach we are therefore also preparing a bank of mutants generated by EMS. By using a concentration of EMS that allows a survival rate of 20-50% of cells, mutants will be generated with multiple point mutations, thus reducing the number of individual lines required to saturate the genome. As EMS usually induces C-to-T transitions this approach is particularly suited to *Chlamydomonas,* given the GC-rich nature of its genome. Genomic DNA will be prepared from individual EMS mutants, and pooled. The population will be screened by denaturing HPLC for point mutations in any gene of interest using the TILLING method (McCallum *et al* 2000). Once isolated the mutants will be fully characterised using both physiological and molecular analyses.

It is therefore our ultimate aim that by using a two edged approach we will be able to identify all genes from *Chlamydomonas* that are involved in photosynthesis, photoprotection and oxidative stress. Although this project will utilize data generated using *Arabidopsis* and *Synechocystis*, *Chlamydomonas* offers unique advantages for further analysis of photosynthetic mutants at the biochemical, biophysical, physiological and genetic levels (Dent *et al* 2001).

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