CHAPTER FIVE

Gene Hunting by Complementation of Pooled *Chlamydomonas* Mutants

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Abstract We have generated a library of 34 photosynthesis mutants of *Chlamydomonas reinhardtii*, using insertional mutagenesis with the antibiotics resistance markers *ble* and *aphVIII*. To clone the affected genes, we transform the mutants by electroporation with an indexed cosmid library. Because the recipient strain has been selected for high efficiency transformation, we are able to perform transformations of mutants in pools of 4. Once a cosmid pool has been identified that yields phototrophic transformants, the individual mutant is identified. The individual cosmid is then pooled out by transforming with preparations from rows and columns of the 96-well cosmid plate. Using this strategy, we have analyzed a first pool of four mutants (one ATPase mutant and three *ac* mutants with no fluorescence phenotype). One gene has been cloned, and two others are at the stage of de-pooling. We are now embarking on a full-size screen of the library, with the aim of identifying new genes involved in photosynthesis or biogenesis of the photosynthetic apparatus.

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Introduction

Over the last 50 years, hundreds of photosynthesis mutants have been isolated in the green alga *Chlamydomonas reinhardtii*, owing to the ease with which they can be grown on plates containing acetate. Mutants were found to reside both in the chloroplast and in the nuclear genome. The latter lack either a component of the photosynthetic apparatus or a protein involved in co-factor assembly or in the expression of a specific chloroplast gene. Identification of the affected genes has been achieved either by cloning the flanking DNA or by complementation with an indexed DNA library. We have used the latter approach to clone factors involved in the biogenesis of the cytochrome c_i of cytochrome b_6f (Kuras et al. 2007): the mutant is transformed with pools of cosmids until one is found that complements the acetate requirement. We used the cosmid library of (Purton and Rochaix 1994) which provides 2.3× coverage of the genome in hundred 96-well plates (average 30 kb inserts).

The main drawback in applying this technique to our collection of fully-characterized mutants is that these have been obtained by a variety of procedures in all sorts of genetic backgrounds, often many years ago, so that each cloning requires tailoring the transformation conditions to the particular strain being used. To overcome this drawback, and to gain access to genes that have not been characterized thus far, we have generated a series of mutants in an optimized cell-wall-less arginine-requiring strain. We take advantage of its high transformation rate to pool mutants before transformation so as to limit the number of screenings.

Materials and methods

Strains and plasmids. The recipient strain XS1, with the *arg7 cw15 mt+* genotype and optimum mating and transformation ability, was derived from a cross of WT-S24 *mt-* strain with the mutant *ccb4 arg7 cw15 mt+* (Kuras et al. 2007). To generate insertional photosynthesis mutants, three plasmids were used: pMS188 (Schroda et al. 2002) containing the *ble* gene under control of the *HSP70A/RBCS2* fusion promoter; pBC2 (Sizova et al. 2001) and pSL72 (S. Lemaire, personal communication) containing the *aphVIII* gene under the same promoter and under the *PSAD* promoter, respectively. « Cassettes » containing the promoter, resistance gene and 3´ UTR were generated by digestion and gel-isolated. The ScaI-linearised pSL72 and pBC2 were also used. The linearized plasmid pABG2, containing the *ARG7* gene, was used to complement the *arg* phenotype in transformation tests. Strains resistant to paromomycin (Sigma) were selected at 5–15 µg/mL. Resistance to zeocin (Invitrogen) was in the range of 0.5–3 µg/mL.

Transformation. XS1 was cultured in 200 mL minimal medium (MIN) with 50 µg/mL arginine. Cultures were transformed in the exponential phase (2×10^6 cells/mL) using an electroporation protocol adapted from (Shimogawara et al. 1998). Cells, concentrated to 5×10^7 cells/mL in 250 µL of a 80% TAP 40 mM sucrose (ToS) solution, were transformed with 300–450 ng DNA using a voltage of 10 V and high capacitance. After transformation, cells were diluted 100× in ToS supplemented with arginine, incubated for 18–24 h shaking at 125 rpm with 1,000 lux. Cells were centrifuged (4,500 rpm, 5 min) and plated onto ten TARG plates containing a range of antibiotic concentrations. To enrich for photosynthetic mutants, transformed cells were incubated with metranidazole (Sigma) to a final concentration of 2.4 mg/mL for 10^6 cells/mL resulting in a 3 mL solution per 50 mL Erlenmeyer flask. Incubation was carried out at 6,000 lux with 300 rpm shaking for 24 h, then overnight in dark before plating. All plates were kept in an incubator at 27°C with low light for 4 weeks.

Mutant screens. Colonies were restreaked (100/plates) onto square Petri dishes containing TARG and MIN-ARG and incubated at low light or 2,000 lux, respectively. After 2 weeks, plates were compared to identify acetate requiring (*ac*) transformants. Original plates were also screened directly by digital fluorescence imaging (Bennoun and Beal 1998).

Storage. ac mutants were stored in Petri dishes at 20°C on TARG media for 6 weeks. Mutants were cryopreserved at −180°C in a solution of TAP sucrose with 10% methanol (Crutchfield et al. 1999). Regenerated cultures were checked for maintenance of *cw15* phenotype, transformability and fecundity.

Complementation. Strains grown to mid-log phase were complemented by electroporation with pooled cosmids from each of the 100 plates in the library. Twenty-five microliters (1/10) of the transformation was plated on TAP to control for transformation efficiency, using the *ARG7* marker present in the cosmid vector. The rest was plated on MIN medium supplemented with 25 µg/mL Arg and 100 µg/mL ampicillin (Sigma) to control contamination. They were scored 10–20 days later for the presence of phototrophic colonies. Once a cosmid pool had been identified, cosmids were prepared from the 8 rows and 12 columns of the plate. The cosmid at the intersection of the positive samples was prepared individually after streaking to single colony, and checked for restoration of the *ac+* phenotype. It was end-sequenced and the candidate genes were identified by mapping

to the genome. The experiment was repeated after digestion with a series of restriction enzymes predicted to cut some but not all of the genes, until a single candidate remained.

Results

Mutant collection. Thirty-four insertional mutants unable to grow on MIN medium were obtained in five transformation experiments using zeocin and paromomycin resistance markers (Table 1). When an enrichment step with metronidazole was included, the proportion of *ac* mutants rose from 0.25% to 14%. Mutants with unacceptably high reversion rates, identified by transformation with *ARG7* and plating on MIN plates, were eliminated. Others were frozen in liquid nitrogen for further use. Four classes of mutants could be identified based on their fluorescence induction

Table 1 Summary of mutant status

Mutant pool	Name	Phenotype	b6f pool checked ?	Plasmid used	Metroniadazole enrichment	Library screened	Pool identified	Cosmid identified	Gene identified
A	wcf1	WT		pBC2		done	none		
	wcf2	WT		pSL72		done	21	21.G11	yes
	wcf3	WT		pBC2		done	14	14.5F	yes
	wcf4	ATPase		pMS188		done	none		
	P5.2	cyt b6f	Y	pBC2		done	50		
	ZIF8	cyt b6f	Y	pMS188		done	50		
	BCID3	cyt b6f	Y	pBC2	Y	partial			
	BCID10	cyt b6f	Y	pBC2	Y	partial			
	BCIG10	cyt b6f	Y	pBC2	Y				
	BCIG5	cyt b6f	Y	pBC2	Y				
	BCID2	cyt b6f	Y	pBC2	Y	done	50		
	BCIB2	cyt b6f	Y	pBC2	Y	no			TCA1
	P10B2	cyt b6f	Y	pBC2		done	50		
	P10B5	cyt b6f	Y	pBC2					
	BCIH10	cyt b6f	Y	pBC2	Y				
	P10B6	PQ-?	Y	pBC2		no			PCY1
	wcf5	WT		pBC2					
	Z1B10	PSI		pMS188					
	EP101Ca	PSI		pBC2					
	IP102Ca	PSI		pBC2					
	P10B4	PSI		pBC2					
	P10S1	PSI		pBC2					
	P10S4	PSI		pBC2					
	BCID6	PSI		pBC2	Y				
	BCIE1	PSI		pBC2	Y				
	ZIG4	PSII		pMS188					
	ZID8	PSII		pMS188					
	AP152Ci	PSII		pBC2					
	GP102Ca	PSII		pBC2					
	BCID7	PSII		pBC2	Y				
	BCIH3	PSII		pBC2	Y				
	P10B3	PSII		pBC2					
	BCIH9	PSII		pBC2	Y				
	BCIC1	PSII		pBC2	Y				

phenotype: PSII mutants showed constant high fluorescence; PSI and cytochrome b_6f mutants had similar continuously rising fluorescence curves and were further distinguished by visualizing the CP1 Chlorophyll-protein complex and cytochromes on a TMBZ-strained "green" gel (12% SDS-PAGE at 4°C); ATPase mutants had a WT-like fluorescence induction, but acquired a PSII phenotype when exposed to 3,000 lux for 1 h; these *w*ild type *c*hlorophyll *f*luorescence (*wcf*) mutants were indistinguishable from WT under low light, in spite of their inability to grow in the absence of acetate. We initiated the gene hunt with the latter two categories.

Gene cloning. A first pool of four mutants was screened with the entire cosmid library. Mutant *wcf2* was complemented by two cosmid pools and one was de-pooled to identify the cosmid of interest. Mutants *wcf1* and *wcf3* were complemented by a single pool, and are at the stage of cosmid identification. Mutant *wcf4* was not reliably complemented by any of the cosmid pools and was dropped from this study. Because of the possibility for mutant cells to feed on their dead sisters, and of the difficulty in maintaining strict sterility when using starch for plating wall-less strains, some cosmid pools had to be tested several times. Our turn-around rate is establishing about 4 weeks per mutant pool, and we plan to turn to pools of six or eight mutants in the future. A second pool of four cytochrome b_6f mutants is currently under screening. To insure that they do not reside in already known genes, all mutants with a cytochrome b_6f phenotype have first been transformed individually with a special pool of 13 genomic and cDNA clones, containing most of the genes hitherto identified as necessary for biogenesis of the complex. Compared with the first mutant pool, cytochrome b_6f mutants are easier to study because of the fluorescence signature of the true complemented colonies.

Discussion

Analysis of the genetic network that sustains biogenesis of the photosynthetic apparatus has benefited tremendously from the availability of the draft genome sequence of Chlamydomonas (Merchant et al. 2007). Phylogenomic analysis has thus led to a list of 349 genes with orthologues in Viridiplantae but not in non-photosynthetic eukaryotes, among which many will turn out to be involved in chloroplast biogenesis. Our aim is to expand the current body of knowledge by linking non-photosynthetic phenotypes with genes of hitherto unknown function. Our cloning method limits us to genes whose mutation leads to inefficient photosynthesis or to strong light-sensitivity. Still, our finding that 2 of the 12 mutants with a cytochrome b_6f phenotype were complemented by our pool of 14 known genes, strongly suggests that our approach can indeed unravel novel genes. Compared to the cloning of the flanking DNA (Dent et al. 2005; Gonzalez-Ballester et al. 2005), its main advantages are that (i) the complemented *ac* mutation needs not be linked to the resistance marker; (ii) the gene is easily identified by end-sequencing the cosmid, whereas it is often difficult to clone both flanks of an insertion that is often accompanied by large deletions; and (iii) gene identification is simultaneous with the obtention of the gDNA clone that contains it.

Once a gene has been identified, a complete study of the phenotype is initiated, which can by itself represent a tremendous task. But by ensuring that gene cloning precedes mutant characterization, we try to make this process more efficient. Because the mutant collection can be greatly expanded and our strategy is not limited to insertional mutagenesis, we can envision approaching saturation mutagenesis within a couple years of effort. We hope to identify more genes than our laboratory can characterize, and would like to provide the *Chlamydomonas* photosynthesis community with many interesting gene/mutant pairs.

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