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Minireview

The three genomes of Chlamydomonas

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Abstract

During the past 50 years, the green unicellular alga *Chlamydomonas reinhardtii* has played a key role as model system for the study of photosynthesis and chloroplast biogenesis. This is due to its well-established nuclear and chloroplast genetics, its dispensable photosynthetic function in the presence of acetate, and its highly efficient nuclear and chloroplast transformation systems. Considerable progress has been achieved in our understanding of the structure, function, inheritance, and expression of nuclear, chloroplast, and mitochondrial genes and of the molecular cross-talk between the nuclear, chloroplast, and mitochondrial genetic systems.

Introduction

The green unicellular alga *Chlamydomonas reinhardtii* has emerged as a powerful model system for studying a large set of biological processes, including photosynthesis, chloroplast and mitochondrial biogenesis, flagellar assembly and motility, phototaxis, circadian rhythms, gametogenesis and mating, and cellular metabolism. This historical review deals mostly with photosynthesis and organellar biogenesis. Two main reasons for choosing this alga as a research organism were its ability to grow in the absence of photosynthetic activity with acetate as carbon source and its ability to undergo a well-defined sexual cycle. These properties made it possible to perform an extensive and successful genetic dissection of the structure and function of the photosynthetic apparatus.

The early phase of Chlamydomonas genome studies

The history of research on the Chlamydomonas genomes is especially interesting to follow because the discoveries and the functional studies of these genomes played a key role in establishing this alga as a model system. Like plants and other eukaryotic algae, Chlamydomonas possesses three genetic systems located in the nucleus, the chloroplast, and the mitochondria. While this seems unremarkable today, it was not at all clear 50 years ago. In 1954, Ruth Sager isolated mutants of *C. reinhardtii* resistant to streptomycin that showed Mendelian and non-Mendelian inheritance during crosses (Sager 1954). The identification of the non-Mendelian or uniparental mutants was a seminal finding that started the research history of the Chlamydomonas chloroplast genome, at least at the genetic level. At that time, the existence of chloroplast DNA was still disputed and it was only several years later that an unambiguous proof for this DNA was provided. Ris and Plaut (1962) detected Feulgen-positive regions in the cytoplasm of *C. reinhardtii* that were DNAse sensitive. Chun et al. (1963) fractionated total DNA of *C. reinhardtii* by CsCl density centrifugation and were able to detect a satellite β DNA band at a density of 1.695 g cm^{-3} in addition to the main nuclear α DNA band at 1.724 g cm^{-3}. The β DNA was found to be enriched in chloroplast fractions from this alga and correctly identified as chloroplast DNA (Sager and Ishida 1963). The considerable difference

in density or in GC content between the chloroplast and nuclear DNAs of *C. reinhardtii* made it possible to purify the chloroplast DNA by CsCl density centrifugation and thus to study its physical and chemical properties.

In 1960, Paul Levine (1960) developed a screen for isolating photosynthetic mutants of *C. reinhardtii*. He isolated acetate auxotrophs that were tested for their inability to incorporate radioactive CO_2. This broad and large-scale screen led to the isolation of numerous nuclear photosynthetic mutants affected in photosynthetic electron transfer, photophosphorylation, and carbon metabolism. The mutations were mapped in several linkage groups. A faster screen for photosynthetic mutants was developed later by Pierre Bennoun and Paul Levine (1967), which measured the chlorophyll fluorescence output of live cells and which is still used extensively today. A similar screen was developed by Garnier et al. (1968).

First molecular studies with Chlamydomonas DNA

While the genetic studies were in progress in the 1960s, N. Sueoka and K.-S. Chiang performed an extensive characterization of the nuclear and chloroplast DNA of*C. reinhardtii*. By performing ^{15}N- ^{14}N density shift experiments, they showed that both DNAs replicate semiconservatively during vegetative cell divisions (Sueoka et al. 1967). It was found later by Turmel et al. (1981) that chloroplast DNA replication is dispersive rather than semiconservative, i.e., newly synthesized DNA appears in both strands. Sueoka and Chiang also determined the total DNA content per haploid vegetative cell of *C. reinhardtii* at 1.23×10^{-13} g and estimated that 14% of this DNA was chloroplast DNA (Chiang and Sueoka 1967). However, it was not clear whether this DNA, which represented twice the amount of *E. coli* DNA, consisted of a small number of large chloroplast genomes or whether it was made of a larger number of small chloroplast genomes. The answer came through the study of reassociaton kinetics of denatured chloroplast DNA, which revealed a kinetic complexity of 2×10^8 daltons or 300 kbp (Wells and Sager 1971; Bastia et al. 1971). Although this number exceeds the current size estimate of the chloroplast genome of *C. reinhardtii* by 50%, these early studies clearly indicated that the chloroplast genome is present in multiple copies of a relatively small unit. The organization of the chloroplast genome of *C. reinhardtii* was further investigated by resection of DNA fragments with an exonuclease, which created single-stranded overhangs. Subsequent ligase treatment gave rise to circles, thus indicating that the chloroplast DNA of *C. reinhardtii* contains a large number of repeated DNA sequences (Rochaix 1972). Transcriptional mapping of the chloroplast ribosomal RNA genes showed that 16S and 23 S rDNA are part of an operon, with the 16S rRNA gene transcribed first (Surzycki and Rochaix 1971). At the same time, sophisticated genetic mapping techniques were developed for mapping chloroplast genes (reviewed by Gillham 1994). This resulted in a linkage group of uniparental markers that included mostly antibiotic resistance markers. This linkage group was first thought to correspond to the entire chloroplast genome (Singer et al. 1976). However, further studies showed that it corresponds only to a small segment of the plastid genome, specifically the rRNA genes within the chloroplast inverted repeat and the ribosomal protein gene *rps12* in the unique sequence region (reviewed by Harris 1989). The technical tools were rather crude at that time and it was not possible to prove unambiguously that the uniparental linkage group and the chloroplast genome were the same.

It was recognized early that chloroplasts contain 70S ribosomes which are distinct from the cytosolic 80S ribosomes and that these ribosomes are sensitive to different sets of antibiotics. By treating cells with inhibitors of 70S ribosomes, it was possible to show that the synthesis of several photosynthetic activities depends on chloroplast translation (Armstrong et al. 1971). Since it was correctly assumed that mRNAs cannot be imported into the chloroplast, these results indicated that chloroplast-encoded factors were involved in these activities. A further refinement was to pulse-label cells with ^{14}C-acetate in the presence of chloramphenicol or cycloheximide, specific inhibitors for 70S or 80S ribosomes, respectively, and to reveal the proteins synthesized under these conditions by polyacrylamide gel electrophoresis and autoradiography. In this way the nuclear or chloroplast genetic origin of several photosynthetic components could be determined (Chua and Gillham 1977).

Identification of genes involved in photosynthesis

With the availability of restriction enzymes and the advent of the recombinant DNA technology in the

1970s, rapid progress in the study of the organization of nuclear, chloroplast and mitochondrial genes was achieved. The physical map of the chloroplast genome of *C. reinhardtii* was established showing that this DNA consists of circles of ca. 200 kbp, with two large 21 kbp inverted repeats containing the rRNA genes (Rochaix 1978). The chloroplast DNA circles were also observed by electron microscopy (Behn and Herrmann 1977).

In the late 1970s, a large number of uniparental mutants had been isolated (for review see Gillham 1994). In crosses these mutations are normally transmitted to all four meiotic products from the mating-type plus (mt+) parent. However, a few exceptional zygotes occur in which the chloroplast genomes from both parents are transmitted to the progeny. Because these markers re-combine, they can be mapped genetically and they were found to be part of a unique linkage group (reviewed by Gillham 1994). A great deal of work was devoted to develop methods for the specific induction with 5-fluorodeoxyuridine and selection with metronidazole of non-photosynthetic mutants. Because all these mutations affected chloroplast structure or function, it seemed likely that they were located on the chloroplast genome. The unambiguous proof that the chloroplast DNA of *C. reinhardtii* is uniparentally inherited came through the restriction enzyme analysis of the DNA of the progeny from reciprocal crosses between wild-type and mutant strains containing deletions in their chloroplast DNA (Grant et al. 1980). In spite of these findings, it was still not clear whether the chloroplast DNA was indeed the carrier of the uniparental mutations. The 10-6C mutation affecting *rbcL*, the chloroplast gene of the large subunit of ribulose 1,5 bisphosphate carboxylase, which had been shown to be linked to other genetic markers in the established uniparental linkage group (Mets and Geist 1983), provided the first direct link between the uniparental linkage group and chloroplast DNA (Dron et al. 1983). Rapid advances folllowed and led to the identification of all the antibiotic resistance mutations within the chloroplast rRNA genes and the adjacent *rps12* gene in the unique sequence region, and to a correlation between the physical and genetic map of this part of the chloroplast genome (Harris et al. 1989). In addition, many chloroplast genes were identified that were either involved in photosynthesis or plastid gene expression. It is a paradox that the chloroplast genome from *C. reinhardtii* was the first to be identified genetically, but the determination of its sequence has only been completed recently, while a dozen of other plastid genomes have already been fully sequenced (see 'Genomes' section on the Internet at http://www.ncbi.nlm.nih.gov). The chloroplast DNA of *C. reinhardtii* consists of 203′337 bp and contains 34 genes involved in photosynthesis, 31 genes involved in chloroplast transcription and translation, 1 protease gene, 29 tRNA genes, and 9 genes of unknown function (J. Maul, J. Lilly and D. Stern, unpublished data).

Transformation of the chloroplast compartment

Development of the biolistic chloroplast transformation system for *C. reinhardtii* by Boynton et al. (1988) opened a new area in chloroplast DNA research. Because of the efficient homologous chloroplast recombination system, it became possible to perform specific gene disruptions and hence chloroplast reverse genetics. This could be achieved by using the bacterial *aadA* gene fused to appropriate chloroplast regulatory sequences as selectable marker expressing resistance to streptomycin and spectinomycin (reviewed by Goldschmidt-Clermont 1998a). The sequencing of several chloroplast genomes from plants and algae had revealed the existence of a set of conserved genes called *ycf* genes (*y*pothetical *c*hloroplast open reading *f*rame) of unknown function. By disrupting specifically each of these genes, the phenotype of the resulting mutants could be examined and in several cases the role of the disrupted *ycf* could be inferred (Table 1; reviewed by Rochaix 1997). The chloroplast transformation system was also used for performing site-directed mutagenesis of several genes involved in photosynthesis. This approach was particularly successful for the elucidation of the structure–function relationship of the thylakoid protein–pigment complexes Photosystem (PS) I and PS II and the cytochrome b_6f complex each of which consists of both chloroplast and nucleus-encoded subunits (reviewed by Hippler et al. 1998). Key findings were the identification of the ligands of P700, a chlorophyll dimer that acts as the primary electron donor of Photosystem I, of the 4Fe–4S clusters F_X and the terminal electron acceptors F_A and F_B. More recently, the established crystal structure of PS I revealed two symmetrical branches between P700 and F_X. One of the branches includes the primary electron acceptors A_0, a chlorophyll *a* and A_1, a phylloquinone while the other branch includes their counterparts A_0' and A_1' (Schubert et al. 1997). Chloroplast transformation and site-directed mutagen-

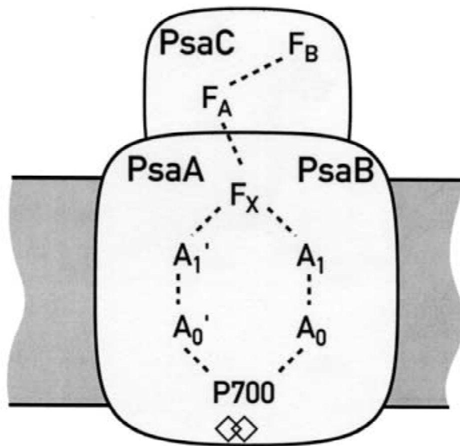

Figure 1. Both electron transfer branches are active in the PS I reaction center. The redox cofactors of the two large PS I reaction center polypeptides PsaA and PsaB are indicated: P700, a chlorophyll dimer, the primary electron donor; A_0, a chlorophyll *a*, the first electron acceptor; A_1, a phylloquinone; F_X, a 4Fe–4S center. A_0' and A_1' represent the symmetrical counterparts of $A_0$and A_1. The two terminal electron acceptors F_A and F_B, both 4Fe–4S centers, are liganded to the PsaC subunit. The two electron transfer branches from P700 to F_X are indicated by broken lines.

esis coupled to *in vivo* spectrophotometric analysis was used to demonstrate that both symmetrical electron pathways are used from P700 to F_X (Figure 1; Guergova-Kuras et al. 2001). This contrasts with the bacterial photosynthetic reaction centers in which only one of the branches in the complex is used for electron transfer. Other examples are in the identification of key aminoacids in the functioning of donor and acceptor side of Photosystem II (see e.g., Roffey et al. 1994; Xiong et al. 1998).

Besides providing numerous new insights into the function of chloroplast genes, the transformation technology was also instrumental in studying chloroplast gene expression. In particular, it was possible to dissect chloroplast promoters, 5′ and 3′ UTRs (untranslated regions) and to elucidate the maturation and processing of plastid mRNAs (reviewed by Goldschmidt-Clermont 1998b).

Unusual properties of the mitochondrial genome of *C. reinhardtii*

Among the three genomes of Chlamydomonas, the mitochondrial genome is most unusual. A first surprise was the finding that it is uniparentally inherited from the mating type minus (mt−) parent, in contrast to the chloroplast DNA which is generally inherited from the mt+ parent. This was demonstrated in reciprocal crosses between *C. reinhardtii* and *C. smithii*, which differ in their mitochondrial DNA restriction patterns (Boynton et al. 1987). These two strains also differ in chloroplast restriction patterns, making it possible to show unambiguously that the two organellar genomes are transmitted by opposite mating types (reviewed by Remacle and Matagne 1998). A second surprise was the small size of the mitochondrial genome: it consists of linear 15.8 kb DNA molecules with a very reduced gene content in comparison with other green algal and land plant mitochondrial genomes (Grant and Chiang 1980). The genome was fully sequenced (Gray and Boer 1988; Michaelis et al. 1990) and shown to contain seven respiratory genes, a reverse-transcriptase-like gene and only 3 tRNA genes. The other tRNAs have to be presumably imported into the mitochondria, although it has not yet been possible to prove this directly. The two rRNA genes are arranged in an unusual way on the mitochondrial genome and are fragmented and scrambled, i.e., the gene pieces are intermingled with other coding regions, and they do not follow the 5′–3′ order of the normal rRNA genes (Boer and Gray 1988). They give rise to small transcripts that can pair with each other to form a discontinuous secondary structure that resembles that of the conventional continuous rRNAs. Transformation of mitochondria of *C. reinhardtii* has been achieved although with very low efficiency (Randolph-Anderson et al. 1993).

Table 1. Chloroplast reverse genetics in *C. reinhardtii*: function of *ycf*s

	Functions of gene product
ycf1	Essential, function unknown
ycf2	Essential, function unknown
ycf3	Assembly of PS I
ycf4	Assembly of PS I
ycf5	Heme attachment to c-type cytochromes
ycf7	PetL, subunit of cytochrome $b_6 f$; required for optimal efficiency and dimerization of the complex
ycf8	PsbT, subunit of PS II; required for optimal activity under adverse growth conditions
ycf9	Associated with PS II; possible role in the connection of the antennae to the PS II reaction center
ycf10	Envelope protein, facilitates inorganic carbon uptake into the chloroplast

Note: Further details can be obtained from Rochaix (1997).

The nuclear genome of *C. reinhardtii*: excitements and frustrations

Sueoka (1960) was the first to characterize the nuclear genome of *C. reinhardtii* and to show that it has an unusually high GC content of 62%. Current estimates of the size of this genome range around 100 Mbp (see Harris 1989). Nearly 200 loci were mapped on the 17 nuclear linkage groups (Harris 1993). A molecular map was constructed from the segregation analysis of molecular markers amongst the progeny of a cross between *C. reinhardtii* and an interfertile Chlamydomonas strain divergent in genomic DNA sequence. This map is still under construction and contains currently 170 molecular markers (Silflow 1998).

Among the highlights of research on the nuclear genome of *C. reinhardtii*, the cloning and molecular characterization of the mating-type locus represents one of the most remarkable achievements. The mating-type locus is not only involved in the mating behavior of the cells, but also determines which of the two parents transmits its chloroplast and mitochondrial DNA to the progeny. The mating type locus was cloned through an extensive chromosomal walk of 1.1 Mbp (Ferris and Goodenough 1994). The locus was found to comprise at least 830 kbp that could be further subdivided into three domains. The centomere-proximal C domain of 525 kbp and the telomere-proximal T domain of 109 kbp, both of which are colinear in the mt+, and have mt– genomes flanking the central domain of 190 kbp. This region differs markedly between the mt+ and mt− loci as a result of extensive rearrangements due to four intrachromosomal translocations, two inversions and large deletions and duplications. This central domain also includes regions that are unique to the mt+ and mt− loci and that encode mt functions. Of particular interest was the identification of the *Ezy1* gene present in multiple copies in the C domain that is zygote-specific and transcribed upon zygote formation (Armbrust et al. 1993). The Ezy1 protein is directed to the chloroplast of both mt cells where it associates with the nucleoids. Irradiation with UV light, a treatment known to interfere with uniparental inheritance, represses expression of *Ezy1* in mt+ cells, but not in mt− cells. These findings raise the possibility that *Ezy1* is involved directly or indirectly in the selective degradation of the mt− chloroplast DNA which normally occurs in zygotes, and they also both open the door for understanding the molecular basis of chloroplast uniparental inheritance, a phenomenon which was discovered nearly 50 years ago by Ruth Sager.

The first evidence for nuclear transformation in Chlamydomonas was reported in 1982 using a yeast gene as the selectable marker (Rochaix and van Dillewijn 1981). It took several years until authentic Chlamydomonas selectable markers, such as the genes of nitrate reductase and of argininosuccinate lyase, could be incorporated into transformation vectors and used for efficient and reliable transformation of the auxotrophic *nit1* and *3* mutants (reviewed by Kindle 1998). It was soon noticed that homologous recombination occurs at a very low frequency and that in most cases the transforming DNA integrates randomly into the nuclear genome. Thus, nuclear transformation could be used as a mutagen for tagging nuclear genes (Tam and Lefebvre 1993). This method has proven to be very powerful for isolating nuclear genes of *C. reinhardtii*.

A problem that has frustrated Chlamydomonas researchers for many years is the difficulty of expressing foreign genes in the nuclear compartment of this alga. One possibility is that this may be due to the highly biased codon usage towards G and C observed in most nuclear genes of this organism. It was indeed possible to express foreign genes with a high GC content in *C. reinhardtii* (Stevens et al. 1996; Sizova et al. 2001). Furthermore, the GFP (green fluorescent protein) gene could be expressed after reconstruction of the gene with the biased *C. reinhardtii* codon usage (Fuhrmann et al. 1999). The presence of introns also appears to be important since many nuclear genes of this alga contain introns and their corresponding cDNAs are usually not expressed. A third possibility is gene silencing, as shown by the lack of stable expression of the bacterial spectinomycin resistance gene after this gene had been inserted into *C. reinhardtii* cells (Cerutti et al. 1997). Remarkably, it was possible to select mutants that were deficient in this post-transcriptional gene silencing activity and to isolate one of the genes involved in this process (Wu-Scharf et al. 2000). The gene encodes a protein that belongs to the DEAH-box RNA helicase family (DEAH refers to a conserved motif in these proteins) and is most likely required for the degradation of aberrant RNA that is produced by some transgenes and transposons. Understanding this silencing process will undoubtedly help in achieving high levels of expression of foreign proteins in *C. reinhardtii*.

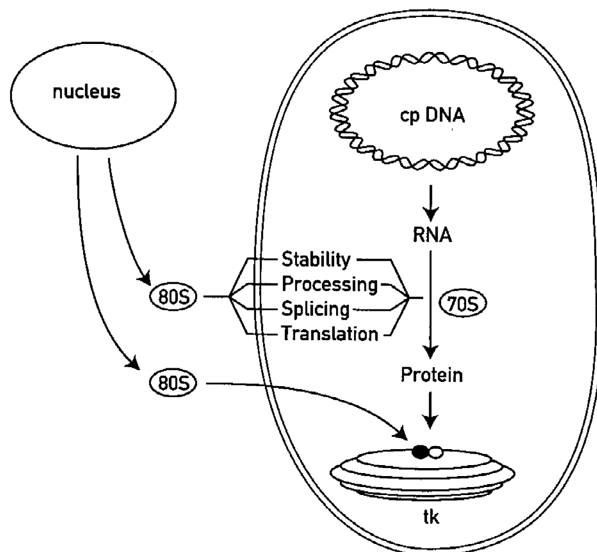

Figure 2. The biosynthesis of the photosynthetic apparatus depends on the concerted interactions between the nuclear and chloroplast genomes. Photosynthetic complexes in the thylakoid membranes (tk) consist of nucleus (●) and chloroplast (○) encoded subunits. The former are translated on cytosolic 80S ribosomes and imported post-translationally into the chloroplast. The chloroplast-encoded subunits are translated on 70S ribosomes to form, together with their nucleus-encoded partner polypeptides, functional complexes in the thylakoid membranes. To express individual chloroplast genes, a large number of nucleus-encoded factors are required for the different post-transcriptional steps that include RNA stability, RNA processing, RNA splicing and translation.

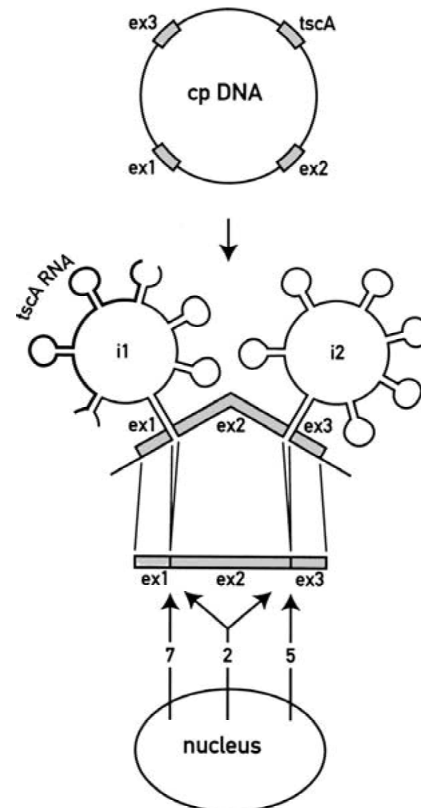

Figure 3. Maturation of the chloroplast *psaA* mRNA of *C. reinhardtii*. The chloroplast *psaA* gene consists of three exons that are transcribed independently to give rise to three transcripts that are *trans*-spliced with the help of the *trans*-acting *tscA* RNA that completes the groupII intron structure of the first intron i1. At least 14 nucleus-encoded factors are required for the assembly of the *psaA* mRNA: 7 for the first exon1-exon 2 *trans*-splicing reaction, 5 for the second exon 2-exon 3 *trans*-splicing reaction and 2 for both reactions. Exon sequences are not drawn to scale.

Cross-talk among the genomes of Chlamydomonas

One of the most surprising results of the analysis of mutants of *C. reinhardtii* deficient in photosynthesis was the discovery of a large number of nuclear loci involved in chloroplast gene expression. The factors encoded by these loci are required for the expression of specific plastid genes and act at post-transcriptional steps, such as the stability, the maturation, the splicing and the translation of individual chloroplast mRNAs (Figure 2; see Goldschmidt-Clermont 1998b). As an example, one of these factors is involved in the stability of *psbD* mRNA encoding a PS II reaction center polypeptide and at least two others are specifically required for the translation of this mRNA. Mutants deficient in these factors are only affected in *psbD* expression, as shown by the fact that the expression of the other chloroplast genes is normal. An extreme example of nuclear participation in chloroplast gene expression is the maturation of the *psaA* mRNA used to encode one of the large reaction center polypeptides of PS I. This gene was found to consist of three independently transcribed exons that are widely separated on the chloroplast genome and flanked by group II intron sequences (Kück et al. 1987). Maturation of the *psaA* mRNA thus depends on two trans-splicing reactions. Quite surprisingly, it was found that splicing of the two first exons of *psaA* requires an additional *trans*-acting RNA, *tscA* RNA, which provides the missing link between the 5′ and 3′ part of the first *psaA* intron (Figure 3; Goldschmidt-Clermont et al. 1991). The genetic analysis of *psaA* mRNA assembly revealed that at least 14 nucleus-encoded factors are required. Because the chloroplast genome is estimated to contain over 100 genes, there could be thousands or more of these factors. It is interesting to note that the recent sequencing of the Arabidopsis nuclear genome has revealed the existence of nearly 3000 nuclear genes encoding

chloroplast-destined proteins, many of which could be involved in regulating the expression of plastid genes (The Arabidopsis Genome Initiative 2000). Thus, there is an extensive cross-talk between the nuclear and chloroplast genomes. It is only recently that the molecular identification of these factors was achieved, mainly because of the development of efficient nuclear transformation methods that allowed these factors to be cloned through genomic complementation or through insertional mutagenesis (reviewed by Barkan and Goldschmidt-Clermont 2000).

Recent evidence suggests that genetic interactions also exist between the chloroplast and mitochondrial genomes. A search for chloroplast suppressors of a chloroplast missense mutation within the plastid *rbcL* gene revealed a suppressor that was also able to suppress mitochondrial mutations (Bennoun and Delosme 1999). Although this suppressor was exclusively characterized genetically, it is likely that it is an informational suppressor such as a ribosomal protein or a tRNA. Because the mitochondrial genome contains only three tRNA genes, it is possible that it uses chloroplast tRNAs that would need to be shuttled between the two organelles.

Perspectives

The past years have witnessed substantial advances in our understanding of the structure, organization and function of the nuclear, chloroplast, and mitochondrial genomes of Chlamydomonas. The availability of a well-defined genetic system and the development of efficient transformation systems for the nuclear and chloroplast compartments have provided powerful tools for studying the mechanisms of gene expression in these cellular compartments as well as for understanding the structure–function relationship of protein complexes. While the nuclear influence on the chloroplast genetic system has been explored quite intensively, less is known about the influence of the state of the chloroplast on nuclear gene activity, although recent results indicate that this interaction pathway exists as well in Chlamydomonas (Kropat et al. 1997). Finally, the interactions between the chloroplast and mitochondrial genetic systems constitute another promising area that is still largely unexplored.

Very recently a genomic Chlamydomonas project was initiated thanks to Japanese (http://www.kazusa.or.jp/en/plant/chlamy/ES T/) and US (http://www.biology.duke.edu/chlamy_genome/ EST.html) initiatives. At this time, 136′604 ESTs (expressed sequence tags) have been produced and are available on the World Wide Web. This has provided a tremendous boost to the Chlamydomonas research community and considerably facilitated and accelerated research. It is hoped that a nuclear genomic sequencing project will soon be started. Its completion would further enhance the power of *C. reinhardtii* as a unique eukaryotic and photosynthetic model system.

I end this minireview by showing a photograph of some of the people involved in Chlamydomonas research.

Figure 4. Chlamydomonas researchers at Cold Spring Harbor (1988). From left to right: Robert Togasaki, Stefan Surzycki, Ursula Goodenough, Jean-David Rochaix, and Nicholas Gillham.

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