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Transformation of the mitochondrial genome

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ABSTRACT Although mitochondrial transformation is highly desirable in mammals and plants, it is only possible in two unicellular organisms, the budding yeast *Saccharomyces cerevisiae* and the unicellular green alga *Chlamydomonas reinhardtii*. Here, we give an overview of the attempts made to transform mitochondria of mammals and plants and the possible reasons for their failure. This review briefly describes the mitochondrial transformation principles in yeast and describes in more detail the transformation and its applications in *Chlamydomonas*.

KEY WORDS: *mitochondria, transformation, Chlamydomonas, respiration, mutation*

Introduction

Mitochondria are the main sites where the biological energy is generated in eukaryotes. As semiautonomous organelles, they possess their own genome, the mitochondrial DNA (mtDNA), that differs in form and size among the various organisms and occurs in multiple copies, varying from a few dozen up to several thousand copies per cell. The mtDNA can form circular molecules in mammals (Boore, 1999) and in some plants, such as bryophytes (liverworts, hornworts and mosses) (Terasawa *et al*., 2007). In contrast, some organisms, such as the unicellular green alga *Chlamydomonas reinhardtii,* show linear mtDNAs that end with defined terminal structures, called mitochondrial telomeres (Vahrenholz *et al*., 1993).

Concerning the mtDNA size, the genomes are generally small, such as in humans in which the mtDNA is a circular double-stranded molecule of 16 kb (Anderson *et al*., 1981), whereas those of seed plants are much larger, ranging from 221 kb for the smallest mitochondrial genome sequenced to date (Handa, 2003) to more than 2,000 kb for the Cucurbitaceae or Silene families (Ward *et al*., 1981; Sloan *et al*., 2012). The size enlargement does not reflect an increase in the gene content, but rather additional noncoding sequences, such as introns, DNA repeat motifs, and inserted nuclear and chloroplast fragments (Knoop, 2004). For example, the mtDNA of *Arabidopsis Thaliana* is 22-fold larger than that of *Homo sapiens*, but its coding capacity is only 2.5-fold stronger (Unseld *et al*., 1997). In addition, seed plants possess a complex pool of frequently recombining molecules, of which the stoichiometry is controlled by nuclear genes (Arrieta-Montiel *et al*., 2009).

Even though the mtDNA varies in form and shape, as far as we know it plays the same fundamental role in all eukaryotes and it encodes a limited number of ribosomal (r)RNAs, transfer (t)RNAs, and proteins essential for respiration (Gray, 1999).

Mitochondrial diseases and defects: transformation capacity and incapacity

Mammals

In humans, mtDNA mutations are found in subunits of the respiratory chain complexes or in tRNAs and rRNAs (listed at www.mitomap.org) and are known to cause a wide array of clinical disorders related to defects in the energy metabolism. It is generally accepted that 1 on 3,500 to 6,000 individuals harbors mtDNA mutations (Taylor and Turnbull, 2005). There is increasing evidence from mammalian models (Trifunovic *et al*., 2004) that acquired mtDNA mutations and mitochondrial dysfunctions are involved in ageing and age-related diseases, such as diabetes (Petersen *et al*., 2003). However, the mtDNA mutations are difficult to analyze for the reasons cited below.

As the mitochondrial genome is present in multiple copies, some mutations can affect all the copies (homoplasmic mutation), whereas others occur only in some of the copies (heteroplasmic mutation). These heteroplasmic mutations can escape detection because the impact of the mutation might be hidden by the wild-type copies. In addition, the mitochondrial genome harbors large numbers of polymorphic base changes without pathogenic significance (DiMauro, 2007). Furthermore, mitochondrial diseases can also arise from nuclear gene disorders, because most proteins involved in the mitochondrial metabolism and all those involved in the mtDNA maintenance are encoded in the nucleus. Therefore, a system is highly needed and desirable that would allow us to study the mutations located in the mitochondrial genome and, hence,

Abbreviations used in this paper: mtDNA, mitochondrial DNA; ; rRNA, ribosomal RNA; tRNA, transfer RNA.

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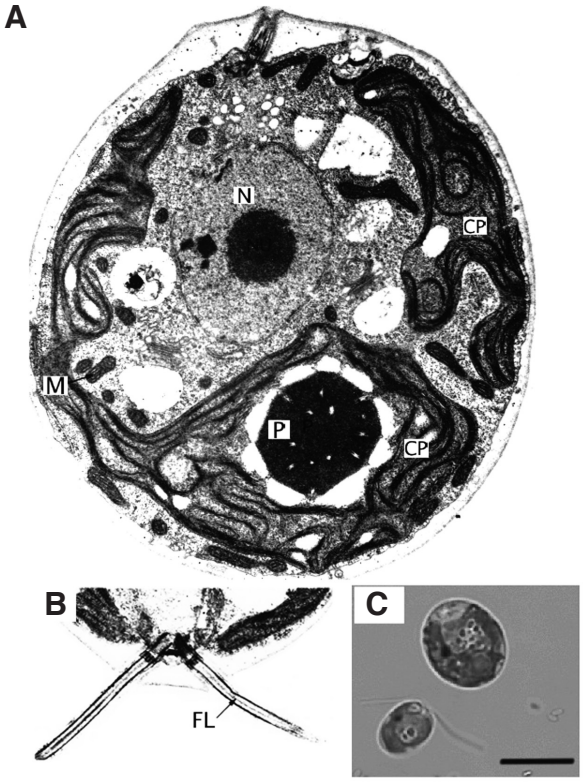

Fig. 1. Microscopic images of *C. reinhardtii*. (A) *Electron microscopy. CP, chloroplast; M, mitochondria; N, nucleus; P, pyrenoid (site of CO_2 fixation).* **(B)** *Electron microscopy. FL, flagella.* **(C)** *Optical microscopy. The black bar represents 10 μm.*

transform mtDNAs of mammalian cells.

In fact, in mammalian cells, small linear DNA fragments, such as oligonucleotides (Vestweber and Schatz, 1989) or double-stranded DNA (Seibel *et al.*, 1995; Koulintchenko *et al.*, 2006) can be transported into mitochondria (Chinnery *et al.*, 1999; Geromel *et al.*, 2001; Flierl *et al.*, 2003). Much enlarged plasmid DNA molecules have been introduced also into isolated mammalian mitochondria by electroporation (Collombet *et al.*, 1997), but the electroporation conditions used in these procedures appear to damage irreparably the mitochondria, thus preventing their reincorporation into the mammalian cells (Yoon and Koob, 2005). Introduction of an exogenous DNA into the mitochondria of human cells by means of a process called 'protofection', a system involving bacteria-to-mitochondria conjugation (Yoon and Koob, 2005), had been tried, but was inconclusive. Some successes were obtained by transfer of *in vitro* mitochondria from a patient, suffering from mitochondrial pathology, into cultured mtDNA-less human cells (Chomyn *et al.*, 1991; Hayashi *et al.*, 1991). Moreover, viable mitochondria, isolated from human somatic cells, were microinjected *in vitro* in mouse (*Mus musculus*) cells, but these analyses were not followed up (Irwin *et al.*, 1999; Takeda *et al.*, 2005). However, a promising approach is the use of adeno-associated viruses to express human mitochondrial genes in mice (Yu *et al.*, 2012a). By means of this technique, a human mitochondrial mutation responsible for vision loss, the Leber Hereditary Optic Neuropathy, has been introduced into the ocular mitochondria of mice (Yu *et al.*, 2012b) and expression and translation of the construction has been detected. Unfortunately, the construction did not seem to be integrated into the mitochondrial genome and, thus far, *in vivo* direct engineering of the mtDNA in mammalian mitochondria is still impossible.

Plants

Regarding plants, mitochondrial disorders have been identified also in subunits of the respiratory-chain complexes, leading to visible phenotypes, such as the cytoplasmic male sterility in tobacco (*Nicotiana sylvestris*) (Gutierres *et al.*, 1997) or the non-chromosomal stripe phenotype in maize (*Zea mays*) (Karpova and Newton, 1999). Although such genotypes might represent good candidates for mitochondrial transformation, it is still impossible to stably transform plant mitochondria. Like in mammals, *in vitro* import of DNA, electroporation of isolated mitochondria with DNA/RNA, and *in vivo* transformation experiments have been performed, but not successful (reviewed in Remacle *et al.*, 2012). The reasons for the failure are probably multiple and could be linked to the instability of the plant mitochondrial genomes and to the lack of strong selection markers that can be maintained throughout the development of the adult plant after the zygote formation. Indeed, in higher plants, subgenomes are found and their presence is due to high-frequency intermolecular and intramolecular recombinations on large repeated sequences. When these repeated sequences are in direct orientation, they allow the genome to be subdivided into a collection of molecules, each containing only a portion of the genetic information. These molecules of plant mitochondrial DNA can vary in copy number over time, a process called substoichiometric shifting (Small *et al.*, 1987; Janska *et al.*, 1998; Abdelnoor *et al.*, 2003) that leads to heteroplasmic complexity (Woloszynska, 2010). Silencing of genes that control such shifting, such as the DNA mismatch repair protein MSH1 that is homologous to mutator protein MutS in *Escherichia coli* (Abdelnoor *et al.*, 2003), might be a good approach to solve the problem of plant transformation.

Nevertheless, it is worth mentioning that manipulation of mitochondrial expression might be possible via the antisense strategy. Indeed, knockdown of the expression of the mitochondrial subunit 9 ATP-ase gene *ATP9* of *Arabidopsis* plants has been demonstrated via stable transformation of the nuclear genome. To this end, a chimeric catalytic RNA was constructed that consisted of a specifically designed *trans*-cleaving hammerhead ribozyme and a tRNA mimic targeting the construction to the mitochondria, because many cytosolic tRNAs are imported to the mitochondria to ensure proper mitochondrial translation in seed plants (Val *et al.*, 2011).

In conclusion, even though technologies to study mitochondria, such as isolation of mitochondria, biochemical and microscopic analyses, and genome sequencing have evolved and been improved, *in vivo* and stable mitochondrial transformation is still feasible in two unicellular organisms only, namely the budding yeast *Saccharomyces cerevisiae* (Fox *et al.*, 1988; Johnston *et al.*, 1988) and the green alga *Chlamydomonas reinhardtii* (Remacle *et al.*, 2006). High-efficiency transformation methods relying on biolistic delivery have been developed, in which respiratory-deficient mutants serve as recipient strains for mitochondrial transformation.

Mitochondrial transformation of *Saccharomyces cerevisiae*

In *S. cerevisiae*, the mitochondrial transformation process is possible thanks to the fact that DNA sequences can be delivered

into the yeast mitochondria by microprojectile bombardment (biolistic transformation) and be incorporated successively into the mtDNA by the highly active homologous recombination machinery present in the organelle (Bonnefoy and Fox, 2007). As recipient strains, *S. cerevisiae* cells that lack part (*rho*–) or all (*rho*0) of the mtDNA are routinely used for transformation. In fact, *rho*– mtDNAs replicate independently of the protein synthesis and do not require a specific replication origin sequence. These strains can be transformed with bacterial plasmid DNAs that subsequently propagate defined mtDNAs as 'synthetic' *rho*– molecules (Fox *et al.*, 1988) or can be transformed also with linear DNA molecules obtained either from plasmid clones or polymerase chain reaction (PCR) amplification (Bonnefoy and Fox, 2002). Alternatively, when a mutated DNA sequence provides a function that can be selected phenotypically, direct transformation of *rho*+ strains bearing deletions in the region of interest can be used to integrate mutations into the mtDNA (Bonnefoy and Fox, 2000; Bonnefoy *et al.*, 2001). In yeast, auxotrophic markers that correspond to nuclear genes encoding mitochondria-targeted enzymes are attractive for the development of a selection method. Indeed, expression of the biosynthetic arginine gene *ARG8* from the mitochondrial genome allows nuclear *arg8* mutants to grow without arginine (Steele *et al.*, 1996). The ARG8 protein is normally imported into mitochondria from the cytoplasm, but also functions when synthesized within the organelle in the mitochondrial transformants. Thus, arginine prototrophy might become a mitochondrial gene expression-dependent phenotype dependent.

Mitochondrial transformation of *C. reinhardtii*

Principles

The second organism in which stable mitochondrial transformation is feasible is *C. reinhardtii*, also called green yeast (Fig. 1). This two-flagellar microalga is 10 μm long and consists of a haploid nuclear genome of 120 Mb (Merchant *et al.*, 2007), a very large horseshoe-shaped chloroplast containing circular DNA molecules of 220 kb (Maul *et al.*, 2002), and small mitochondria containing linear DNA molecules of 15.8 kb. These molecules are terminated by telomeres made of inverted repeats of approximately 500 bp (Vahrenholz *et al.*, 1993). Thirteen genes are present and encode five subunits of the NADH:ubiquinone oxidoreductase or complex I (*nd1*, *nd2*, *nd4*, *nd5,* and *nd6*), apocytochrome *b* of the bc_1 complex or complex III (*cob*), subunit 1 of cytochrome *c* oxidase or complex IV (*cox1*), a reverse transcriptase-like protein (*rtl*), three tRNAs (*trnW*, *trnQ*, and *trnM*), and the rRNAs (Fig. 2A). The rRNA genes are discontinuous and split into mini-sequences that encode four small subunit (*S*) and eight large subunit (*L*) rRNA modules, interspersed with one another and with protein and tRNA genes. The small rRNA segments of the two ribosomal subunits are believed to interact through an extensive intermolecular pairing to form conventional rRNA molecules (Boer and Gray, 1988).

The mitochondrial genome is a multicopy system of approximately 50-100 copies organized into 20–30 nucleoids (Hiramatsu *et al.*, 2006). The nucleoids and the mitochondria seem extremely dynamic and undergo changes in size and shape during the cell cycle (Hiramatsu *et al.*, 2006).

The first successful mitochondrial transformation in *Chlamydomonas* was a dark uniparental minus (*dum1*) mutant (Fig. 2B) in which the left telomere and *cob* gene (1.5 kb deletion) were deleted. Such a respiratory mutant, isolated after random mutagenesis with acriflavine (Remacle *et al.*, 2001), has lost the capacity to grow under heterotrophic conditions, i.e. in the dark, with acetate as carbon source, because it lacks the cytochrome pathway of respiration (Fig. 2C). In this first round of transformation experiments, the respiratory competence was restored in *dum1* by means of a biolistic device and partially purified mtDNA from *C. reinhardtii* or *C. smithii* (Randolph-Anderson *et al.*, 1993). Later, biolistics was again used successfully to transform the same recipient strain with purified mtDNA or cloned mtDNA fragments (Yamasaki *et al.*, 2005). In both cases, the wild-type mitochondrial sequence of the transformed DNA replaced the deleted genome in the transformants selected under heterotrophic conditions (dark+acetate) and the transformation efficiency was low (0.4–3 transformants/μg DNA).

Subsequently, the biolistic transformation was optimized by using cloned mtDNA or PCR fragments as transforming molecules (Remacle *et al.*, 2006) and a deletion mutant carrying a 1.2-kb deletion, including the left telomere and part of the *cob* gene (*dum11*) (Fig. 2B). This mutant also lacks the cytochrome pathway

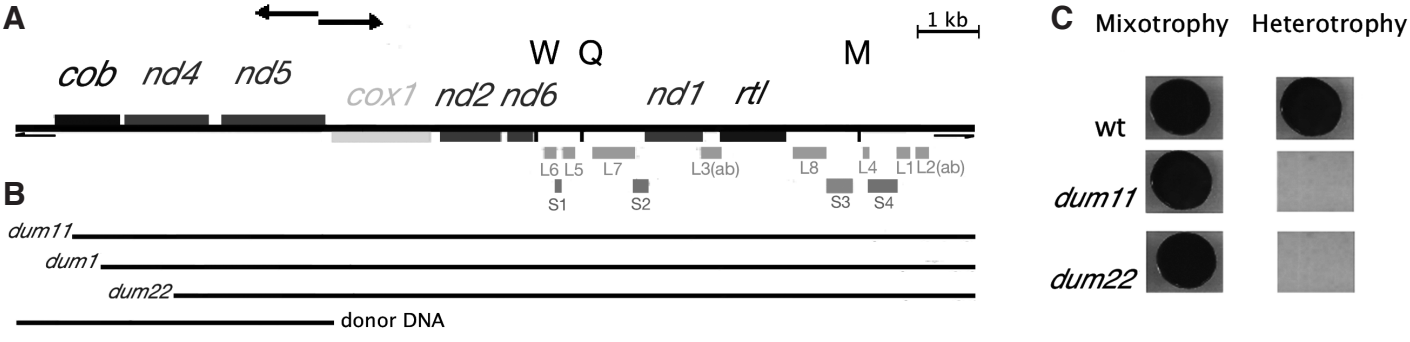

Fig. 2. Mitochondrial genome and mutants of *C. reinhardtii.* (A) *Physical map of the 15.8-kb linear mitochondrial genome (GenBank accession U03843). The rectangles represent protein-coding genes:* cob*, gene encoding apocytochrome* b *of complex III;* nd1, nd2, nd4, nd5, *and* nd6*, genes encoding the corresponding subunits of complex I;* cox1*, gene encoding the subunit 1 of complex IV;* rtl*, gene encoding a reverse transcriptase-like protein;* W, Q, *and* M*, tRNA genes encoding tryptophan, glutamine, and methionine, respectively. L1-L8 and S1-S4: rRNA gene fragments of the large (L) and small (S) subunits. Short and long arrows indicate the inverted telomeric ends and the bidirectional origin of transcription between* nd5 *and* cox1*, respectively.* **(B)** *Schematic represention of the deleted mitochondrial genome from the* dum1, dum11, *and* dum22 *mutants and one DNA fragment used during the mitochondrial transformation.* **(C)** *Growth phenotype of the* dum11 *and* dum22 *mutants under mixotrophic (light + acetate) and heterotrophic (dark + acetate) conditions.*

of respiration and could not grow under heterotrophic conditions (Fig. 2C). Mitochondrial transformants obtained from *dum11* could be rescued after selection in the dark with a mtDNA fragment covering the deletion and the *cob/nd4/nd5* genes as donor DNA (Fig. 2B). Homologous recombination occurred between the introduced DNA and the endogenous mitochondrial genome and homologous sequences as short as 28 nucleotides could recombine (Remacle *et al*., 2006). The mitochondrial transformants were homoplasmic for the 15.8 kb wild-type genome. Moreover, high transformation efficiency was achieved (100-250 transformants/μg DNA) and the best results were obtained with linearized plasmid DNA.

Using the strategy described above, we were able to introduce nondeleterious mutations and also loss-of-function molecular lesions into the mitochondrial genome. Myxothiazol-resistant transformants were generated by introducing the *mud2* mutation at codon 129 that is present in the *cob* gene of the strains displaying myxothiazol resistance (Remacle *et al*., 2006). Similarly, an in-frame deletion of 23 codons was reconstructed in the *nd4* gene (Remacle *et al*., 2006). During selection in the dark, recombination events resulted in *cob* gene cointegration and *nd4* deletion in some molecules of the mitochondrial genome, despite the negative effects of the *nd4* mutation on the complex-I assembly and activity and on the total cellular respiration (Remacle *et al*., 2006). After a 2-month selection in the dark, heteroplasmy could still be detected in some transformants, suggesting that the segregation process of the mitochondrial molecules was extremely slow. To circumvent the heteroplasmy problem, we used a mutant with a deletion extending up to *nd4* (*dum22*) (Fig. 2B) (Remacle *et al*., 2001) to force mutations to be inserted into *nd4* by recombination. The *dum22* mutant depends totally on glycolysis and chloroplasts for ATP formation, because it lacks the three phosphorylating enzymes of the respiratory chain (complexes I, III, and IV). This mutant has proven to be a good recipient strain to isolate homoplasmic *nd4* transformants, although the transformation efficiency is very low (1-2 transformants/μg DNA) (see below).

Applications

These results opened the way to reverse genetics in *Chlamydomonas* mitochondria. The three recipient strains cited above, *dum1*, *dum11,* and *dum22*, were efficiently used in transformations (i) to reconstruct a human mitochondrial complex-I mutation in *Chlamydomonas*, (ii) to elucidate the tRNA import in mitochondria, and (iii) to express transgenes in mitochondria.

Reconstruction of a human mitochondrial complex-I mutation in Chlamydomonas

As described above, the pathogenicity of a given mitochondrial mutation can be difficult to analyze because the mitochondrial genome harbors large numbers of polymorphic base changes without pathogenic significance (DiMauro, 2007). In addition, mitochondrial mutations are usually found in the heteroplasmic state, by which the biochemical effects of the mutation could be hidden. Human mitochondrial mutations can be reconstructed in the *S. cerevisiae* (Blakely *et al*., 2005), except for complex-I mutations because it lacks complex I and oxidizes NADH via a monomeric type-II NADH dehydrogenase. As a substitute, bacterial systems have been used to reconstruct human pathogenic mutations (Blakely *et al*., 2005; Torres-Bacete *et al*., 2007); however, they are not ideal, because the membrane domain of the eukaryotic complex I is much more complex than the corresponding arm of the bacterial complex I (28 subunits versus 7 subunits). Therefore, we proposed that *Chlamydomonas* could represent an attractive eukaryotic system to study such mutations (Larosa *et al*., 2012) because (i) respiratory-deficient mutants are viable and mitochondrial mutations are found in the homoplasmic state (Remacle *et al*., 2001), (ii) transformation of the mitochondrial genome is feasible (Remacle *et al*., 2006), and (iii) the *Chlamydomonas* complex I is close to that of humans (Cardol, 2011). Indeed, of the 42 subunits of the *Chlamydomonas* complex I, 40 are homologous to those of the human complex I (Cardol, 2011). As an illustration, a Leu_{157}Pro (Fig. 3A) substitution was introduced into the ND4 subunit of the *Chlamydomonas* complex I of two different recipient strains (*dum11* and *dum22*) by biolistic transformation. This substitution did not lead to any defective respiratory enzymes when occurring at the heteroplasmic state in a patient presenting chronic progressive external ophthalmoplegia. When present at the homoplasmic state in the alga, the mutation did not prevent the assembly of the 950-kDa whole complex I that conserves nearly all the NADH dehydrogenase activity of the peripheral arm, but the NADH:duroquinone oxidoreductase activity was strongly reduced. Due to its nature, the introduced proline could disturb the organization of the transmembrane domain (Fig. 3B), in which the substitution is found and affects the ubiquinone fixation to the membrane domain. The *in vitro* defects were correlated *in vivo* with decreased respiration and growth rates.

Coevolution of mitochondrial tRNA Import and codon usage determines translational efficiency in Chlamydomonas

The mitochondrial translational machinery of *Chlamydomonas* strongly depends on cytosolic tRNA import, because only three tRNA genes are encoded by the mitochondrial genome (Fig. 2). Indeed, of the 49 cytosolic tRNA isoacceptors, 34 are found in mitochondria (Vinogradova *et al*., 2009). In addition, this import is very specific because only necessary cytosolic tRNAs are introduced. The mitochondrial localization extent for each tRNA species is highly

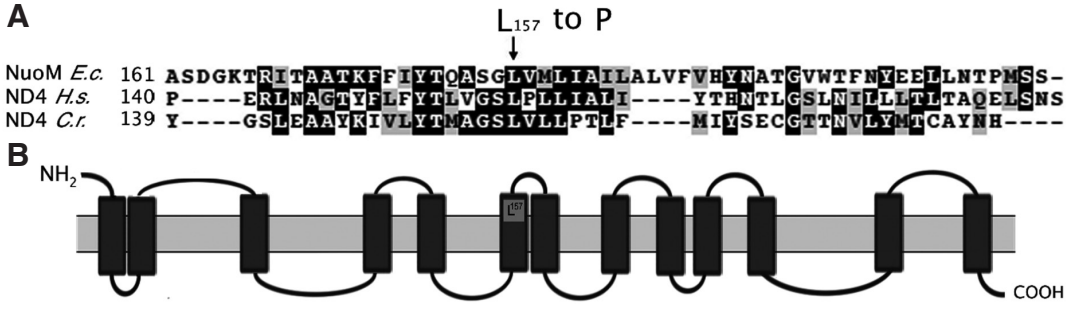

Fig. 3. ND4 subunit topology and alignment. (A) *Partial alignment between the* Escherichia coli *NuoM subunit, humane, and* C. reinhardtii *ND4 subunit. Arrow marks the leucine (at position 157 in* Chlamydomonas*) substituted by a proline.* **(B)** *Hypothetical model for the folding of the* Chlamydomonas *ND4 subunit, including 13 putative transmembrane helices. The Leucine$_{157}$ is highlighted in the sixth helix.*

variable: the percentage for most of them ranges from 0.2% to 26%, whereas three cytosolic tRNAs are nearly exclusively located inside the mitochondria. The correlation of the import efficiency with the mitochondrial codon usage (Vinogradova *et al.*, 2009) led to the hypothesis that the tRNA import might be a dynamic process capable of adaptation to the mitochondrial genome content. By manipulating the *Chlamydomonas* mitochondrial genome with the two recipient strains *dum11* and *dum22*, we introduced point mutations and found that the codon usage modifications resulted in reduced mitochondrial translation levels and in subsequently decreased respiratory complex levels and activities. These effects were linked to the consequential limitations of the tRNA pools in mitochondria. This observation indicated that the tRNA mitochondrial import cannot be regulated rapidly in response to a novel genetic context and, thus, is seemingly not a dynamic process, but rather suggested that the steady-state levels of imported tRNAs in mitochondria result from a coevolutive adaptation between the tRNA import mechanism and the requirements of the mitochondrial translation machinery (Salinas *et al.*, 2012).

Transgene expression in Chlamydomona mitochondria

The gene encoding the enhanced green fluorescent protein *egfp* was inserted between the *cob* gene and the left terminal repeat, with the *dum1* strain as recipient. The *egfp* gene was successfully expressed in the mitochondria of the transformants, as demonstrated by confocal microscopy and Western blot analyses (Hu *et al.*, 2011). Similarly, the zeomycin resistance conferring gene *ble* from *Streptoalloteichus hindustanus* was inserted at the same site and with the same recipient strain. After 2 months of selection in the dark, the *ble* gene was expressed and the transformants grew on zeomycin. However, the concentrations used were much lower (3-5 μg/ml) than those usually reported for algae, yeasts, and mammals (100 μg/ml) (Hu *et al.*, 2012). Nevertheless, the expression of the *ble* gene in mitochondria could be developed as a resistance marker for transgenic expression of other kinds of recombinant proteins in *Chlamydomonas* mitochondria.

Perspectives

Our century tends to study genes and their engineering. Indeed, forward and reverse genetics approaches are currently developed to address a lot of biological questions (Barbieri *et al.*, 2011; Huang *et al.*, 2012; Rahrmann *et al.*, 2013). Moreover, the evolution and the democratization of high-throughput sequencing enriched such a research.

Thus far, genome manipulations can be achieved in all organisms, except for mitochondrial transformation that is still a challenge in multicellular eukaryotes. The complexities of these organisms and the absence of selection criteria are major obstacles that need to be solved.

Concerning the mitochondrial transformation of *Chlamydomonas*, an obvious limitation is the selection process that requires a 2-month incubation period in the dark before any molecular characterization can be performed. Although the expression of antibiotic resistance genes in the mitochondria is attractive, it is still necessary to apply the dark selection before addition of the antibiotic to the culture (Hu *et al.*, 2011, 2012). Therefore, the development of a more rapid selection using phototrophic growth for the generation of mitochondrial transformants is a high priority. Currently, we are testing several strategies in our laboratory, such as cotransformation and new recipient strains affected both in chloroplasts and mitochondria to maximize the light dependence of the cellular respiration.

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