Chapter 19

Transformation and Nucleic Acid Delivery to Mitochondria

Claire Remacle*, Veronique Larosa, and Thalia Salinas
Laboratory of Genetics of Microorganisms, Institute of Botany B22, University of Liège, B-4000 Liège, Belgium

Patrice Hamel, and Nitya Subrahmanian
Department of Molecular Genetics and Department of Molecular and Cellular Biochemistry, 500 Aronoff Laboratory, 318 W. Ave, Columbus, OH 43210, USA and Plant Cellular and Molecular Biology Graduate Program, The Ohio State University, Columbus, OH USA

Nathalie Bonnefoy
Centre National de la Recherche Scientifique, Centre de Génétique Moléculaire FRE3144, FRC3115, Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France

Frank Kempken*
Abteilung Botanische Genetik und Molekularbiologie, Botanisches Institut, Christian-Albrechts-Universität zu Kiel, Olshausenstr. 40, D-24098 Kiel, Germany

Summary 444
I. Mt Transformation 444
A. Introduction 444
B. Mt Transformation in the Unicellular Green Alga *Chlamydomonas* 446
1. The Mt Genome of *Chlamydomonas* 446
2. The Mt Mutants of *Chlamydomonas* 446
3. Recombination and Segregation of mt DNA 447
4. Mt Transformation 447
5. Toward a Selection Independent of the Restoration of Heterotrophic Growth 448
II. DNA and RNA Delivery into Plant Mitochondria 449
A. Cytosolic tRNA Import into Plant Mitochondria 449
B. In Vitro Import of DNA 450
C. Electroporation of Isolated Mitochondria with DNA and RNA 450

*Authors for correspondence, e-mail:c.remacle@ulg.ac.be; fkempken@bot.uni-kiel.de

R. Bock and V. Knoop (eds.), Genomics of Chloroplasts and Mitochondria, Advances in Photosynthesis and Respiration 35, pp. 443–458,
DOI 10.1007/978-94-007-2920-9_19, © Springer Science+Business Media B.V. 2012

D. In organello Analysis 452
1. DNA Replication 452
2. Transcription and RNA Processing 452
3. Translation 453
III. Conclusion 453
Acknowledgements 454
References 454

Summary

Genomic, transcriptomic and proteomic approaches have yielded considerable information, which impacted our understanding of the interactions between the nucleus and the mitochondria. Plant mitochondrial (mt) genomes are very large (220–2,000 kb) and often occur as complex pools of recombined molecules whose stoichiometry is tightly controlled by the nucleus. Unlike their mammalian and fungal counterparts, plant mt transcripts undergo complex post-transcriptional modifications such as editing and trans-splicing. Due to the impossibility to stably transform plant mitochondria and hence to manipulate mt gene expression, the genetic regulation of plant mt genomes has remained poorly understood. In this chapter, we will review the experimental data concerning the unicellular green alga *Chlamydomonas reinhardtii*, the only photosynthetic organism for which mt transformation has been achieved. Although *Chlamydomonas* harbors an extremely compact linear mt genome (15.8 kb) that differs from the one typically found in vascular plants, this system could bring novel insights on the role of the few subunits of the respiratory chain that are encoded in the mt genome. This is particularly relevant for the nd genes, which encode subunits of complex I since the yeast *Saccharomyces cerevisiae*, the other unicellular organism where mt transformation is performed nearly at will, is deprived of complex I. Moreover, because the *Chlamydomonas* mt genome only encodes three tRNAs, genetic manipulation of the organellar genome is a promising avenue to dissect the import of cytosolic tRNAs, a process that is now known to take place in plant and also human mitochondria. We also present alternative approaches such as the in vitro import of DNA or RNA and electroporation of isolated mitochondria followed by *in organello* synthesis that have been developed. These approaches have generated fruitful information about transcription and post-transcriptional processing of plant mt RNAs.

I. Mt Transformation

A. Introduction

Two types of mt DNA are present in the embryophytes (land plants). The mitochondria of the three clades of bryophytes (liverworts, hornworts and mosses, see chapter "Promiscuous Organellar DNA") possess circular genomes of around 105–185 kb resembling that of their green algal relatives like *Chara* and even the early-branching nongreen eukaryote, *Reclinomonas americana* (Li et al. 2009). They are therefore considered to be of the ancestral type despite the acquisition of several additional features such as moderate size increase, intron gain and RNA editing, which are generally absent in algal genomes (Wang et al. 2009). On the contrary, the mitochondria of seed plants (see Chap. 10 "Horizontal Gene Transfer in Eukaryotes: Fungi-to-Plant and Plant-to-Plant Transfers of organellar DNA") harbor

Abbreviations: mt – Mitochondrial; tRNA – Transfer RNA; VDAC – Voltage-dependent anion channel

much larger genomes, from 221 kb for the smallest mt genome sequenced to date (Handa 2003) to more than 2,000 kb in the Cucurbitaceae family (Ward et al. 1981). The size increase does not reflect an increase of the gene content but addition of noncoding sequences such as introns, DNA repeat motifs and insertion of nuclear and chloroplast fragments (Knoop 2004). In addition seed plants possess a complex pool of frequently recombining molecules, the stoichiometry of which is controlled by nuclear genes (Abdelnoor et al. 2003; Arrieta-Montiel et al. 2009). Plant mt transcription is also complex and plant mt RNA transcripts undergo unique post-transcriptional modifications such as editing and trans-splicing (see section "DNA and RNA Delivery into Plant Mitochondria" of this chapter). Investigations of such processes remain difficult due to the impossibility to stably transform plant mitochondria and to manipulate mt gene expression. A plant mt transformation system would therefore be of great value.

Several significant seed plant mtDNA mutations (see chapter "Expression Profiling of Organellar Genes") were identified. Among them, some are found in subunits of the respiratory-chain complexes and are responsible for visible phenotypes such as the cytoplasmic male sterility in tobacco (Pla et al. 1995; Gutierres et al. 1997; Pineau et al. 2005) or the nonchromosomal stripe phenotype in maize (Karpova and Newton 1999; Kubo and Newton 2008).

In principle, and similarly to the situation in other unicellular organisms like the yeast *Saccharomyces cerevisiae* (Fox et al. 1988; Johnston et al. 1988) and in the green alga *Chlamydomonas* (see below for details), such respiratory deficient mutants could serve as recipient strains for mt transformation. A few years ago, cucumber was described as a promising system for mt transformation for several reasons: (1) the mt genome is inherited by the paternal parent and microspores possess a few huge mitochondria that could be transformed before ovule fertilization, (2) mutants with mt deletions, responsible for a mosaic phenotype, exist and could serve as non-reverting recipient strains for mt transformation (Havey et al. 2002). However, despite several attempts, no successful transformation of mitochondria in cucumber or any plant system has been reported to date. The reasons for the failure are probably multiple: (1) the lack of a method to transform either microspores or pollen cells or the ovule for plants with maternal transmission of mitochondria; (2) the fact that plant mt genomes are extremely complex and unstable and can rapidly evolve via substoichiometric shifting due to recombination (Small et al. 1987, 1989; Mackenzie 2007); (3) the lack of a selection that is maintained throughout the formation of the adult plant after zygote formation. Auxotrophic markers corresponding to nuclear genes encoding enzymes targeted to mitochondria are attractive for the development of a selection method. In yeast, expression of the synthetic gene $ARG8^{m}$ from the mt genome allows nuclear *arg8* mutants to grow without arginine (Steele et al. 1996). The Arg8p protein is normally imported into mitochondria from the cytoplasm, but also functions when synthesized within the organelle in the mt transformants. Thus, arginine prototrophy can become a phenotype dependent on mt gene expression.

Unfortunately, there are very few auxotrophic markers that could function within mitochondria of plants. For example, the *Chlamydomonas* or *Arabidopsis* ortholog of *ARG8* cannot be used as a mitochondrial marker since it actually encodes a plastid protein (Remacle et al. 2009). One notable exception is the BIO2 protein, an iron-cluster enzyme responsible for the last step of biotin synthesis inside the mitochondria. The *bio2* mutants defective for the BIO2 protein have been characterized in *Arabidopsis* and they survive if supplemented with biotin (Patton et al. 1998; Arnal et al. 2006). The *bio2* mutants may represent a promising system for mitochondrial transformation, by transforming either protoplasts with subsequent regeneration of plants or the ovule before pollen fertilization, using as selection a medium devoid of biotin.

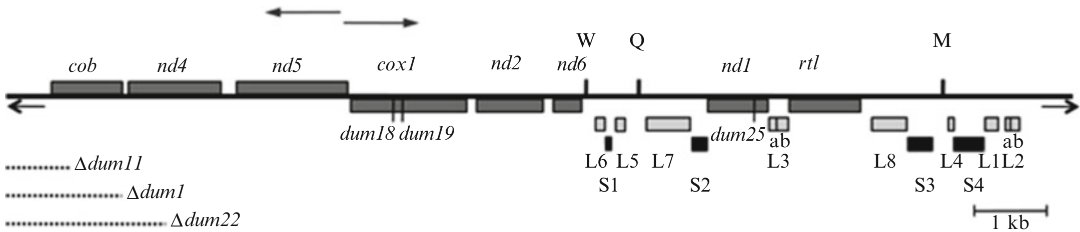

Fig. 19.1. Physical map of the 15.8 kb mt genome of *C. reinhardtii*. The *rectangles* represent protein-coding genes: *cob*, gene encoding *apocytochrome b* of complex III; *nd1*, *2*, *4*, *5*, and *6*, genes encoding the corresponding subunits of complex I; *cox1*, gene encoding subunit 1 of complex IV, *rtl*: reverse transcriptase-like protein. L and S represent modules encoding segments of rRNAs of the large and the small ribosomal subunits, respectively. W, Q, and M represent tRNAs for Trp, Gln, and Met, respectively. The inverted telomeric ends are represented by *short arrows* and the bidirectional origin of transcription between *nd5* and *cox1* by *longer arrows*. Positions of the *dum1*, *dum11* and *dum22* deletions and of the *dum18*, *dum19* and *dum25* mutations are indicated.

B. Mt Transformation in the Unicellular Green Alga Chlamydomonas

1. The Mt Genome of Chlamydomonas

The mt genome of *C. reinhardtii* is a 15.8-kb linear molecule containing at each extremity telomeres corresponding to inverted repeats of about 500 bp, with 40-bp single-stranded extensions (Vahrenholz et al. 1993). Thirteen genes that 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*, *trnM*) and the ribosomal RNAs (rRNAs) are present in the mt genome (Fig. 1). The rRNA genes are discontinuous and split into mini-sequences encoding 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 by way of extensive intermolecular pairing between one another to form conventional rRNA molecules (Boer and Gray 1988).

The mt genome is a multicopy system of around 50–100 copies organized into about 20–30 nucleoids (Nishimura et al. 1998; Hiramatsu et al. 2006). The nucleoids and the mitochondria seem extremely dynamic and undergo changes in their size and shape during the cell cycle (Ehara et al. 1995; Hiramatsu et al. 2006).

2. The Mt Mutants of Chlamydomonas

Several mutations altering the mt *cob*, *cox1* and *nd* genes have been isolated following random mutagenesis with acriflavine (Remacle et al. 2001b). Phenotypically, the mutants in the *cob* and *cox1* genes have lost the capacity to grow under heterotrophic conditions i.e. in the dark, with acetate as carbon source because they lack the cytochrome pathway of respiration. In contrast, mutants altered in the *nd* genes, which encode subunits of complex I, are able to grow in the dark, but considerably more slowly than the wild-type strain (Remacle et al. 2001a; Cardol et al. 2002). Most of the mutations located in the *cob* gene are deletions covering not only the coding sequence but also the left telomere, whereas mutations in the *cox1* and *nd* genes are usually frameshifts caused by deletion or insertion of one thymidine (Remacle et al. 2001b). In addition, the *mud2* mutation at codon 129 (Phe TTC → Leu CTC) of the *cob* gene confers resistance to myxothiazol and mucidine, inhibitors of the cytochrome bc_1 complex (Bennoun et al. 1991).

All the mt point mutants studied so far were found to be homoplasmic, i.e. they contain only mutated mt genomes. However, the

mt DNA present in the deletion mutants exists as a mixture of both deleted monomers and dimers arising from head-to-head fusions between deleted monomers (Matagne et al. 1989; Dorthu et al. 1992; Duby and Matagne 1999). The total amount of mt DNA in such mutants is generally lower than in the wild type. The deletion mutants do not revert and are ideally suited as recipients for mt transformation experiments.

3. Recombination and Segregation of mt DNA

In *Chlamydomonas*, homologous mt DNA recombination is only detected after crosses between *mt*$^+$ and *mt*$^-$ strains in mitotic zygotes that do not undergo meiosis. In such zygotes, mt DNA is transmitted by both parents and recombination between the parental genomes is frequent (Remacle et al. 1990; Remacle and Matagne 1993). This demonstrates that enzymes involved in homologous recombination are active in *Chlamydomonas* mitochondria, an important feature for mt transformation as stable integration of the transforming DNA relies on recombination. Segregation of the mt genomes occurs in the zygotes and their mitotic progeny and after 15–20 divisions, most of the diploid cells are homoplasmic for a mt genome, either recombined or parental.

4. Mt Transformation

a. Deletion Mutants as Recipient for mt Transformation

In the first report of mt transformation in *Chlamydomonas*, a mutant (*dum1*) deleted for the left telomere and *cob* gene (1.5 kb deletion) was successfully transformed to respiratory competence with partially purified mt DNA from *C. reinhardtii* or *Chlamydomonas smithii* using a biolistic device (Randolph-Anderson et al. 1993). *C. reinhardtii* and *C. smithii* are two interfertile species, which harbor identical mt genomes with the exception of a 1-kb group I intron located in the *cob* gene that is present in *C. smithii* but absent in *C. reinhardtii* (Boynton et al. 1987; Remacle et al. 1990). Later, biolistics was again used successfully to transform the same recipient strain, with purified mt DNA or cloned mt DNA fragments (Yamasaki et al. 2005). In both cases, the wild-type mt sequence of the transforming DNA had replaced the deleted genome in the transformants selected under heterotrophic conditions (dark+acetate). Transformation efficiency was low (0.4–3 transformants/μg DNA). This precluded any genetic manipulation of the mt genome, since isolation of transformants with the desired genotype usually requires the screening of many colonies, as this is also the case in *Saccharomyces* mt transformation (Bonnefoy et al. 2007).

Subsequently, biolistic transformation was optimized using cloned mt DNA or PCR fragments as transforming molecules (Remacle et al. 2006). Another deletion mutant carrying a 1.2 kb deletion including the left telomere and part of the *cob* gene (*dum11*) could be rescued after selection in the dark using a mt DNA fragment covering the deletion and the *cob* gene as donor DNA (Fig. 1). Homologous recombination occurred between the introduced DNA and the endogenous mt genome and homologous sequences as short as 28 nucleotides could direct recombination (Remacle et al. 2006). Mt transformants were homoplasmic for the 15.8 kb wild-type genome and did not exhibit the dimeric forms of the mt genome that were present in the recipient strain. Moreover, a high transformation efficiency was achieved (100–250 transformants/μg DNA), the best results being obtained with linearized plasmid DNA.

Interestingly, a strain lacking the *cob* gene and the left telomere could be rescued, although at a very low rate, when the transforming DNA is nearly completely devoid of the left telomere (Remacle et al. 2006). This indicated that the right telomere can be copied to reconstruct the left telomere by recombination. Using the strategy described above, we were able to introduce non-deleterious mutations and also loss-of-function molecular lesions in the mt genome. Myxothiazol-resistant

transformants were generated by introducing the nucleotide substitution 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 with a frequency of one homoplasmic *nd4* transformant among 90 transformants analyzed (Remacle et al. 2006). During selection in the dark, recombination events resulted in the co-integration of the *cob* gene and the deletion in *nd4* in some molecules of the mt genome despite the negative effect of the *nd4* mutation on complex I assembly and activity as well as on whole cell respiration (Fig. 1) (Remacle et al. 2006). These results open the way to reverse genetics in *Chlamydomonas* mitochondria and more specifically, to site-directed mutagenesis of mitochondrially encoded subunits of complex I (ND subunits). This is of special interest because the yeast *S. cerevisiae*, whose mt genome can be manipulated virtually at will, lacks complex I.

After a 2 month selection in the dark, heteroplasmy could still be detected in some transformants, suggesting that the segregation process of the mt molecules was extremely slow. To circumvent the problem of heteroplasmy, we recently used a deletion mutant with a deletion extending up to *nd4* (the *dum22* mutant) (Remacle et al. 2001b) to force the insertion of mutations into *nd4* by recombination. The *dum22* mutant is absolutely dependent on glycolysis and the chloroplast for ATP formation since it lacks the three key protein complexes of the respiratory chain (complex I, complex III and IV). This mutant has proven to be a good recipient strain for isolation of homoplasmic *nd4* transformants, although transformation efficiency is very low (1–2 transformants/μg of DNA). For example, using the *dum22* mutant as recipient strain, we have been recently able to isolate two homoplasmic transformants affected in *nd4*. One transformant bears a point mutation (Leu TTG → Pro CCA) corresponding to a human disease (Chronic Progressive External Ophthalmoplegia) at codon 158 of *nd4* (V. Larosa, unpublished). The other one has been created to study mt codon usage and bears a set of 11 codons, which differ from the usual mt codon usage (T. Salinas and C. Remacle, unpublished). It is worth mentioning that a similar transformant remained heteroplasmic when using the *dum11* mutant only affected in *cob* as a recipient strain (T. Salinas, unpublished).

b. Frameshift Mutants as Recipients for mt Transformation

Point mutants can also be used for mt transformation. A double frameshift mutant in both the *cox1* and *nd1* genes (*dum19 dum25*) (Fig. 1) could be rescued for heterotrophic growth and the 23 codon in-frame deletion in *nd4* cited above could be reconstructed (Remacle et al. 2006). Another frameshift mutant (*dum18*) in the *cox1* gene was also employed as a recipient strain for transformation (Colin et al. 1995). This mutant seems best suited for site-directed mutagenesis of the *nd* genes since the *cox1* gene lies between the *nd4* and *nd5* genes on one end and the *nd2* and *nd6* genes on the other end of the genome (Fig. 1). Unfortunately, the high frequency of reversion of this frameshift mutation precluded the use of this strain as a recipient for transformation experiments.

5. Toward a Selection Independent of the Restoration of Heterotrophic Growth

An obvious limitation to mt transformation in *Chlamydomonas* is the selection process that requires a 2-month incubation period in the dark before any molecular characterization can be performed. The development of a more rapid selection using phototrophic growth for the generation of mt transformants is therefore a high priority. We first tested a co-transformation strategy using a primary selection in the light, by bombarding the recipient strain with a plasmid carrying a nuclear marker and another construct containing the mt DNA of interest. Nuclear transformants were first selected in the light and then tested for their respiratory competence to detect mt transformation events

(Remacle et al. 2006). This type of selection, commonly used for yeast transformation (Bonnefoy et al. 2007), was not successful when applied to *Chlamydomonas*, presumably because simultaneous transformation of the nuclear and mitochondrial genomes is a extremely rare event (Remacle et al. 2006). A novel method of selection for mt transformation based on the use of the maize URF13 protein is currently being tested. URF13 is a chimeric mt inner membrane protein that arose from the recombination of mt molecules (Hanson 1991). The presence of URF13 in the mitochondria causes cytoplasmic male sterility and susceptibility to methomyl, an insecticide commonly used for crops (Hanson 1991; Levings and Siedow 1992; Rhoads et al. 1995). Interestingly, the expression of a mitochondrially targeted URF13 in the fungi *S. cerevisiae* and *Pichia pastoris* also confers methomyl sensitivity (Glab et al. 1990; Huang et al. 1990; Soderholm et al. 2001). A *Chlamydomonas* recipient strain carrying a codon-optimized version of URF13 in place of the *nd4* gene will be created by biolistic transformation using dark selection (*nd4::URF13*). We expect the *nd4::URF13* strain to be methomyl sensitive (and deficient for complex I) based on the fact that expression of URF13 in the mitochondria of *Saccharomyces*, *Pichia* and tobacco confers this trait (Glab et al. 1990; Huang et al. 1990; von Allmen et al. 1991; Chaumont et al. 1995; Soderholm et al. 2001). As a proof of concept, the methomyl sensitive *nd4::URF13* recipient strain will be transformed with a construct containing the wild-type *nd4* gene and selection of methomyl resistant transformants will be attempted in the light. Such transformants are expected to arise from homologous recombination between the *nd4::URF13* region in the mt DNA and the transforming DNA carrying the wild-type *nd4* gene. If such a selection is successfully established, mutations in the *nd4* gene resulting in complex I deficiency in humans will be reconstructed in the *Chlamydomonas* mt genome of the *nd4::URF13* strain using the methomyl resistance selection. The same methodology can be applied to manipulate other mt *nd* genes.

II. DNA and RNA Delivery into Plant Mitochondria

A. Cytosolic tRNA Import into Plant Mitochondria

Mitochondria perform protein biosynthesis. Therefore, they require a complete set of transfer RNAs (tRNAs). The availability of several complete mt genomes of land plants and algae allowed the identification of mt *trn* genes encoding tRNAs (O'Brien et al. 2009). In algae (with the exception of *C. reinhardtii* and *Polytomella capuana*) and in bryophytes, the number of *trn* genes seems sufficient or nearly sufficient for mt translation (O'Brien et al. 2009). In contrast, in seed plants the number of *trn* genes is clearly insufficient as *trn* genes for tRNAs corresponding to 5–7 amino acids are absent from the mt genomes. Experimental studies in a number of these organisms showed that this lack is compensated by the import of the corresponding cytosolic tRNAs (Glover et al. 2001; Vinogradova et al. 2009). Transport of tRNAs from the cytosol to mitochondria is not restricted to plants but is a widespread process that also occurs in organisms that would not need to import tRNAs to sustain mt translation (Salinas et al. 2008; Alfonzo and Soll 2009; Lithgow and Schneider 2010). An important feature in plants is that the number and the identity of imported tRNAs vary from one species to another and are not always consistent with the assigned phylogenetic position. The significance of this observation is currently unclear. Interestingly, with a few notable exceptions, the mt population of nuclear-encoded tRNAs was found to be primarily complementary to those encoded in the mt genome. Therefore, tRNA import in plant mitochondria represents a highly specific process as only a subset of cytosolic tRNAs appears to be routed to the mitochondria. In vivo studies in tobacco demonstrated the involvement of aminoacyl-tRNA synthetases in tRNA import (Dietrich et al. 1996). These studies also revealed the presence of different import determinants within tRNAs depending on the tRNA studied,

showing the complexity and selectivity of the import process (Delage et al. 2003b; Salinas et al. 2005). The development of an in vitro tRNA import system (Delage et al. 2003a) together with biochemical approaches in potato mitochondria allowed the identification of some components of the translocation machinery of tRNAs through the mt membranes. These investigations implicated the Voltage Dependent Anion Channel (VDAC), a known player in metabolite transport, as the major component of the tRNA transport system through the outer mitochondrial membrane. Moreover, TOM20 and TOM40, two major components of the TOM (Translocase of the Outer mitochondrial Membrane) complex, are likely to be important for tRNA binding at the surface of mitochondria (Salinas et al. 2006).

B. In Vitro Import of DNA

Isolated plant mitochondria are able to take up double-stranded DNA without sequence specificity. This was first documented using a 2.3 kb linear DNA plasmid originally described in maize mitochondria (Leon et al. 1989). Additional experiments indicate the ability of mitochondria to take up DNA molecules of more than 10 kb. This uptake appeared to be an active transmembrane potential-dependent mechanism and DNA was found to be transcribed *in organello* after import in the mt matrix (Koulintchenko et al. 2003). Mitochondria from mammalian and yeast cells also display the ability to take up DNA, which subsequently is transcribed *in organello* (Koulintchenko et al. 2006; Weber-Lotfi et al. 2009). The authors concluded that the process of DNA uptake may involve VDAC and the adenine nucleotide translocator, which are core components of the mt permeability transition pore complex in animal cells (Zamzami and Kroemer 2001). At the same time the authors ruled out mt membrane permeabilization as a possible mechanism for the DNA uptake in the organelle. In a yeast system, it was shown that DNA import is inhibited by VDAC effectors. Yeast strains deleted for the VDAC-1 or VDAC-2 gene are severely reduced in mt DNA import (Weber-Lotfi et al. 2009), supporting the role of this component in DNA import into mitochondria. Understanding the mechanism that mitochondria use to import DNA in vitro may be useful for the genetic engineering of plant or animal mitochondria in vivo. This has not yet been possible, even with vectors designed for mt gene expression. If VDACs are able to import DNA into mitochondria in living cells, this would greatly increase the chances to establish mt transformation (Weber-Lotfi et al. 2009).

C. Electroporation of Isolated Mitochondria with DNA and RNA

Electroporation changes the conductance and molecular permeability of cell membranes and has been used for delivery of nucleic acids into a broad spectrum of cells (see literature in Rao et al. 2009). Delivery of small RNAs into plant mitochondria has been reported upon electroporation of protoplasts with tRNAs, which subsequently were imported into mitochondria (Wintz and Dietrich 1996). The first reports of successful electroporation of isolated organelles were published some 20 years ago. One group demonstrated the introduction of RNA into isolated chloroplasts and used this system to study the control of mRNA stability in chloroplasts by 3′ inverted repeats (Adams and Stern 1990), while another group used mitochondria from maize and rice (Mulligan et al. 1989). These attempts were not continued with plant organelles at first, but some work was done using mitochondria isolated from mice liver cells (Collombet et al. 1997). Electroporation of maize, *Sorghum* and wheat mitochondria was successfully established a few years later (Farré and Araya 2001; Staudinger and Kempken 2003). Recently, isolated mitochondria from *Arabidopsis* and cauliflower were also used for electroporation (Bolle and Kempken 2006; Kempken et al. 2009) and the details of the methodology have

been published (Farré et al. 2007; Kempken et al. 2007).

The data from the mt electroporation systems differ with respect to efficiency. While in animal and wheat mitochondria, electroporation at 1.4 kV/cm (Collombet et al. 1997) and 1.3 kV/cm (Farré and Araya 2001), respectively, gave optimal results, in maize, 1.8–2.0 kV/cm appeared to be most efficient (Staudinger and Kempken 2003). In contrast, Mulligan et al. (1989) reported 8.8 kV/cm to be optimal. It is possible that these older data may be due to differences in the type of apparatus used, while the deviation of the newer data may be due to differences in the amount of mitochondria and plasmid DNA used.

There appears to be a size limit for the DNA to be introduced. While plasmid DNA of up to 11 kb could be introduced into mitochondria via electroporation, larger DNA molecules of about 30 kb were not (Staudinger and Kempken 2003). Moreover, while both linear and covalently closed circular (ccc) plasmid DNA can be imported into the mt matrix, the ccc DNA is mostly converted to open circular and linear DNA during the process (Collombet et al. 1997; Staudinger and Kempken 2003). Furthermore, there is no evidence for integration of the introduced DNA into the mt chromosome (Staudinger and Kempken 2003). The use of a mt plasmid from *Chenopodium album* for electroporation (Backert et al. 1997) gave some indication that replication of mt plasmids may be possible in isolated mitochondria (Kempken, unpublished data, see Fig. 2).

In addition to DNA, RNA can also be transformed into mitochondria using electroporation. This was demonstrated recently for maize and cauliflower mitochondria (Hinrichsen et al. 2009). The data presented demonstrate that plant mt RNA processing appears to be independent of both transcription and respiratory regulation. Moreover, introduction of RNA offers interesting experimental strategies to investigate RNA processing events.

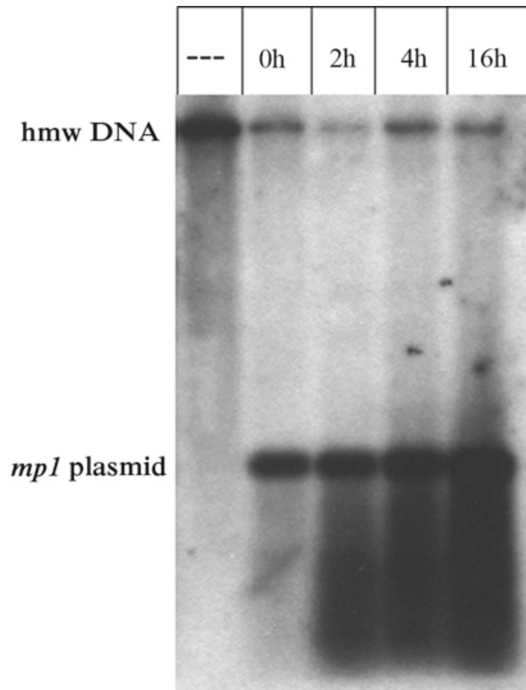

Fig. 19.2. Introduction of *mp1* plasmid DNA (Backert et al. 1997) in maize mitochondria via electroporation. DNA was introduced in mitochondria as described in (Staudinger and Kempken 2003), and *in organello* incubation was carried out in the presence of α-^{32}P-dCTP for up to 16 h. DNA was isolated after incubation and subjected to agarose gel electrophoresis. Over time, an increase in the *mp1* signal is observed in the autoradiogram.

Electroporation has been used for transformation of isolated organelles only. However, it may be possible to employ electroporation for genetic engineering of organelles in intact cells as well. Recently, a mathematical cell model was established, which indicates the possibility to use electric fields for intracellular manipulations (Esser et al. 2010). It was indeed demonstrated that high-intensity electric pulses induce mitochondria-dependent apoptosis in mice cells (Li et al. 2008). Therefore electroporation may be useful in the future for the in vivo transformation of mitochondria. This certainly will have applications not only in plant science but also in the design of therapies for human mt disorders (Cwerman-Thibault et al. 2010).

D. In organello Analysis

1. DNA Replication

Plant and fungal mt *in organello* systems have been used to analyze replication (Bedinger and Walbot 1986; Kempken et al. 1989) and transcription of mt genomes (Carlson et al. 1986). One specific type of genetic elements, the so-called linear plasmids, has been of particular interest in these studies. Linear plasmids are several thousand bases in size, characterized by long terminal inverted repeats and the presence of 5′-end bound terminal proteins. They are believed to be remnants of bacteriophages with similar structures (Meinhardt et al. 1990; Kempken et al. 1992). Studies on plant mitochondria undergoing *in organello* replication while using radiolabeled nucleotides support the existence of DNA repair and DNA replication activities within the organelle. Linear plasmids showed much higher incorporation of radiolabeled nucleotides than the high molecular weight DNA, which might be due to the activity of DNA polymerases encoded by linear plasmids. Similar results were obtained in a fungal *in organello* system employing mitochondria from *Ascobolus immersus*. End fragments from the plasmid were found to be more heavily labeled than internal fragments, indicating a potential role of the terminal proteins in DNA replication. It is assumed that terminal proteins function as primers for the replication of linear plasmids, as is the case for certain viruses with linear plasmids (Bedinger and Walbot 1986; Kempken et al. 1989). However, the fungal system provided stronger evidence for true DNA replication versus DNA repair, as the use of strand specific probes gave evidence for protein-primed DNA replication (Kempken et al. 1989).

2. Transcription and RNA Processing

Molecular analysis of plant mt transcription and RNA processing was long hampered due to the inability to transform plant mitochondria. Alternatively, in vitro systems have successfully been employed (e.g. Takenaka et al. 2004; Kühn et al. 2005) and *in organello* systems provide another substitute for *in planta* experiments (e.g. Farré and Araya 2001; Staudinger and Kempken 2003).

Two processes have mainly been studied using *in organello* systems: RNA editing and splicing. RNA editing is a post-transcriptional process that alters the information content of RNA (see chapter "Organellar Proteomics: Close Insights into the Spatial Breakdown and Functional Dynamics of Plant Primary Metabolism"). Several functional types are known (Knoop 2010), one of which occurs mostly by conversion of specific cytidine residues to uridine residues in the RNAs of higher plant mitochondria (Covello and Gray 1989; Gualberto et al. 1989; Hiesel et al. 1989). In addition, in several plant clades such as hornworts or ferns, many U-to-C changes have also been observed to occur in mitochondria (for reviews see Shikanai 2006; Takenaka et al. 2008). RNA editing is also observed in mosses, some liverworts and in gymnosperms (Knoop 2010). The number of RNA editing sites differs, but there are up to maybe more than 500 in higher plant mitochondria (Takenaka et al. 2008; Bruhs and Kempken 2010; Knoop 2010), and even more than 1,500 in lycophyte mitochondria (Grewe et al. 2011).

In attempts to elucidate higher plant mt RNA editing and the mechanism by which the C-to-U transition is achieved, *in organello* systems based on mitochondria from pea seedlings, potato tubers (Yu and Schuster 1995) or maize seedlings (Rajasekhar and Mulligan 1993) were developed. Taken together, the studies have excluded nucleotide excision and base exchange as possible editing reactions, while deamination or transamination reactions both are possible mechanisms. However, so far no conclusive evidence for either reaction has been obtained (Takenaka et al. 2008).

In organello assays in combination with uptake of DNA via electroporation have been successfully employed to analyze RNA processing (Farré et al. 2001; Staudinger and

Kempken 2003). Using biotinylated Uridine Tri-Phosphate (UTP), de novo transcription and RNA processing were observed *in organello*. The *in organello* systems were shown to faithfully reflect the *in planta* situation. Transcripts that are fully edited *in planta* are fully edited *in organello*, while those partially edited *in planta* are also partially edited in the *in organello* system (Staudinger and Kempken 2003, 2004). Using a wheat *in organello* system, a core sequence including 16 nucleotides upstream and six nucleotides downstream of the edited nucleotide was defined for recognition of two editing sites (Farré et al. 2001; Choury et al. 2004). However, for efficient editing, an upstream sequence of 40 nucleotides appeared to be required as shown by in vitro experiments (Takenaka et al. 2004). While it is now established that pentatricopeptide proteins (see chapter "Organellar Proteomics: Close Insights into the Spatial Breakdown and Functional Dynamics of Plant Primary Metabolism") participate in the recognition of mt RNA editing sites (e.g. Verbitskiy et al. 2009; Zehrmann et al. 2009), experiments using cauliflower and maize *in organello* systems also suggest an influence of a RNA secondary or tertiary structure (Bolle and Kempken 2006).

The way splicing and editing function in cross-species analyses is not fully predictable. While the *cox2* RNA is spliced and edited in mono- and di-cotyledonous mitochondria regardless of the mono- or dicot origin of the gene (Staudinger and Kempken 2003), the *rps10* RNA from potato (dicot) is neither spliced nor edited in wheat (monocot) mitochondria (Choury et al. 2005). The *atp6* mRNA from sorghum (monocot) is not even edited in maize (monocot) mitochondria despite very high sequence similarity. However, a chimeric *atp6* transcript composed of maize and sorghum DNA sequences gave rise to partial RNA editing, which may be due to presence of a RNA editing recognition sequence in the *atp6* transcript (Staudinger and Kempken 2003). An *in organello* system has also demonstrated the inability of plant mitochondria to recognize chloroplast editing sites (Bolle et al. 2007).

RNA editing can be essential for subsequent RNA splicing (Farré and Araya 2002; Castandet et al. 2010). However, both RNA splicing and editing are independent of the transcription machinery, as demonstrated using the *cox2* mRNA electroporated into mitochondria (Hinrichsen et al. 2009).

3. Translation

In organello translation of proteins employing ^{35}S labeled amino acids has long been established (Grohmann 1995). It has been employed for analysis of cytoplasmic male sterility (e.g. Horn et al. 1991; Moneger et al. 1994) and for the analysis of translation of partially edited transcripts. Amino acid sequencing of immunoprecipitated NAD9 protein indicated that only proteins from fully edited *nad9* mRNAs accumulate *in organello* (Grohmann et al. 1994).

While transcription and RNA processing can occur from DNA electroporated into isolated mitochondria and subsequent *in organello* incubation, there is no experimental evidence supporting that introduced RNA can be translated. In animal mitochondria, RNAs from a mt reporter gene (modified luciferase) could be stably maintained in the presence of functioning mitochondrial protein synthesis. However, the RNAs were not translated (McGregor et al. 2001). Likewise there is no proof for translation of RNA transcribed from introduced DNA into higher plant mitochondria (Kempken, unpublished data). As the introduced mRNA is faithfully processed (Hinrichsen et al. 2009), the reason for why translation cannot occur remains unclear.

III. Conclusion

Mt transformation of the unicellular alga *C. reinhardtii* can be achieved with surprisingly high efficiency and is of great value for the analysis of mt gene functions. Unfortunately comparable attempts to transform vascular plant mitochondria have been unsuccessful so far. While plant mt

transformation has not yet been achieved, mt *in organello* systems and the ability of mitochondria to uptake DNA provide an attractive alternative to study transcription and RNA processing mechanisms, such as RNA splicing and RNA editing. However, although *in organello* translation has long been established, the translation of RNA either directly introduced into isolated mitochondria, or transcribed from DNA electroporated into mitochondria, has not been reported yet. It is conceivable that a close coupling between transcription and translation as well as the proximity of the mt membrane could be necessary to synthesize polypeptides within mitochondria, but other parameters could be important, too. In addition, there could be a connection between the failure to translate imported nucleic acids within mitochondria and the inability to transform plant mitochondria. Therefore, understanding what might be the reason(s) for this lack of translation represents a major challenge that will provide clues to successful transformation of plant mitochondria.

Acknowledgements

Work in the lab of C. Remacle is supported by 'Fonds National de la Recherche Scientifique' from Belgium (F.R.S.-FNRS) 1.5.255.08 and 2.4601.08 and by Action de Recherche Concertée ARC07/12 04 and a joint United Mitochondrial Disease Foundation Grant (UMDF) to C. Remacle and P. Hamel. T. Salinas is supported by the European Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement n° 220808 (Marie Curie Fellowship). V. Larosa is supported by 'Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture' (FRIA). Work in the lab of F. Kempken its supported by the German Research Foundation (DFG). N. Bonnefoy is the recipient of an ANR contract (JC-JC-06-0163). We warmly thank Dr R. Lamb (Ohio State University, OH) for careful reading of the manuscript.

References

Abdelnoor RV, Yule R, Elo A, Christensen AC, Meyer-Gauen G, Mackenzie SA (2003) Substoichiometric shifting in the plant mitochondrial genome is influenced by a gene homologous to MutS. Proc Natl Acad Sci USA 100:5968–5973

Adams CC, Stern DB (1990) Control of mRNA stability in chloroplasts by 3′ inverted repeats: effects of stem and loop mutations on degradation of psbA mRNA in vitro. Nucleic Acids Res 18:6003–6010

Alfonzo JD, Soll D (2009) Mitochondrial tRNA import – the challenge to understand has just begun. Biol Chem 390:717–722

Arnal N, Alban C, Quadrado M, Grandjean O, Mireau H (2006) The *Arabidopsis* Bio2 protein requires mitochondrial targeting for activity. Plant Mol Biol 62:471–479

Arrieta-Montiel MP, Shedge V, Davila J, Christensen AC, Mackenzie SA (2009) Diversity of the *Arabidopsis* mitochondrial genome occurs via nuclear-controlled recombination activity. Genetics 183:1261–1268

Backert S, Meißen K, Börner T (1997) Unique features of the mitochondrial rolling circle-plasmid *mp1* from the higher plant *Chenopodium album* (L.). Nucleic Acid Res 25:582–589

Bedinger P, Walbot V (1986) DNA synthesis in purified maize mitochondria. Curr Genet 10:631–637

Bennoun P, Delosme M, Kuck U (1991) Mitochondrial genetics of *Chlamydomonas reinhardtii*: resistance mutations marking the cytochrome b gene. Genetics 127:335–343

Boer PH, Gray MW (1988) Scrambled ribosomal RNA gene pieces in *Chlamydomonas reinhardtii* mitochondrial DNA. Cell 55:399–411

Bolle N, Kempken F (2006) Mono- and dicotyledonous plant-specific RNA editing sites are correctly edited in both *in organello* systems. FEBS Lett 580:4443–4448

Bolle N, Hinrichsen I, Kempken F (2007) Plastid mRNAs are neither spliced nor edited in maize and cauliflower mitochondrial *in organello* systems. RNA 13:2061–2065

Bonnefoy N, Remacle C, Fox TD (2007) Genetic transformation of *Saccharomyces cerevisiae* and *Chlamydomonas reinhardtii* mitochondria. Methods Cell Biol 80:525–548

Boynton JE, Harris EH, Burkhart BD, Lamerson PM, Gillham NW (1987) Transmission of mitochondrial and chloroplast genomes in crosses of *Chlamydomonas*. Proc Natl Acad Sci USA 84:2391–2395

Bruhs A, Kempken F (2010) RNA editing in higher plant mitochondria. In: Kempken F (ed) Plant

mitochondria. Springer, New York/Dordrecht/Heidelberg/London, pp 157–176

Cardol P, Matagne RF, Remacle C (2002) Impact of mutations affecting ND mitochondria-encoded subunits on the activity and assembly of complex I in *Chlamydomonas*. Implication for the structural organization of the enzyme. J Mol Biol 319:1211–1221

Carlson JE, Brown GL, Kemble RJ (1986) *In organello* mitochondrial DNA and RNA synthesis in fertile and cytoplasmic sterile *Zea mays* L. Curr Genet 11:151–160

Castandet B, Choury D, Bégu D, Jordana X, Araya A (2010) Intron RNA editing is essential for splicing in plant mitochondria. Nucleic Acids Res 38:7112–7121

Chaumont F, Bernier B, Buxant R, Williams ME, Levings CS III, Boutry M (1995) Targeting the maize T-urf13 product into tobacco mitochondria confers methomyl sensitivity to mitochondrial respiration. Proc Natl Acad Sci USA 92:1167–1171

Choury D, Farre JC, Jordana X, Araya A (2004) Different patterns in the recognition of editing sites in plant mitochondria. Nucleic Acids Res 32:6397–6406

Choury D, Farré JC, Jordana X, Araya A (2005) Gene expression studies in isolated mitochondria: *Solanum tuberosum rps10* is recognized by cognate potato but not by the transcription, splicing and editing machinery of wheat mitochondria. Nucleic Acids Res 33:7058–7065

Colin M, Dorthu MP, Duby F, Remacle C, Dinant M, Wolwertz MR, Duyckaerts C, Sluse F, Matagne RF (1995) Mutations affecting the mitochondrial genes encoding the cytochrome oxidase subunit I and apocytochrome *b* of *Chlamydomonas reinhardtii*. Mol Gen Genet 249:179–184

Collombet J-M, Wheeler VC, Vogel F, Coutelle C (1997) Introduction of plasmid DNA into isolated mitochondria by electroporation. J Biol Chem 272:5342–5347

Covello PS, Gray MW (1989) RNA editing in plant mitochondria. Nature 341:662–666

Cwerman-Thibault H, Sahel JA, Corral-Debrinski M (2010) Mitochondrial medicine: to a new era of gene therapy for mitochondrial DNA mutations. J Inherit Metab Dis 34:327–344

Delage L, Dietrich A, Cosset A, Maréchal-Drouard L (2003a) In vitro import of a nuclearly encoded tRNA into mitochondria of *Solanum tuberosum*. Mol Cell Biol 23:4000–4012

Delage L, Duchene AM, Zaepfel M, Maréchal-Drouard L (2003b) The anticodon and the D-domain sequences are essential determinants for plant cytosolic tRNA(Val) import into mitochondria. Plant J 34:623–633

Dietrich A, Maréchal-Drouard L, Carneiro V, Cosset A, Small I (1996) A single base change prevents import of cytosolic tRNA(Ala) into mitochondria in transgenic plants. Plant J 10:913–918

Dorthu MP, Remy S, Michel-Wolwertz MR, Colleaux L, Breyer D, Beckers MC, Englebert S, Duyckaerts C, Sluse FE, Matagne RF (1992) Biochemical, genetic and molecular characterization of new respiratory-deficient mutants in *Chlamydomonas reinhardtii*. Plant Mol Biol 18:759–772

Duby F, Matagne RF (1999) Alteration of dark respiration and reduction of phototrophic growth in a mitochondrial DNA deletion mutant of *Chlamydomonas* lacking *cob*, *nd4*, and the 3′ end of *nd5*. Plant Cell 11:115–125

Ehara T, Osafune T, Hase E (1995) Behavior of mitochondria in synchronized cells of *Chlamydomonas reinhardtii* (Chlorophyta). J Cell Sci 108(Pt 2): 499–507

Esser AT, Smith KC, Gowrishankar TR, Vasilkoski Z, Weaver JC (2010) Mechanisms for the intracellular manipulation of organelles by conventional electroporation. Biophys J 98:2506–2514

Farré J-C, Araya A (2001) Gene expression in isolated plant mitochondria: high fidelity of transcription, splicing and editing of a transgene product in electroporated organelles. Nucleic Acids Res 29:2484–2491

Farré JC, Araya A (2002) RNA splicing in higher plant mitochondria: determination of functional elements in group II intron from a chimeric *cox II* gene in electroporated wheat mitochondria. Plant J 29:203–213

Farré JC, Leon G, Jordana X, Araya A (2001) *cis* recognition elements in plant mitochondrion RNA editing. Mol Cell Biol 21:6731–6737

Farré JC, Choury D, Araya A (2007) *In organello* gene expression and RNA editing studies by electroporation-mediated transformation of isolated plant mitochondria. Methods Enzymol 424:483–500

Fox TD, Sanford JC, McMullin TW (1988) Plasmids can stably transform yeast mitochondria lacking endogenous mtDNA. Proc Natl Acad Sci USA 85: 7288–7292

Glab N, Wise RP, Pring DR, Jacq C, Slonimski P (1990) Expression in *Saccharomyces cerevisiae* of a gene associated with cytoplasmic male sterility from maize: respiratory dysfunction and uncoupling of yeast mitochondria. Mol Gen Genet 223:24–32

Glover KE, Spencer DF, Gray MW (2001) Identification and structural characterization of nucleus-encoded transfer RNAs imported into wheat mitochondria. J Biol Chem 276:639–648

Grewe F, Herres S, Viehöver P, Polsakiewicz M, Weisshaar B, Knoop V (2011) A unique transcriptome: 1782

positions of RNA editing alter 1406 codon identities in mitochondrial mRNAs of the lycophyte *Isoetes engelmannii*. Nucleic Acids Res 39:2890–2902

Grohmann L (1995) *In organello* protein synthesis. Methods Mol Biol 49:391–397

Grohmann L, Thieck O, Herz U, Schröder W, Brennicke A (1994) Translation of *nad9* mRNAs in mitochondria from *Solanum tuberosum* is restricted to completely edited transcripts. Nucleic Acids Res 22:3304–3311

Gualberto JM, Lamattina L, Bonnard G, Weil JH, Grienenberger JM (1989) RNA editing in wheat mitochondria results in the conservation of protein sequences. Nature 341:660–662

Gutierres S, Sabar M, Lelandais C, Chetrit P, Diolez P, Degand H, Boutry M, Vedel F, de Kouchkovsky Y, De Paepe R (1997) Lack of mitochondrial and nuclear-encoded subunits of complex I and alteration of the respiratory chain in *Nicotiana sylvestris* mitochondrial deletion mutants. Proc Natl Acad Sci USA 94:3436–3441

Handa H (2003) The complete nucleotide sequence and RNA editing content of the mitochondrial genome of rapeseed (*Brassica napus* L.): comparative analysis of the mitochondrial genomes of rapeseed and *Arabidopsis thaliana*. Nucleic Acids Res 31:5907–5916

Hanson MR (1991) Plant mitochondrial mutations and male sterility. Annu Rev Genet 25:461–486

Havey MJ, Lilly JW, Bohanec B, Bartoszewski G, Malepszy S (2002) Cucumber: a model angiosperm for mitochondrial transformation? J Appl Genet 43:1–17

Hiesel R, Wissinger B, Schuster W, Brennicke A (1989) RNA editing in plant mitochondria. Science 246:1632–1634

Hinrichsen I, Bolle N, Paun L, Kempken F (2009) RNA processing in plant mitochondria is independent of transcription. Plant Mol Biol 70:663–668

Hiramatsu T, Nakamura S, Misumi O, Kuroiwa T, Nakamura S (2006) Morphological changes in mitochondrial and chloroplast nucleoids and mitochondria during the *Chlamydomonas reinhardtii* (Chlorophyceae) cell cycle. J Phycol 42:1048–1058

Horn R, Kohler RH, Zetsche K (1991) A mitochondrial 16 kDa protein is associated with cytoplasmic male sterility in sunflower. Plant Mol Biol 17:29–36

Huang J, Lee SH, Lin C, Medici R, Hack E, Myers AM (1990) Expression in yeast of the T-urf13 protein from Texas male-sterile maize mitochondria confers sensitivity to methomyl and to Texas-cytoplasm-specific fungal toxins. EMBO J 9:339–347

Johnston SA, Anziano PQ, Shark K, Sanford JC, Butow RA (1988) Mitochondrial transformation in yeast by bombardment with microprojectiles. Science 240:1538–1541

Karpova OV, Newton KJ (1999) A partially assembled complex I in ND4-deficient mitochondria of maize. Plant J 17:511–521

Kempken F, Meinhardt F, Esser K (1989) *In organello* replication and viral affinity of linear, extrachromosomal DNA of the ascomycete *Ascobolus immersus*. Mol Gen Genet 218:523–530

Kempken F, Hermanns J, Osiewacz HD (1992) Evolution of linear plasmids. J Mol Evol 35:502–513

Kempken F, Bolle N, Forner J, Binder S (2007) Transcript end mapping and analysis of RNA editing in plant mitochondria. Methods Mol Biol 372:177–192

Kempken F, Bolle N, Bruhs A (2009) Higher plant *in organello* systems as a model for RNA editing. Endocyt Cell Res 19:1–10

Knoop V (2004) The mitochondrial DNA of land plants: peculiarities in phylogenetic perspective. Curr Genet 46:123–139

Knoop V (2010) When you can't trust the DNA: RNA editing changes transcript sequences. Cell Mol Life Sci 68:567–586

Koulintchenko M, Konstantinov Y, Dietrich A (2003) Plant mitochondria actively import DNA via the permeability transition pore complex. EMBO J 22: 1245–1254

Koulintchenko M, Temperley RJ, Mason PA, Dietrich A, Lightowlers RN (2006) Natural competence of mammalian mitochondria allows the molecular investigation of mitochondrial gene expression. Hum Mol Genet 15:143–154

Kubo T, Newton KJ (2008) Angiosperm mitochondrial genomes and mutations. Mitochondrion 8:5–14

Kühn K, Weihe A, Börner T (2005) Multiple promoters are a common feature of mitochondrial genes in *Arabidopsis*. Nucleic Acids Res 33:337–346

Leon P, Walbot V, Bedinger P (1989) Molecular analysis of the linear 2.3 kb plasmid of maize mitochondria: apparent capture of tRNA genes. Nucleic Acids Res 17:4089–4099

Levings CS III, Siedow JN (1992) Molecular basis of disease susceptibility in the Texas cytoplasm of maize. Plant Mol Biol 19:135–147

Li C, Hu LN, Dong XJ, Sun CX, Mi Y (2008) High-intensity electric pulses induce mitochondria-dependent apoptosis in ovarian cancer xenograft mice. Int J Gynecol Cancer 18:1258–1261

Li L, Wang B, Liu Y, Qiu YL (2009) The complete mitochondrial genome sequence of the hornwort *Megaceros aenigmaticus* shows a mixed mode of conservative yet dynamic evolution in early land plant mitochondrial genomes. J Mol Evol 68:665–678

Lithgow T, Schneider A (2010) A Evolution of macromolecular import pathways in mitochondria, hydrogenosomes and mitosomes. Philos Trans R Soc Lond B Biol Sci 365:799–817

Mackenzie SA (2007) The unique biology of mitochondrial genome instability in plants. In: Logan DC (ed) Plant mitochondria. Blackwell Publishing, Singapore

Matagne RF, Michel-Wolwertz MR, Munaut C, Duyckaerts C, Sluse F (1989) Induction and characterization of mitochondrial DNA mutants in *Chlamydomonas reinhardtii*. J Cell Biol 108: 1221–1226

McGregor A, Temperley R, Chrzanowska-Lightowlers ZM, Lightowlers RN (2001) Absence of expression from RNA internalised into electroporated mammalian mitochondria. Mol Genet Genomics 265:721–729

Meinhardt F, Kempken F, Kämper J, Esser K (1990) Linear plasmids among eukaryotes: fundamentals and application. Curr Genet 17:89–95

Moneger F, Smart CJ, Leaver CJ (1994) Nuclear restoration of cytoplasmic male sterility in sunflower is associated with the tissue-specific regulation of a novel mitochondrial gene. EMBO J 13:8–17

Mulligan RM, Leon P, Calvin N, Walbot V (1989) Introduction of DNA into maize and rice mitochondria by electroporation. Maydica 34:207–216

Nishimura Y, Higashiyama T, Suzuki L, Misumi O, Kuroiwa T (1998) The biparental transmission of the mitochondrial genome in *Chlamydomonas reinhardtii* visualized in living cells. Eur J Cell Biol 77:124–133

O'Brien EA, Zhang Y, Wang E, Marie V, Badejoko W, Lang BF, Burger G (2009) GOBASE: an organelle genome database. Nucleic Acids Res 37:D946–D950

Patton DA, Schetter AL, Franzmann LH, Nelson K, Ward ER, Meinke DW (1998) An embryo-defective mutant of *arabidopsis* disrupted in the final step of biotin synthesis. Plant Physiol 116:935–946

Pineau B, Mathieu C, Gerard-Hirne C, De Paepe R, Chetrit P (2005) Targeting the NAD7 subunit to mitochondria restores a functional complex I and a wild type phenotype in the *Nicotiana sylvestris* CMS II mutant lacking *nad7*. J Biol Chem 280:25994–26001

Pla M, Mathieu C, De Paepe R, Chetrit P, Vedel F (1995) Deletion of the last two exons of the mitochondrial *nad7* gene results in lack of the NAD7 polypeptide in a *Nicotiana sylvestris* CMS mutant. Mol Gen Genet 248:79–88

Rajasekhar VK, Mulligan RM (1993) RNA editing in plant mitochondria: α-phosphate is retained during C-to-U conversion in mRNAs. Plant Cell 5:1843–1852

Randolph-Anderson BL, Boynton JE, Gillham NW, Harris EH, Johnson AM, Dorthu MP, Matagne RF (1993) Further characterization of the respiratory deficient *dum-1* mutation of *Chlamydomonas reinhardtii* and its use as a recipient for mitochondrial transformation. Mol Gen Genet 236:235–244

Rao AQ, Bakhsh A, Kiani S, Shahzad K, Shahid AA, Husnain T, Riazuddin S (2009) The myth of plant transformation. Biotechnol Adv 27:753–763

Remacle C, Matagne RF (1993) Transmission, recombination and conversion of mitochondrial markers in relation to the mobility of a group I intron in *Chlamydomonas*. Curr Genet 23:518–525

Remacle C, Bovie C, Michel-Wolwertz MR, Loppes R, Matagne RF (1990) Mitochondrial genome transmission in *Chlamydomonas* diploids obtained by sexual crosses and artificial fusions: role of the mating type and of a 1 kb intron. Mol Gen Genet 223:180–184

Remacle C, Baurain D, Cardol P, Matagne RF (2001a) Mutants of *Chlamydomonas reinhardtii* deficient in mitochondrial complex I: characterization of two mutations affecting the *nd1* coding sequence. Genetics 158:1051–1060

Remacle C, Duby F, Cardol P, Matagne RF (2001b) Mutations inactivating mitochondrial genes in *Chlamydomonas reinhardtii*. Biochem Soc Trans 29:442–446

Remacle C, Cardol P, Coosemans N, Gaisne M, Bonnefoy N (2006) High-efficiency biolistic transformation of *Chlamydomonas* mitochondria can be used to insert mutations in complex I genes. Proc Natl Acad Sci USA 103:4771–4776

Remacle C, Cline S, Boutaffala L, Gabilly S, Larosa V, Barbieri RM, Coosemans N, Hamel PP (2009) The *ARG9* gene encodes the plastid-resident *N*-acetyl ornithine aminotransferase in the green alga *Chlamydomonas reinhardtii*. Eukaryot Cell 8:1460–1463

Rhoads DM, Levings CS III, Siedow JN (1995) URF13, a ligand-gated, pore-forming receptor for T-toxin in the inner membrane of cms-T mitochondria. J Bioenerg Biomembr 27:437–445

Salinas T, Schaeffer C, Maréchal-Drouard L, Duchene AM (2005) Sequence dependence of tRNA(Gly) import into tobacco mitochondria. Biochimie 87:863–872

Salinas T, Duchene AM, Delage L, Nilsson S, Glaser E, Zaepfel M, Maréchal-Drouard L (2006) The voltage-dependent anion channel, a major component of the tRNA import machinery in plant mitochondria. Proc Natl Acad Sci USA 103:18362–18367

Salinas T, Duchene AM, Maréchal-Drouard L (2008) Recent advances in tRNA mitochondrial import. Trends Biochem Sci 33:320–329

Shikanai T (2006) RNA editing in plant organelles: machinery, physiological function and evolution. Cell Mol Life Sci 63:698–708

Small ID, Isaac PG, Leaver CJ (1987) Stoichiometric differences in DNA molecules containing the *atpA* gene suggest mechanisms for the generation of mitochondrial genome diversity in maize. EMBO J 6:865–869

Small I, Suffolk R, Leaver CJ (1989) Evolution of plant mitochondrial genomes via substoichiometric intermediates. Cell 58:69–76

Soderholm J, Bevis BJ, Glick BS (2001) Vector for pop-in/pop-out gene replacement in *Pichia pastoris*. Biotechniques 31:306–310

Staudinger M, Kempken F (2003) Electroporation of isolated higher-plant mitochondria: transcripts of an introduced *cox2* gene, but not an *atp6* gene, are edited *in organello*. Mol Genet Genomics 269:553–561

Staudinger M, Kempken F (2004) *In organello* editing of mitochondrial *atp9*, *cox2*, and *nad9* transcripts. Endocyt Cell Res 15:551–560

Steele DF, Butler CA, Fox TD (1996) Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. Proc Natl Acad Sci USA 93:5253–5257

Takenaka M, Neuwirt J, Brennicke A (2004) Complex *cis*-elements determine an RNA editing site in pea mitochondria. Nucleic Acids Res 32:4137–4144

Takenaka M, Verbitskiy D, van der Merwe JA, Zehrmann A, Brennicke A (2008) The process of RNA editing in plant mitochondria. Mitochondrion 8:35–46

Vahrenholz C, Riemen G, Pratje E, Dujon B, Michaelis G (1993) Mitochondrial DNA of *Chlamydomonas reinhardtii*: the structure of the ends of the linear 15.8-kb genome suggests mechanisms for DNA replication. Curr Genet 24:241–247

Verbitskiy D, Zehrmann A, van der Merwe JA, Brennicke A, Takenaka M (2009) The PPR protein encoded by the LOVASTATIN INSENSITIVE 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. Plant J 61:446–455

Vinogradova E, Salinas T, Cognat V, Remacle C, Maréchal-Drouard L (2009) Steady-state levels of imported tRNAs in *Chlamydomonas* mitochondria are correlated with both cytosolic and mitochondrial codon usages. Nucleic Acids Res 37:1521–1528

von Allmen JM, Rottmann WH, Gengenbach BG, Harvey AJ, Lonsdale DM (1991) Transfer of methomyl and HmT-toxin sensitivity from T-cytoplasm maize to tobacco. Mol Gen Genet 229:405–412

Wang B, Xue J, Li L, Liu Y, Qiu YL (2009) The complete mitochondrial genome sequence of the liverwort *Pleurozia purpurea* reveals extremely conservative mitochondrial genome evolution in liverworts. Curr Genet 55:601–609

Ward BL, Anderson RS, Bendich AJ (1981) The mitochondrial genome is large and variable in a family of plants (Cucurbitaceae). Cell 25:793–803

Weber-Lotfi F, Ibrahim N, Boesch P, Cosset A, Konstantinov Y, Lightowlers RN, Dietrich A (2009) Developing a genetic approach to investigate the mechanism of mitochondrial competence for DNA import. Biochim Biophys Acta 1787:320–327

Wintz H, Dietrich A (1996) Electroporation of small RNAs into plant protoplasts: mitochondrial uptake of transfer RNAs. Biochem Biophys Res Commun 223:204–210

Yamasaki T, Kurokawa S, Watanabe KI, Ikuta K, Ohama T (2005) Shared molecular characteristics of successfully transformed mitochondrial genomes in *Chlamydomonas reinhardtii*. Plant Mol Biol 58:515–527

Yu W, Schuster W (1995) Evidence for a site-specific cytidine deamination reaction involved in C-to-U RNA editing of plant mitochondria. J Biol Chem 270:18227–18233

Zamzami N, Kroemer G (2001) The mitochondrion in apoptosis: how Pandora's box opens. Nat Rev Mol Cell Biol 2:67–71

Zehrmann A, Verbitskiy D, van der Merwe JA, Brennicke A, Takenaka M (2009) A DYW domain-containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. Plant Cell 21:558–567