Regulation and retrograde coordination of mitochondrial gene expression

Adnan Khan Niazi

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Adnan Khan NIAZI

Régulation et coordination rétrograde de l'expression génétique mitochondriale

Regulation and retrograde coordination of mitochondrial gene expression

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Pr. David MACHEREL (Université d'Angers)	Rapporteur externe
Pr. Mizuki TAKENAKA (Université de Ulm)	Rapporteur externe
Dr. Pascale ROMBY (CNRS - Université de Strasbourg)	Examinateur
Dr. André DIETRICH (CNRS - Université de Strasbourg)	Directeur de thèse

Membre invité : Frédérique WEBER-LOTFI (Université de Strasbourg)

Unité de Recherche

Institut de Biologie Moléculaire des Plantes - CNRS UPR2357

Strasbourg, FRANCE

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INTRODUCTION

RESULTS, CHAPTER I

RESULTS, CHAPTER II

RESULTS, CHAPTER III

DISCUSSION AND CONCLUSIONS

MATERIALS AND METHODS

LITERATURE CITED

SYNTHESE EN FRANCAIS

ABBREVIATIONS

A and mA	Ampere and milliampere
aaRS	Aminoacyl-tRNA synthetase
ABC	ATP-binding cassette
ABI4	Abscisic acid insensitive 4
AOX	Alternative oxidase
b, bp and kb	Base, base pairs and kilo base pairs
BSA	Bovine serum albumin
°C	Degree (s) Celsius
CaMV	Cauliflower Mosaic Virus
CATMA	Complete Arabidopsis Transcriptome Micro Array
cm, µm and nm	Centimeter, micrometer and nanometer
CMS	Cytoplasmic male sterility
CPEO	Chronic progressive external ophtalmoplegia
CREB	Response element-binding protein
CRR	Chloroplast retrograde regulation
CT	Threshold cycle
DNA/cDNA/mtDNA	Deoxyribonucleic acid / Complementary/Mitochondrial
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetate
mtExo	Mitochondrial exosome
mtTFAM/mtTFBM	Mitochondrial transcription factor A/B
g, mg, µg and ng	Gram, milligram, microgram and nanogram
h and min	Hour and minute
HSP	Heat shock proteins
HDV	Hepatitis Delta Virus
HSPs/LSP	Heavy/light strand promoter
IMS	Mitochondrial intermembrane space
KSS	Kearns-Sayre syndrome
L, mL and µL	Liter, milliliter and microliter
LB	Luria Bertani medium
LHCII	Light-harvesting complex II
LHON	Leber's hereditary optic neuropathy
mmol and µmole	Millimole and micromole
M and mM	Molar and millimolar
MELAS	Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis and Stroke-like episodes
MERRF	Myoclonic epilepsy and ragged-red fibers
MRP	Mitochondrial RNA Peptidase
MRR	Mitochondrial retrograde regulation
MTS	Mitochondrial targeting presequence
NAA	Naphthalene acetic acid
NARP	Neuropathy, ataxia, and retinitis pigmentosa

NCS	Non-chromosomal stripe
NO	Nitric oxide
NRF	Nuclear respiratory factor
O_H / O_L	Origins of replication of heahy/light-strand
OGDRAW	OrganellarGenomeDRAW
orf	Open reading frame
OXPHOS	Oxidative phosphorylation
PAP	3'-phosphoadenosine 5'-phosphate
PCR / qPCR	Polymerase Chain Reaction / quantitative
PGE	Plastid gene expression
PKTLS	Pseudoknot tRNA-Like Structure
Polγ or Polγ2	Mitochondrial DNA polymerase
POLRMT	Mitochondrial RNA polymerase
PNPase	Phosphorolytic polynucleotide phosphorylase
PPARγ	Peroxisome proliferator-activated receptor γ
PPR	Pentratricopeptide repeat
RpoT/RpoTm/RpoTp/RpoTmp	Nuclear-encoded RNA polymerase / mitochondria- / plastid- / dual-targeted
PS	Passenger sequence
PSI	Photosystem I
PVP	Polyvinylpyrrolidone
mtRF	Mitochondrial release factor
RIC	RNA import complex
tRNA / mRNA and rRNA	Transfer ribonucleic acid / messenger and ribosomal
sc-mtRNAP	Nuclear-encoded RNA polymerase
ROS	Reactive Oxygen Species
sec and ms	Second and millisecond
Stat3	Signal transducer and activator of transcription 3
T3	Thyroid hormone tri-iodothyronine
mTERF1	Mitochondrial termination factor 1
TLS	tRNA-Like Structure
TOR	Target of rapamycin
Tris	N-tris-(hydroxymethyl) aminomethane
TYMV	Turnip Yellow Mosaic Virus
UPS	Ubiquitin proteasome system
UPSK	Upstream pseudoknot structure
UTRs	Untranslated regions
V and kV	Volt and kilovolt
VDAC	Voltage-dependent anion channel
v/v	volume / volume
w/v	weight/volume
WD-repeats	Tryptophan-aspartate repeats

INTRODUCTION

I. Organization of mitochondrial genomes

The mitochondria are derived from the endocytosis of a prokaryotic-like α-proteobacterium and their primary function is the production of energy as ATP. Their origin and function are common but their evolution has resulted in large differences in the structure and expression of their genomes. This is due to different constraints imposed by the various organisms. During evolution, the mitochondrial genome has decreased in size and gene content and its expression has become more complex.

In most of the organisms, the mitochondrial genome encodes subunits of the respiratory chain complexes and of the ATP synthase, the complex responsible for the production of ATP driven by the inner membrane proton gradient. ATP production is linked to different needs depending on metabolic conditions or growth. As the nucleus and the mitochondrion are both associated with these phenomena, a good coordination between the genomes of these two compartments is necessary.

In this first part, I will present the structure of the different mitochondrial genomes and the key knowledge on the expression of these genomes, mainly in yeast, mammals and plants, the most studied organisms on this topic.

I.1. Organization of the mitochondrial genome in yeast

I.1.1. Structure

The mitochondrial genome of yeast is relatively small, ranging from 11 kb in *Hanseniaspora uvarum* (Pramateftaki *et al.*, 2006) to 85 kb in *Saccharomyces cerevisiae* (Foury *et al.*, 1998). In *H. uvarum*, this 11 kb basic genome, excluding telomeric inverted repeats (3.5 kb long), encodes all essential yeast mitochondrial genes. It contains 32 genes encoding RNAs involved in the mechanisms of translation (2 rRNAs, 23 tRNAs) and seven genes encoding proteins of the respiratory chain (cox1, cox2, cox3, cob, atp6, atp8, atp9). The mitochondrial DNA (mtDNA) of *H. uvarum* also exists as a multimeric replicating form in concentric circles through the mechanism known as "rolling circle" (Han and Stachow, 1994; Pramateftaki *et al.*, 2006). A single intron of group IB interrupts the cox1 gene. There are many remarkable reasons that contributed towards its strikingly small size. The size of the ribosomal RNA (rRNA) genes is reduced by more than 40%. Most transfer RNA (tRNA) genes and five of the seven protein-coding genes are the shortest among known yeast homologs and non-coding regions are restricted to 5.1% of the genome (Pramateftaki *et al.*, 2006).

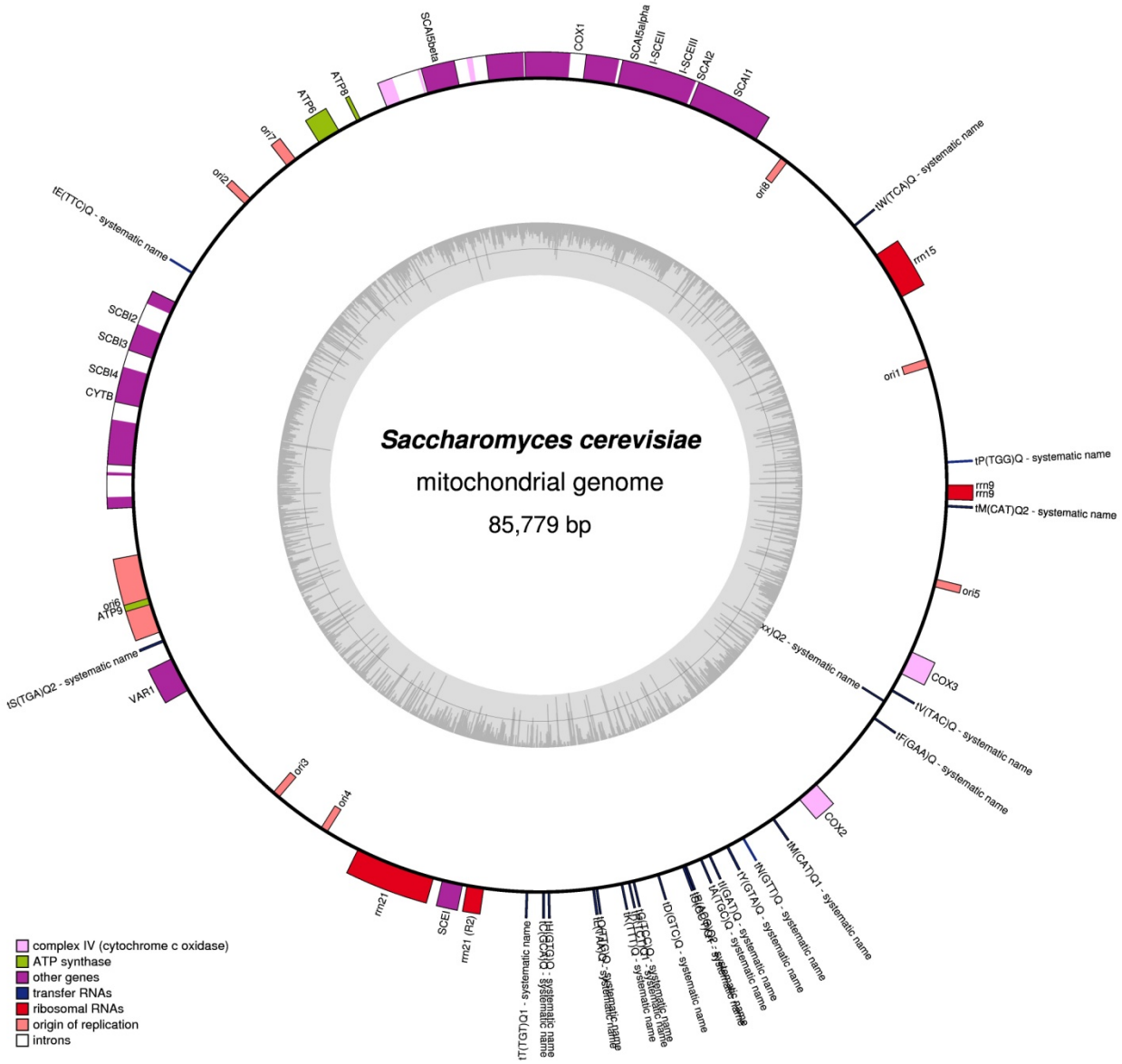

Figure 1: Physical map of the yeast *Saccharomyces cerevisiae* mitochondrial genome Obtained from the OrganellarGenomeDRAW website (ogdraw.mpimp-golm.mpg.de) Lohse *et al.*, 2007). GC content represented by the grey circle in the middle. GenBank: AJ011856

The 85 kb double-stranded circular mtDNA of *S. cerevisiae* (Figure 1) is much larger than mammalian or *H. uvarum* mtDNA (Foury *et al*., 1998; Pramateftaki *et al*., 2006; Jung *et al*., 2012). This is due to the presence of large introns and intergenic regions. The genes coding for proteins are generally highly conserved among different strains of *S. cerevisiae* and are the same in other yeast species. In addition to the genes encoded by *H. uvarum* mtDNA, *S. cerevisiae* mtDNA encodes a protein of the mitochondrial small ribosomal subunit, the RNA component of RNase P and one more tRNA (Foury *et al.*, 1998).

Because it is not organized as chromatin, like the nuclear DNA, it has long been thought that the mtDNA is naked, but spheroid bodies with a diameter of ~200-400 nm in aerobic and ~600–900 nm in anaerobic cells can be observed microscopically in the mitochondria of yeast (Malka *et al.*, 2006), while a single mtDNA molecule has a length of ~25 μm. The mtDNA is actually packaged with non-histone proteins to form so-called nucleoids. Yeast cells have approximately 40-60 nucleoids, each containing one or two mtDNA copies in aerobic cells and ~20 copies in anaerobic cells (Chen and Butow, 2005), so many fewer copies of mtDNA than humans (see below).

The first mitochondrial gene sequenced was the *atp9* gene of *S. cerevisiae* and the presence of introns in mitochondrial genes was first demonstrated in this organism (Hensgens *et al.*, 1979; Macino and Tzagoloff, 1979; Tzagoloff *et al.*, 1975). Several open reading frames have also been identified in group I and group II introns (Lambowitz and Belfort, 1993). Through their mobility, they are responsible for rearrangements and evolution of the mitochondrial genomes of yeast (Foury *et al.*, 1998). The mitochondrial genome of *S. cerevisiae* relies on homologous recombination for its maintenance (Contamine and Picard, 2000). This characteristic trait makes the mtDNA system of *S. cerevisiae* much more similar to that of higher plant mtDNA than to that of vertebrate mtDNA. In 1955, Ephrussi and Slonimski pointed towards another feature of the yeast mitochondrial system, the occurrence of the so-called cytoplasmic "petite" mutants. Cytoplasmic petite mutants are characterized by the absence of functional (rho^{+}) mtDNA, either through a complete loss (rho^{0}), or extensive deletions followed by reamplification of the remaining fragment (rho^{-}). They have a pleiotropic phenotype resulting from the loss of respiratory capacity due to the absence of mitochondrial gene expression.

I.1.2. Expression

To date, *S. cerevisiae* is the most extensively studied model organism for research on the mitochondrial genome maintenance and expression. In *S. cerevisiae*, thirteen main primary mitochondrial transcripts are synthesized from several (up to 13) promoters. These transcripts are not polyadenylated and contain long untranslated regions (UTRs). Most of them are polycistronic, with various combinations of rRNAs, tRNAs and messenger RNAs (mRNAs). All genes are transcribed from the same strand, except a $tRNA^{Thr}$ gene, and driven by the presence of nonanucleotide promoter sequences (Foury *et al.*, 1998; Szczesny *et al.*, 2013; Tzagoloff and Myers, 1986). In *S. cerevisiae,* transcription in mitochondria is achieved by the nuclear-encoded RNA polymerase sc-mtRNAP. The catalytic subunit Rpo41p is combined with the transcription factor Mtf1p (mt-TFB) (Cliften *et al.*, 1997; Karlok *et al.*, 2002; Mangus *et al.*, 1994).

It is speculated that the expression of mitochondrial genes in yeast is partly controlled by the ATP concentration in the organelle (Amiott and Jaehning, 2006). On the other hand, no correlation has been found between the abundance of particular transcripts or proteins and the relative transcription rates, which rather implies a post-transcriptional regulation pattern. Regulation usually occurs during processing and translation of mitochondrial transcripts and involves mostly nuclear-encoded factors, with few exceptions (Lipinski *et al.*, 2010).

Polycistronic RNAs are processed through different steps. Intron splicing proceeds with the assistance of intron-encoded maturases and nuclear-encoded splicing factors. Maturation of the 5' and 3' ends of mRNAs in polycistronic transcripts usually starts by excision of a tRNA at one end of the transcript. The tRNA is recognized and excised by tRNA processing enzymes such as homologs of RNase P and RNase Z. The tRNA and mRNA precursors are then both released for further processing. The biogenesis of tRNAs through co-transcription with protein-coding regions and subsequent removal from the polycistronic RNA units is a common feature of yeast and mammalian mitochondrial systems. This mode of maturation is called tRNA punctuation mode (Foury *et al.*, 1998; Richard *et al.*, 1998).

During maturation of the 3' end, a conserved dodecamer sequence is recognized by the mitochondrial exosome (mtExo), also known as the degradosome, forming a complex that includes the ATP-dependent RNA helicase Suv3p, encoded by the SUV3 gene, and an RNase II-like exoribonuclease (Dss1p). These two proteins are tightly interdependent and together create an ATP-dependent 3′-to-5′ exoribonuclease activity. In *S. cerevisiae*

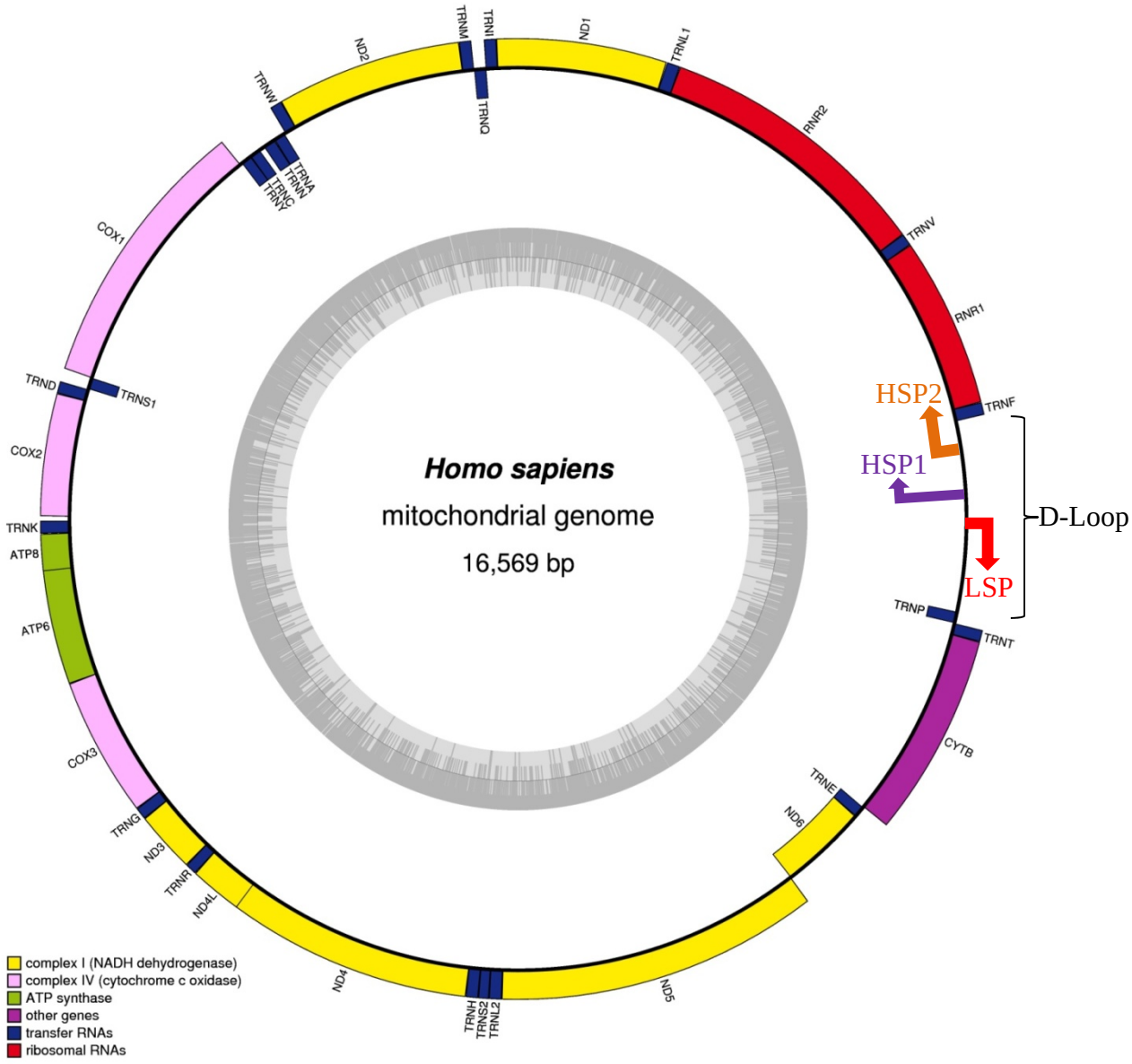

Figure 2: Physical map of the Human mitochondrial genome

Obtained from the OrganellarGenomeDRAW website (ogdraw.mpimp-golm.mpg.de) Lohse *et al.*, 2007). GenBank: NC_012920. GC content represented by the grey circle in the middle.

mitochondria, mtExo is the main ribonuclease involved in RNA turnover, processing and surveillance (Dziembowski *et al.*, 1998; Malecki *et al.*, 2007; Szczesny *et al.*, 2012). In *Schizosaccharomyces pombe*, this complex is termed as processosome, and its helicase and RNase subunits are named Pah1p and Par1p, respectively (Hoffmann *et al.*, 2008). The mRNA 3′-end processing mechanisms in *S. cerevisiae* and *S. pombe* not only differ from each other but also from those in plant and animal mitochondria. Yeast mitochondria do not polyadenylate mRNA precursors because they lack the poly(A) polymerase and phosphorolytic polynucleotide phosphorylase (PNPase) activities (Gagliardi *et al.*, 2004). The mtExo is characteristic of fungi and trypanosomes, even though one of its components, Suv3p, is found in all eukaryotes (Lipinski *et al.*, 2010; Szczesny *et al.*, 2012).

The 5'-to-3' exoribonuclease activity dependent on the Pet127 protein is required for 5'-end processing of several transcripts (Fekete *et al.*, 2008; Wiesenberger and Fox, 1997). Maturation of 5′-ends can also be achieved through a variety of other processes, including removal of tRNAs, cleavage at the upstream dodecamer site or transcription initiation without processing requirement (Lipinski *et al.*, 2010). A characteristic feature of the yeast mitochondrial mRNAs is the presence of long 5′-UTR sequences that provide binding sites for a plethora of gene-specific translation activation factors. The latter coordinate translation and regulate mRNA stability (Lipinski *et al.*, 2010).

Transcription and translation in yeast mitochondria are coupled and colocalized near the inner membrane, which suggests that the steps of mitochondrial gene expression probably take place nearly simultaneously. During the process, spatial organization and physical distribution of protein factors are thought to play a crucial role. As most of the proteins involved in this system are located in the mitochondrial inner membrane, it is speculated that they could form a large multiprotein machinery known as the translational integrator complex (Krause *et al.*, 2004).

I.2. Organization of mammalian mitochondrial genomes

I.2.1. Structure

The mammalian mitochondrial genome consists of a circular double-stranded DNA that varies in size from 13 to 19 kb, depending on the species, which ranks it amongst the small mitochondrial genomes. Mitochondria of human cells revealed a highly coding genome of 16.6 kb with no introns and no intergenic regions (Figure 2, Anderson *et al.*, 1981). The 37 genes that it carries encode 2 rRNAs, 22 tRNAs and 13 out of about 90 proteins that are part

of the respiratory chain complexes located in the mitochondrial inner membrane. These include NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5 and 6, cytochrome c oxidase subunits 1, 2 and 3, ATP synthase subunits 6 and 8, and cytochrome *b*. Beside these genes, a majority of which are being encoded by the Heavy (H) strand, the only non-coding region is the "displacement loop" (D-loop). This 1.1 kb region spans between the $tRNA^{Phe}$ gene and the $tRNA^{Pro}$ gene and contains important sequences for mtDNA replication and transcription: the replication origin for the H strand, the promoter regions of the H and light (L) strands and a termination-associated sequence (Shadel and Clayton, 1997). The mitochondrial proteins that are not encoded by the mitochondrial genome are encoded by the nuclear genome and are imported into the organelles through a specific transport system. Upon import, they combine with the proteins encoded by the mitochondrial genome (Mokranjac and Neupert, 2005).

As in yeast, the mtDNA of mammals takes on a higher structure in which multiple copies are packaged with non-histone proteins to form spherical nucleoids with a diameter of 70 nm. The nucleoids are associated with the inner mitochondrial membrane and spaced along the mitochondrial reticulum. Mitochondrial nucleoids have been demonstrated for example in mouse myoblasts (Chen and Butow, 2005) and in human cells (Iborra *et al.*, 2004). Within a single organelle, up to 800 nucleoids can be counted, while a nucleoid can itself contain multiple copies of mtDNA. In the case of human mitochondria, 10 copies can be compacted to form a nucleoid (Iborra *et al.*, 2004).

Many proteins associated with the nucleoids have been described as participating both in the regulation of mitochondrial gene expression and in the packaging of the mtDNA. Unlike bacterial nucleoid-associated proteins, whose function is essentially the maintenance of the chromosomal DNA, nucleoid proteins in mammalian organelles have multiple functions. For example, one of the major proteins involved in the packaging of mammalian mtDNA turned out to be mitochondrial transcription factor A (mtTFAM, often called TFAM). TFAM is encoded in the nucleus and imported into mitochondria, where it both activates mtDNA transcription and packages the mtDNA into nucleoids (Kukat *et al.*, 2011). TFAM alone is capable of structuring and compacting several mtDNA molecules into nucleoids (Kaufman *et al.*, 2007). Hence TFAM plays a structural role for mtDNA, similar to histones in nucleosomal DNA, which explains the emerging term of “mitochondrial chromatin” (Holt *et al.*, 2007). Two studies on this issue revealed that TFAM shapes the mtDNA into a sharp U-turn, providing a molecular mechanism for its dual role in mtDNA expression and

maintenance (Hallberg and Larsson, 2011). In mice, this protein has the ability to package and organize the double-stranded mtDNA by creating special forms such as Ω/loops or X structures. TFAM has the capacity to be the principal organizer of the mtDNA in the nucleoid, the formation of which requires the compaction of multiple DNA copies into a single spheroid structure. TFAM mediates two types of linkages between mtDNAs: the formation of X structures at an early point in the compaction process and the joining of compacted mtDNAs. The X structures, as well as Ω or loop structures, could occur during replication as nascent nucleotides are exposed, whereas the fusion of compacted molecules might occur as part of nucleoid dynamics (Kaufman *et al.*, 2007). Although the current knowledge on the regulation of mammalian mtDNA replication is still not complete, the data collectively points towards the fact that the main control of mtDNA copy number depends on the mitochondrial abundance of the proteins directly involved in mtDNA replication, *i.e.* the mitochondrial DNA polymerase (Polγ and Polγ2), the helicase Twinkle and TFAM. Other cited factors (ATAD3, PHB1, TFB2M) might have a secondary effect on mtDNA organization and copy number (Renard *et al.*, 2012).

In human mitochondria, TFAM does not bind only to promoters but on almost the entire mtDNA (Ohgaki *et al.*, 2007). Other proteins with multiple functions have been described as being associated with nucleoids. This is the case of enzymes involved in metabolic pathways, such as the ß subunit of ATP synthase or the aspartate aminotransferase (AAT) (Cheng *et al.*, 2005; He *et al.*, 2007).

I.2.2. Expression

In the classical annotation of mammalian mtDNAs, the individual strands are denoted heavy (H) strand and light (L) strand because of their different buoyant densities in a cesium chloride gradient. The observed difference is due to the respective base composition of the two strands: the H-strand is guanine-rich, whereas the L-strand is guanine-poor (Clayton, 2000). Their expression is different. Indeed, mammalian mtDNA transcription is bidirectional and starts from the heavy strand promoters (HSPs) and light strand promoter (LSP) located in the D-loop, generating polycistronic transcripts. The LSP contains one identified initiation site, while the HSP region contains two transcription start sites, noted HSP1 and HSP2. HSP2 is located approximately 100 bp upstream of HSP1 (Fernández-Silva *et al*., 2003).

The genes coding for proteins and rRNAs are interspersed with tRNA genes in mammalian mtDNA, so that transcript processing follows a "tRNA punctuation model": the polycistronic transcripts are processed by an RNase that excises tRNAs to release the mRNAs and rRNAs (Ojala *et al.*, 1981). The LSP promoter allows the synthesis of a transcript that is processed into 1 mRNA and 8 of the 22 tRNAs. Through a mechanism still not well understood, transcription of the light strand would be directly linked to mtDNA replication, as prematurely truncated transcripts serve as primers for the initiation of the heavy strand replication (Guja and Garcia-Diaz, 2012; Montoya *et al.*, 1982). On the other strand, HSP1 and HSP2 do not generate the same transcripts. HSP1 promotes the synthesis of an RNA ranging from 19 nucleotides before the $tRNA^{Phe}$ to the 3'-end of the 16S rRNA, while HSP2 allows the synthesis of a polycistronic transcript corresponding to the entire H strand and generates 2 rRNAs, 14 tRNAs and 12 mRNAs (Asin-Cayuela and Gustafsson, 2007; Christianson and Clayton, 1986).

Contrary to the nuclear transcription machinery, which is composed of a large complex containing numerous proteins, the mitochondrial basal transcription machinery in mammals has only a few members: the RNA polymerase POLRMT (also known as h-mtRPOL, Tiranti *et al*., 1997), mitochondrial transcription factor A (TFAM) (Fisher and Clayton, 1985, 1988), mitochondrial transcription factor B2 (TFB2M) (Falkenberg *et al.*, 2002; McCulloch *et al.*, 2002) and a termination factor (mTERF1) (Daga *et al.*, 1993; Fernandez-Silva *et al.*, 1997). All of the components necessary for transcription are encoded by nuclear genes and imported into mitochondria. Transcription initiation is provided by the assembly of POLRMT and TFAM on a specific sequence located upstream of HSP and LSP promoters, resulting in structural modifications of the mtDNA that allow the likely start of transcription. Direct interactions between TFAM and TFB2M participate in the formation of the TFBM-POLRMT heterodimer (McCulloch and Shadel, 2003). It was demonstrated by Gaspari *et al*. (2004) that this heterodimer protects the region from -4 to +10 of the LSP promoter. Initially, it was also thought that mitochondrial transcription factor B1 (TFB1M) is also essential for initiation, but recent studies indicate that only TFB2M is required for efficient, promoter-specific transcription *in vitro* (Litonin *et al.*, 2010).

Regulation of mitochondrial gene expression is poorly characterized relative to that of the nucleus (Asin-Cayuela and Gustafsson, 2007). Nuclear-encoded transcriptional regulatory proteins have been detected in mammalian mitochondria. They may regulate mitochondrial gene expression in two quite different ways. They can act as 'indirect regulators' by

regulating the transcription of nuclear-encoded genes relevant to mitochondrial function and biogenesis. Alternatively, they can be imported into the mitochondria and alter transcription from the mitochondrial genome as 'direct regulators' of mitochondrial gene expression. Whereas the majority of mitochondrial transcriptional regulators act indirectly, a handful of nuclear transcription factors appear to act in both environments and have been partly characterized as direct regulators of mitochondrial gene expression (Leigh-Brown *et al.*, 2010).

Indirect regulators include the nuclear respiratory factors 1 and 2 (NRF-1, NRF-2), which regulate the expression of nuclear-encoded components of the mitochondrial respiratory chain and the basal transcription machinery (Scarpulla, 2006).

The nuclear transcription factors best characterized as direct regulators include five nuclear factors that have been shown to have distinct mitochondrial roles. These are all involved in signaling pathways (Leigh-Brown *et al.*, 2010). Among them the estrogen receptor and the T3 (thyroid hormone tri-iodothyronine) receptor named p43 are nuclear hormone receptors activated by a hormone ligand. The CREB (Cyclic adenosine monophosphate (cAMP) response element-binding protein) is activated upon cyclic-AMP-dependent phosphorylation (Acin-Perez *et al.*, 2009). Stat3 (Signal transducer and activator of transcription 3) is stimulated by growth hormone signaling pathways (Zhong *et al.*, 1994) and transcription of the tumor suppressor p53 is triggered in response to cell stress (Erster and Moll, 2004). Furthermore, p43 and CREB are transcription factors that can bind the mtDNA to regulate gene expression. p53, Stat3, and the estrogen receptor are thought to act as co-regulators, affecting mitochondrial gene expression through protein-protein interactions (Leigh-Brown *et al.*, 2010).

Some other nuclear transcription factors like the glucocorticoid receptor (Koufali *et al.*, 2003), the heterodimeric transcription factor AP-1 (Ogita *et al.*, 2002), and the peroxisome proliferator-activated receptor γ (PPARγ) (Casas *et al.*, 2000) have been localized to mitochondria but their role is still not well studied. There is some evidence for binding of the glucocorticoid receptor and the AP-1 factor to the mitochondrial genome, potentially to regulate gene expression (Demonacos *et al.*, 1995; Ogita *et al.*, 2003).

Transcription factor mTERF causes the termination of transcription initiated from the HSP1 promoter. Termination occurs at the 3'-end of the 16S rRNA. *In vitro* experiments have shown that this protein binds specifically to the 3'-end of the $tRNA^{Leu}$ (UUR) gene located

after the 16S rRNA gene. A mutation of this recognition site causes a MELAS syndrome (Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis and Stroke-like episodes) (Chomyn *et al.*, 1992). Work has subsequently demonstrated that the mTERF protein could also participate in the initiation of transcription from HSP1 (Martin *et al.*, 2005). Proteins homologous to mTERF are assumed to bind to the termination site of the transcripts initiated from the HSP2 promoter, upstream of the $tRNA^{Phe}$ gene close to the D-loop (Camasamudram *et al.*, 2003).

The human mitochondrial RNA polymerase POLRMT has an N-terminal region containing a repeated motif of 35 amino acids (pentratricopeptide repeat or PPR). This pattern is characteristic of certain proteins (PPR proteins) known to play a role in RNA editing, maturation or splicing (Rodeheffer *et al.*, 2001; Schmitz-Linneweber and Small, 2008). Wang *et al*. (2007) have characterized the mitochondrial ribosomal protein MRPL12 in mitochondrial extracts from HeLa cells. The recombinant MRPL12 protein binds to RNA polymerase POLRMT and stimulates transcription. This complex was co-immunoprecipitated from HeLa cells expressing the protein MRPL12 marked by a FLAG epitope, suggesting that this association does occur *in vivo* and amplifies transcription. A new regulatory mechanism has thus potentially been unveiled, whose role is to coordinate transcription and translation in human mitochondrial gene expression.

The molecular processes that control mitochondrial translation are unclear. Mitochondrial translation more closely resembles its prokaryotic than its eukaryotic cytosolic counterpart. Adaptations of the genetic code developed to minimize the population of tRNAs are striking. Mitochondria use a simplified decoding mechanism that allows translation of all codons with only 22 tRNAs instead of the 31 predicted by Crick's wobble hypothesis (Anderson *et al.*, 1981; Barrell *et al.*, 1980; Yokobori *et al.*, 2001). Mammalian mitochondria use a single $tRNA^{Met}$ for both the initiation and elongation phases (depending on the presence or absence of a formyl group, respectively), whereas not only in the prokaryotic and eukaryotic cytosolic translation systems but also in the mitochondria of plants and most lower eukaryotes two specialized $tRNA^{Met}$ species exist (Mikelsaar, 1983). UGA is no longer a stop codon but a tryptophan codon and AUA became a methionine codon instead of isoleucine. So, the mammalian mitochondrial translation apparatus would use only two stop codons. However, none of the 22 mtDNA-encoded tRNAs contains an anticodon that could decode the AGA and AGG triplets (conventionally specifying for arginine). As human mitochondria were originally considered not to import tRNAs from the

cytosol, it was assumed that they use an extended repertoire of 4 different stop codons: the regular UAG and UAA stop codons, terminating 11 out of the 13 polypeptide-encoding transcripts, and the AGA and AGG codons to terminate the translation of MTCOI and MTND6, respectively (Lightowlers and Chrzanowska-Lightowlers, 2010; Smits *et al.*, 2010).

However, recent advances regarding mitochondrial translation termination have brought new elements that contradict this view. A transcript translation terminates when a stop codon positioned in the A-site of the ribosome provokes the ribosome arrest, as there is no tRNA recognizing these triplets. Instead, the stop codons are recognized by proteins called "release factors" (RFs) (Lightowlers and Chrzanowska-Lightowlers, 2010). The RFs are the proteins that are responsible for the termination of translation and that promote the release of the newly synthesized protein from the ribosome. In the case of human mitochondria, there is a general agreement to consider mtRF1a as a release factor (Soleimanpour-Lichaei *et al.*, 2007; Zhang *et al.*, 1998). mtRF1a has been characterized experimentally as allowing translation termination at codons UAA/UAG *in vivo* and *in vitro* (Soleimanpour-Lichaei *et al.*, 2007). ICT1 (immature colon carcinoma transcript-1), a second member of the mitochondrial release factor family, is an essential mitochondrial protein necessary for the hydrolysis of prematurely terminated peptidyl-tRNA moieties in stalled ribosomes, rather than a key player in regular translation termination at stop codons (Richter *et al.*, 2010). It was also shown that mtRF1a is active on UAA and UAG codons, but has no specificity for the AGA and AGG codons involved in MTCOI and MTND6 translation termination. As both the MTCOI AGA terminal codon and the MTND6 AGG terminal codon are preceded by an U, a -1 frameshift can position a UAG codon in the A-site of the ribosome, allowing the recruitment of mtRF1a to terminate translation. According to these recent data, human mitochondria would use only 2 stop codons instead of 4 (Lightowlers and Chrzanowska-Lightowlers, 2010).

I.3. Organization of mitochondrial genomes of plants

I.3.1. Structure

Higher plant mitochondrial genomes have a number of unique features, compared to their counterparts in animals or fungi, besides the conserved mitochondrial function in all eukaryotic cells. In a plant cell, there are three genomes: nuclear, mitochondrial and chloroplastic. All three work in a coordinated manner to ensure the essential functions of the

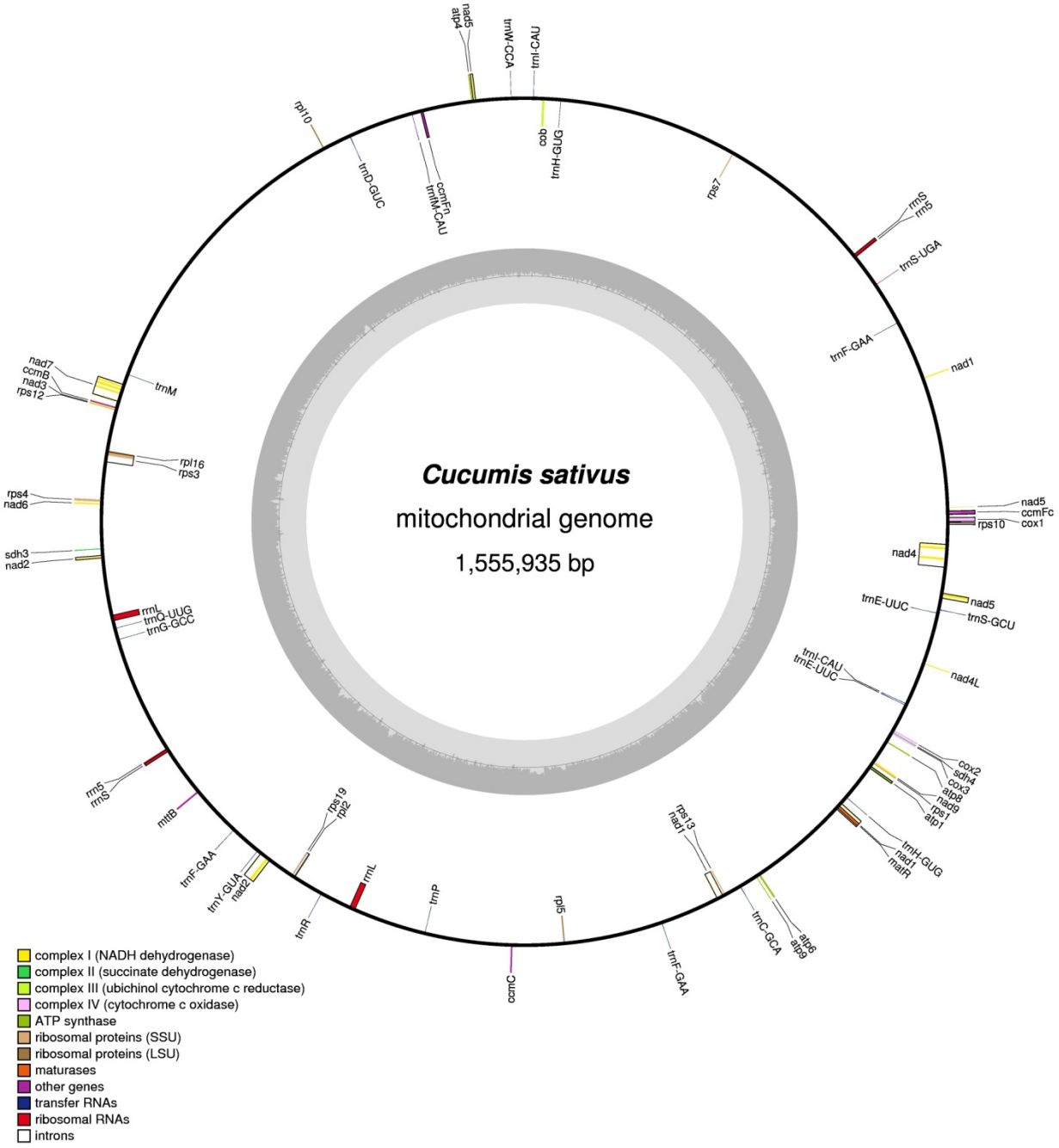

Figure 3: Physical map of the chromosome 1 of *Cucumis sativus* mitochondrial genome
Obtained from the OrganellarGenomeDRAW website (ogdraw.mpimp-golm.mpg.de) Lohse *et al.*, 2007). GenBank: NC_016005. GC content represented by the grey circle in the middle. There are two other chromosomes in this genome. Chromosome 2 (NC_016004) having a size of 83,817 bp codes for rpl5 and rps14. Chromosome 3 (NC_016006) having a size of 44,840 bp does not code for any known gene.

cell such as photosynthesis or respiration, the energy sources of the plant. Unlike those of chloroplasts, mitochondrial genomes in plants differ greatly in size and structure, depending on the species and even between very close species. Moreover, they are larger, variable in size and more complex than the mitochondrial genomes of other organisms (reviewed in Maréchal and Brisson, 2010). In lower plants, the mitochondrial genome of *Marchantia polymorpha* revealed a size of 187 kb (Oda *et al.*, 1992), more than 11 times the size of the human mtDNA. Mitochondrial genomes of higher plants can be much greater, with 221 kb in *Brassica napus* (Handa, 2003), 367 kb in *Arabidopsis thaliana* (Unseld *et al.*, 1997), 369 kb in *Beta vulgaris* (Kubo *et al.*, 2000), 431 kb in *Nicotiana tabacum* (Sugiyama *et al.*, 2005), 569 kb in *Zea mays* (Clifton *et al.*, 2004) and 1685 kb in *Cucumis sativus* (Figure 3, Alverson *et al*., 2011). Physical analyzes were used to estimate the size of the mtDNA of some further cucurbits and predictions range up to 2900 kb in *Cucumis melo*, which represents around 150 times the size of the mitochondrial genomes of *H. uvarum* and mammals.

The physical map of mitochondrial genomes is often represented by a circle of double-stranded DNA, called “master chromosome”, that houses a complete set of genes. However, such a structure could not be experimentally observed by electron microscopy in *Vicia faba* (Negruk *et al.*, 1986). The plant mtDNA actually contains inverted repeat sequences dispersed throughout the genome. They pair up and recombine, generating smaller circular DNA molecules. In *A. thaliana*, two large repeats of 6.5 and 4.2 kb were shown to be involved in recombination (Klein *et al.*, 1994). In the *N. tabacum* mtDNA, there are three pairs of large repeated sequences, with repeat lengths of 18 kb, 6.9 kb and 4.7 kb (Sugiyama *et al.*, 2005). As a consequence, unlike the chloroplast genome and the mitochondrial genomes of other eukaryotes, the mtDNA of plants cannot be physically represented as a unique, organized structure used to determine phylogenetic relationships between species. Recombination and pairing in these genomes are perpetual dynamic systems resulting in significant differences between closely related species. Such a diversity is characteristic in cucurbits, for which it was estimated that the size of the mitochondrial genome would range from one of the smallest of angiosperms (330 kb for watermelon) to the largest (2900 kb for *C. melo*) (Ward *et al.*, 1981). It has subsequently been shown that in the genus *Cucumis*, the large size of mitochondrial genomes is to be linked with the accumulation of small repeated sequences (Lilly and Havey, 2001).

Further studies represent the mitochondrial genome as composed of subgenomic circular or linear molecules. In one notable case, *Z. mays* CMS-S cytoplasm, the mitochondrial genome would exist mainly as multiple linear molecules, and the sequencing data were found to be consistent with this premise (Allen *et al.*, 2007). On the other hand, the apparent morphology of carefully isolated mtDNA was not found to be consistent with a circular model (Oldenburg and Bendich, 1996). Therefore, the entity of a plant mitochondrial genome is likely to be a mixture of various DNA molecules. Nevertheless, the concept of a circular master chromosome remains because the question as to how multipartite and branched DNA molecules are transmitted properly to the next generation is unsolved.

If the size of plant mitochondrial genomes varies depending on the species, the gene content seems similar. Plant mtDNAs carry only 50-60 genes. There are large intergenic regions, as well as multiple group II introns, that lengthen the mtDNA. For example in *A. thaliana,* apart from introns, the 57 identified genes correspond to 10% of the mtDNA (Unseld *et al.*, 1997). Mitochondrial genes in plants encode subunits of the oxidative phosphorylation chain, rRNAs (5S, 18S, and 26S) and tRNAs. With the emergence of second-generation sequencing technologies, the number of completed plant mitochondrial genomes deposited in the GenBank database (http://tinyurl.com/d34jnts) has increased until March of 2013 to 73. Most of them are from Chlorophyta and seed plants. Complete sequencing of the mtDNAs of *A. thaliana, Oryza sativa* or *Beta vulgaris* (Kubo *et al.*, 2000; Notsu *et al.*, 2002; Unseld *et al.*, 1997) and other plants, showed that the mitochondrial genomes of plants possess additional genes *versus* the animal or yeast mtDNA. These additional genes encode polypeptides involved in the biogenesis of respiratory chain complexes or ribosomal proteins. Anyway, the mtDNA of *A. thaliana* contains less than twice the number of genes present in a typical vertebrate mitochondrial genome (37 genes), with a 22 times larger size. Mitochondrial genomes encode just a small part of the organellar proteins. Most of the genes that are lost from the mitochondrion appear to have been transferred to the nuclear genome (Adams and Palmer, 2003). Moreover, it turned out that about 5-20% of the sequences in the mtDNA of higher plants have a chloroplast, nuclear or viral origin and that over 50% are of unknown origin (Unseld *et al.*, 1997).

I.3.2. Expression

The diversity of the mitochondrial genome organization between genera or species makes the study of plant mtDNA expression difficult and specific for each genome. As previously

stated, plant mitochondrial genomes are extraordinarily large and complex, compared to their fungal and animal counterparts, due to the presence of large non-coding regions. Both strands are transcribed, each from many promoters. This was implied in particular for *A. thaliana* (Kühn, 2006; Kühn *et al.*, 2005). Only few genes are co-transcribed from a common promoter into polycistronic precursor RNAs (Gagliardi and Binder, 2007). It appears that transcription initiation is relatively relaxed, since most of the mtDNA is transcribed, including the non-coding sequences (Holec *et al.*, 2006). Control and regulation of gene expression is considered so far to occur primarily during post-transcriptional steps (Giegé *et al.*, 2000). One of these critical steps is the maturation of the 3'-end by an exoribonuclease like the polynucleotide phosphorylase (PNPase) (Perrin *et al.*, 2004).

As stated previously, numerous promoters drive transcription of mitochondrial genes in plants. The 5'-CRTA-3' (R = purine) tetranucleotide has been identified as a core element of major mitochondrial promoters in both monocotyledonous and dicotyledonous species (Fey and Maréchal-Drouard, 1999). In dicotyledonous plants, this sequence is part of a larger conserved region of 9 nucleotides (nonanucleotide), CRTAAGAGA, the penultimate nucleotide being the transcription initiation site (Binder *et al.*, 1996). In *A. thaliana*, apart from the CRTA-type consensus motifs, tetranucleotide core motifs such as ATTA and RGTA were identified, and several promoters showed no consensus sequence at all (Kühn *et al.*, 2005, 2007; Liere and Börner, 2011; Liere *et al.*, 2011; Weihe *et al.*, 2012), making this mechanism more complicated and still under investigation. In monocotyledonous species, mitochondrial promoters have, as a variant of the CRTA motif, degenerated YRTA, AATA, and CTTA sequences and are often less conserved than those of dicotyledonous species (Fey and Maréchal-Drouard, 1999; Weihe *et al.*, 2012). Furthermore, in monocotyledonous plants, most consensus-type promoters share a small sequence element about ten nucleotides further upstream containing an AT-rich region of six nucleotides (Weihe *et al.*, 2012). It has been demonstrated in several monocotyledonous species that multiple promoters could be in charge of the same gene (Lupold *et al.*, 1999). It seems that the existence of multiple start sites of transcription is much more common in monocotyledonous than in dicotyledonous species (Lupold *et al.*, 1999; Mulligan *et al.*, 1988). It was suggested that the presence of multiple promoters along the mtDNA is maintained in order to ensure the expression of individual genes despite frequent recombination in the genome. The opposite idea is that multiple initiation sites of

transcription are the result of a confused machinery of transcription (Lupold *et al.*, 1999; Weihe *et al.*, 2012).

Plant mitochondrial transcription is mediated by T3/T7 phage-type RNA polymerases homologous to the yeast sc-mtRNAP and the human h-mtRNAP encoded by the small RpoT nuclear gene family. In dicotyledonous species such as *A. thaliana*, the RpoT gene family encodes three products that are imported into mitochondria (RpoTm), into plastids (RpoTp) and into both organelles (RpoTmp), respectively. However, RpoTmp is not found in monocotyledonous plants. In dicotyledonous species, RpoTm has to be considered as the basic RNA polymerase required for transcription of most mitochondrial genes, whereas RpoTmp has a specific role for the formation of respiratory chain complexes I and IV (Kühn *et al.*, 2009; Weihe *et al.*, 2012).

Whether there are cofactors for the mitochondrial RNA polymerases in plants remains an open question. Kühn *et al*. (2007) showed that in *A. thaliana* RpoTm and RpoTp recognize a number of promoters, initiate transcription and perform elongation of the transcript without additional co-factors *in vitro* on supercoiled, but not linear DNA. However, in contrast to the RNA polymerases of bacteriophages, auxiliary factors are required for the plant, animal or fungal RpoT polymerases for accurate and efficient transcription initiation *in vivo*. Homologs of mtTFB (and mtTFA), identified in yeast and animal mitochondria, would be potential candidates for mitochondrial specificity factors in plants. mtTFA and mtTFB homologs are encoded in the nuclear genome of *A. thaliana*, but plant mitochondria do not seem to use them as transcription factors (Liere *et al.*, 2011). The mTERF protein family is involved in initiation and termination of transcription in human mitochondria. There exists also a family of mTERF proteins in higher plants. However, transcription termination sequences have not been identified in plant mitochondrial genomes (Forner *et al.*, 2007). A mitochondrially-localized mTERF homologue, MOC1, identified in green alga may be a good candidate, as loss of the MOC1 gene causes a high light-sensitive phenotype (Kühn *et al.*, 2009). Apart from termination of RNA synthesis, however, it is tempting to speculate that plant mTERFs may also have a function in initiating mitochondrial transcription (Liere and Börner, 2011).

Post-transcriptional processes are numerous and complex in the mitochondria of plants and play a major role in determining mitochondrial RNA levels and protein sequence accuracy. First, RNA splicing occurs, due to the presence of introns, essentially from group II, located

almost entirely in genes encoding proteins (Gagliardi and Binder, 2007). Second, plant mitochondrial RNAs are edited, with a number of editing sites that is much higher than in chloroplasts. In flowering plants, editing consists essentially in cytidine (C) to uridine (U) conversions. The reverse can also be found in other plant groups. The modification from C to U requires de- or *trans*-amination. Some editing factors were identified, belonging to the PPR protein family (Arenas-M *et al.*, 2013; Sosso *et al.*, 2012; Takenaka *et al.*, 2008, 2012; Zehrmann *et al.*, 2012; Zhu *et al.*, 2012). The 5'- and 3'-end maturation of plant mitochondrial mRNAs involves stem-loop structures, called t-elements, that can mimic tRNAs (Forner *et al.*, 2007). These structures would be recognized by the endonucleolitic enzymes RNase Z and RNase P that normally cleave the precursors of tRNAs. For example, the *A. thaliana* organellar RNase P is able to cleave the 5'-end of t-elements *in vitro* (Gobert *et al.*, 2010, 2013). However, some mRNA precursors do not possess t-elements, suggesting the existence of other processes.

Mature functional RNAs are not constitutively polyadenylated in plant mitochondria. Conversely, upon polyadenylation, RNA 3'-ends can be processed or degraded by the exoribonuclease PNPase. Two forms of PNPase exist in plants, one targeted into mitochondria, the other located in plastids. The mitochondrial enzyme takes part in degradation of rRNA and tRNA processing intermediates, non-coding RNAs transcribed from intergenic regions, and antisense RNAs. Poly(A) tails trigger rapid exonucleolytic degradation by PNPase, similar to the situation reported in *E. coli* and in chloroplasts (Holec *et al.*, 2006, 2008).

Bioinformatic analyzes have identified a family of at least 550 PPR protein genes in *A. thaliana* (tinyurl.com/d4m8bxt). Much of these proteins are predicted to be targeted to mitochondria or chloroplasts (Lurin *et al.*, 2004). PPR proteins bind to RNAs in organelles and recruit different factors for editing, maturation or translation (Sosso *et al.*, 2012; Zehrmann *et al.*, 2012; Zhu *et al.*, 2012).

Translation in the mitochondria of plants remains the least studied step in gene expression. There is no Shine-Dalgarno-type sequence in the mitochondrial transcripts. Some mRNAs are translated without an AUG initiation codon and translation can end without a stop codon (Raczynska *et al.*, 2006; Unseld *et al.*, 1997). PPR proteins might be involved in the initiation and/or termination of translation in plant mitochondria.

Mitochondrial DNA Disorder	Gene	mtDNA genotype	Clinical phenotype	Reference
LHON	*ND1*	3460G>A	Optic neuropathy	(Howell *et al.*, 1991)
	ND4	11778G>A		(Wallace *et al.*, 1988)
	ND6	14484T>C		(Johns *et al.*, 1992)
NARP	*ATP6*	8993T>G	Neuropathy, ataxia, retinitis pigmentosa	(Holt *et al.*, 1990)
MILS	*ATP6*	8993T>C	Progressive brain-stem disorder	(De Vries *et al.*, 1993)
Exercise intolerance	*CYB*	Individual mutations	Fatigue, muscle weakness	(Andreu *et al.*, 1999)
Fatal, infantile encephalopathy; Leigh/Leigh-like syndrome	*ND3*	10158T>C; 10191T>C	Encephalopathy, lactic acidosis	(McFarland *et al.*, 2004a)
MELAS	*ND1 and ND5*	Individual mutations	Myopathy, encephalopathy lactic acidosis, stroke-like episodes	(Kirby *et al.*, 2004; Santorelli *et al.*, 1997)
	TRNL1	3243A>G; 3271T>C		(Goto *et al.*, 1990)
MIDD	*TRNL1*	3243A>G	Diabetes, deafness	(Van den Ouweland *et al.*, 1992)
Myopathy and diabetes	*TRNE*	14709T>C	Myopathy, weakness, diabetes	(Hao *et al.*, 1995; McFarland *et al.*, 2004b)
Sensorineural hearing loss	*RNR1*	1555A>G	Deafness	(Prezant *et al.*, 1993)
	TRNS1	Individual mutations		(Reid *et al.*, 1994; Sue *et al.*, 1999)
KSS	A single, large-scale deletion	Several deleted genes	Progressive myopathy, ophthalmoplegia, cardiomyopathy	(Holt *et al.*, 1988; Zeviani *et al.*, 1988)
CPEO	A single, large-scale deletion	Several deleted genes	Ophthalmoplegia	(Holt *et al.*, 1988; Moraes *et al.*, 1989)
Pearson syndrome	A single, large-scale deletion	Several deleted genes	Pancytopoenia, lactic acidosis	(Rötig *et al.*, 1990)

Table 1: Clinical disorders that are caused by mutations in mitochondrial DNA
Derived from (Elstner and Turnbull, 2012; Greaves *et al.*, 2012; Taylor and Turnbull, 2005)
LHON, Leber hereditary optic neuropathy; *ND1,3–6*, NADH dehydrogenase subunits 1,3–6; *TRNE,TRNK,TRNL1,TRNS1*, mitochondrial tRNAs; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fibres; *ATP6*, ATPase 6; *CYB*, cytochrome *b*; MIDD, maternally-inherited diabetes and deafness; MILS, maternally-inherited Leigh syndrome; NARP, neurogenic weakness, ataxia and retinitis pigmentosa; *RNR1*, 12*S* ribosomal RNA; KSS, Kearns–Sayre syndrome; CPEO, chronic progressive external ophthalmoplegia;

II. Mutations and rearrangements of the mitochondrial genome, causes and consequences.

The mitochondrial genome, as any genome, can undergo mutations, insertions or deletions under the influence of different factors. Changes in the mtDNA, more or less important, usually lead to dysfunctions. The mitochondrial genetic system is especially exposed to oxidizing molecules such as the ROS (Reactive Oxygen Species) generated by the respiratory activity. Under physiological conditions, ROS production is highly regulated. However, under stress conditions, ROS production is generally increased, which can lead to severe damage with an impairment of the respiratory chain function and mtDNA lesion. Such effects can in turn be the cause for further increased ROS production.

In humans, mutations in the mtDNA cause severe neurodegenerative diseases that are currently incurable and await the development of gene therapy strategies. In plants, when not lethal, mutations affecting the mtDNA mainly lead to cytoplasmic male sterility (CMS), a trait that is widely used in crops for breeding and hybrid maintenance. In this section, I will detail the changes in the mitochondrial genome leading to altered gene expression and their consequences.

II.1. Mutations and rearrangements in the human mitochondrial genome

Due to the key role of the organelles in the cells, mitochondrial dysfunction can cause severe deficiencies. In 1988, mtDNA deletions were first reported in myopathies and KSS (Kearns-Sayre syndrome) and mtDNA point mutations were causatively linked to LHON (Leber's hereditary optic neuropathy) (Holt *et al.*, 1988; Wallace *et al.*, 1988; Zeviani *et al.*, 1988). Since then, over 500 pathogenic mtDNA mutations have been identified (compiled on Mitomap, Human Mitochondrial Genome Database, http://mitomap.org). A few common examples of diseases due to mitochondrial genome abnormalities are presented in Table 1.

The mutation rate in the human mitochondrial genome has been estimated by some authors to be 10-fold higher than that of the nuclear genome. Three types of mutations have been identified in the mtDNA: (1) Missense mutations in protein-coding genes that alter the activity of an oxidative phosphorylation (OXPHOS) protein, (2) Single-base mutations in tRNA or rRNA genes that impair mitochondrial protein synthesis and (3) Rearrangements that consist of duplications and deletions. The phenotypes associated with these mutations may differ, depending on the affected genes, and are often to be linked with an impairment of essential functions of mitochondria. Clinical symptoms can vary widely and may affect

only a specific tissue but can also cause multisystem syndromes. In general, the tissues affected are those that require high energy supply, such as brain, heart or muscles.

mtDNA missense mutations in protein coding genes can be associated with two common ophthalmologic manifestations, LHON and NARP (neuropathy, ataxia, and retinitis pigmentosa). Three primary mtDNA mutations, that are shown in table 1, represent approximately 90% of all LHON cases (Achilli *et al.*, 2012).

Strikingly, more than 50% of the observed pathogenic mutations are concentrated in the tRNA genes, whereas these represent only 5% of the mtDNA sequences (Elson *et al.*, 2009). Pathogenic mtDNA mutations in tRNA and rRNA genes are thought to cause an impairment of the overall mitochondrial protein synthesis, whereas mutations in the protein-encoding genes affect specific respiratory chain complexes (Mariotti *et al.*, 1994). More than 250 mutations in rRNA and tRNA genes have been reported, leading to mitochondrial myopathies (tinyurl.com/cnpmqgr). These mutations cause a variety of metabolic syndromes with several combinations of symptoms involving different tissues. The most common mtDNA mutation in this group is the m.3243A>G point mutation in the MTTL1 ($tRNA^{Leu}$) gene, found in more than 80% of the patients with the clinical features of MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; Mariotti *et al*., 1995; Goto *et al*., 1990). The same mutation is also responsible for a large number of CPEO (chronic progressive external ophtalmoplegia) patients that do not have large-scale mtDNA deletions (Mariotti *et al.*, 1995). The m.8344A>G and m.8356T>C point mutations in the MTTK ($tRNA^{Lys}$) gene have been found in most patients with a MERRF (myoclonic epilepsy and ragged-red fibers) syndrome (Shoffner *et al*., 1990).

Deletions and nucleotide substitutions in the mtDNA also cause many mitochondrial myopathies and seem to have a central role in aging in post-mitotic tissues (Krishnan *et al.*, 2008). The size of the observed deletions may vary from one nucleotide to several kb. Most of the mtDNA deletions share the same characteristics. They are located in the major arc between the two proposed origins of replication (O_H and O_L) and are mostly (about 85%) flanked by short direct repeat sequences (Bua *et al.*, 2006; Samuels *et al.*, 2004). More than 100 mutations of this type along the mtDNA have been characterized, causing varied syndromes. Large deletions are often associated with CPEO, KSS or Pearson's pancreatic syndrome (Alemi *et al.*, 2007). It is generally considered that double-strand breaks, DNA

repair systems and replicative advantage are the likely mechanisms behind deletion formation (Fukui and Moraes, 2009).

In contrast to diseases caused by mutations in the nuclear DNA, mtDNA mutations might not be fully expressed because their expression and segregation are different. Indeed, mtDNA mutations can be homoplasmic or heteroplasmic. In the case of homoplasmic mutations, all mtDNA molecules are mutated. In the heteroplasmic stage, the mutation only affects part of the mtDNA copies and often causes less severe symptoms than in the case of homoplasmy. With mitochondrial diseases, the concept of threshold burden has been proposed. According to this concept, when the percentage of mutated genomes rises above a certain threshold, noticeable symptoms will be observed. When the proportion of mutated DNA far exceeds this threshold level, then an acute (may be lethal) condition will be observed (Berdanier and Everts, 2001).

Transmission of mtDNA mutations is maternal. However, one study showed the existence of a paternal transmission of a microdeletion located in a gene for a protein of complex I, indicating that there must be some rare exceptions (Schwartz and Vissing, 2002). The mitochondrial diseases caused by mutations in the mtDNA show different patterns of inheritance due to three distinctive features of mitochondrial chromosomes: homoplasmy or heteroplasmy, replicative segregation, and maternal inheritance (Zhang and Qi, 2008).

Malfunctions of the OXPHOS may also come from mutations located in nuclear genes encoding proteins that are imported and assembled into the mitochondrial respiratory chain and ATP synthase (Greaves *et al.*, 2012; Smits *et al.*, 2010). About 250 mutations in nuclear genes encoding mitochondrial proteins are responsible of impaired mitochondrial bioenergetics (tinyurl.com/c4ja4pd). Mutations in genes encoding proteins responsible for organelle dynamics were described to cause phenotypes identical to those associated with primary mtDNA mutations, but follow classic Mendelian patterns of dominant-recessive transmission (Elstner and Turnbull, 2012; Liesa *et al.*, 2009).

II.2. Mutations and rearrangements in the plant mitochondrial genome

Like in mammals, targeted mutations and deletions in the plant mitochondrial genome can affect organelle gene expression. However, whereas in mammals a large number of mtDNA point mutations have been described as causing diseases (see § II.1.), only a few cases of nucleotide substitutions affecting mitochondrial functions have been reported in plants. A homoplasmic nonsense mutant producing a shortened form of the COX2 protein has been

characterized in wild beet populations. It results in a 50% reduction of cytochrome c oxidase activity and a male sterile phenotype (Ducos *et al.*, 2001). Comparative analyses of the mitochondrial genome sequences between normal lines and mutant lines showing cytoplasmic male sterility (CMS) were performed in sugar beet (Satoh *et al.*, 2004) and maize (Allen *et al.*, 2007), which revealed the presence of 1 to 24 substitutions in coding regions. Nevertheless, alterations in the plant mtDNA essentially correspond to genome rearrangements (Newton *et al.*, 1995).

In some plant mutants, alterations in mitochondrial gene expression may be due to mtDNA deletions resulting from recombination via rather short (6 to less than 100 nucleotides) repeated sequences. This causes the loss of some mitochondrial genes (Kubo and Newton, 2008), which results in an abnormal phenotype such as growth retardation. More often, the outcome is mottling of leaves caused by a malfunction in the biosynthesis of chlorophyll, itself arising from a malfunction of mitochondrial processes such as respiration. This is the case for example in maize, where deletions may affect some genes such as *cox2* or *nad4* (Lauer *et al.*, 1990; Marienfeld and Newton, 1994). Maize plants with a deletion of the *nad4* gene are both male- and female-sterile and show severe growth problems (Yamato and Newton, 1999). A complete deletion of the mitochondrial *nad7* gene in *Nicotiana sylvestris* causes CMS (Pineau *et al.*, 2005). Multiple recombinations may result in homoplasmic or heteroplasmic deletions of entire genes. In most cases where only growth retardation is observed, the deletion is heteroplasmic. Some angiosperm mitochondrial mutants carry mtDNA deletions that truncate specific genes. These also show abnormal phenotypes, including retarded development or leaf variegation. This is the case in maize 'non-chromosomal stripe' (NCS) and cucumber 'mosaic' (MSC) mutants (Karpova *et al.*, 2002; Lilly *et al.*, 2001).

Finally, rearrangements in the plant mtDNA can also lead to the creation of new chimeric genes that will be expressed thereafter. Such events are predominently associated with the CMS phenotype already mentioned above. CMS actually corresponds to the most common class of mitochondrial mutants in plants. In CMS mutants, male gametophytic development is impaired but the plant is otherwise normal (Chase, 2007; Hanson and Bentolila, 2004). CMS is widely distributed, having been described in over 150 plant species. The phenotype is often associated with the appearance of novel *orfs* in the mitochondrial genome, which are thought to have originated as the result of aberrant recombination events. The protein products of such *orfs* have been identified in a number of species like maize (*Zea mays L.*),

sunflower (*Helianthus annuus L.*), common bean (*Phaseolus vulgaris L.*) or radish (*Raphanus sativus L.*). Nuclear genes known as restorer-of-fertility genes can suppress or counteract this trait (Carlsson and Glimelius, 2011; Chase, 2007).

III. Strategies developed to manipulate the mitochondrial genetic system

Analyzing organelle genetic processes, developing therapies for mitochondrial diseases or obtaining male sterile lines of agronomic interest would be much easier if it would be possible to transform mitochondria. However, at present, conventional methodologies do not allow to transform the mitochondrial genome in plant and animal cells, despite the success of biolistics in the unicellular organisms *S. cerevisiae, Candida glabrata*, and *Chlamydomonas reinhardtii* (Bonnefoy *et al.*, 2007; Zhou *et al.*, 2010). That is why different alternative strategies have been explored, aiming to introduce DNA constructs or RNAs of interest into mitochondria *in vitro* or *in vivo*. Most of these have been developed with mammalian cells, to modelize new gene therapy approaches. Only a few studies have been devoted to plant cells. A number of these new approaches is based on the import of macromolecules into the organelles, to supplement a defective gene or to modify the expression of a given mitochondrial gene. The imported macromolecule either replaces the product of a mutated gene or inhibits the replication of mutated mtDNA copies.

This section is mainly composed of a review paper (Niazi *et al.*, 2012) that we wrote recently. It is entitled "Targeting nucleic acids into mitochondria: Progress and prospects" and is published in "Mitochondrion". In this review, we briefly explain the different strategies explored for mitochondrial delivery of DNA or RNA in living cells and the progress made, especially towards therapeutic purposes to cure mitochondrial disorders. The paper is attached at the end of the Introduction.

Beyond the scope of the review paper, in this section I will give a brief description of mitochondrial RNA import mechanisms and finally explain in detail the molecular strategy used in the present work on the basis of the natural tRNA import pathway.

III.1. Import of 5S rRNA

The 5S rRNA seems to play an important role in translation by binding together the different functional centers of the ribosome, mainly in the 23S rRNA (Kiparisov *et al.*, 2005; Kouvela *et al.*, 2007; Smirnov *et al.*, 2008, 2010, 2011; Smith *et al.*, 2001). It is part

of the large subunit of the ribosome. Trafficking of 5S rRNA is important because translation takes place in several cellular compartments. In mammals, there is no corresponding gene in the mitochondrial genome. In contrast, Yoshionari *et al*. (1994) detected 5S rRNA encoded by the nuclear genome in mitochondria and mitoplasts from beef liver, thus demonstrating its import into the organelles. The observation was further strengthened and it is established that mammalian mitochondria naturally import a significant portion of the nuclear-encoded 5S rRNA (Entelis *et al.*, 2001; Magalhaes *et al.*, 1998). Its function in mitochondria remains unexplained, since attempts to detect 5S rRNA in mitochondrial ribosomes of fungi, animals and some protists failed so far (Koc *et al.*, 2001; Nagaike *et al.*, 2001; Sharma *et al.*, 2003, 2009). Nevertheless, with a level approaching that of 12S and 16S rRNA, 5S rRNA is one of the most abundant RNAs in mammalian mitochondria, suggesting an important role in the organelles, especially in translation (Entelis *et al.*, 2001; Magalhaes *et al.*, 1998).

The *in vitro* import of 5S rRNA in isolated human mitochondria has been demonstrated by Entelis *et al*. (2001). This process is dependent on the presence of ATP and the membrane potential. It seems that receptors on the surface of the outer membrane are involved in mitochondrial import and also protein import channel has some role in this process (Entelis *et al.*, 2001).

In eukaryotes, the 5S rRNA is synthesized by RNA polymerase III in the nucleolus. It is then exported to the cytosol in a complex with the transcription factor TFIIIA (Guddat *et al.*, 1990; Pelham and Brown, 1980; Rudt and Pieler, 1996). Part of the cytosolic pool of 5S rRNA exchanges TFIIIA for ribosomal protein L5, which allows it to return to the nucleolus and be incorporated into the ribosomal 60S pre-subunit (Steitz *et al.*, 1988; Szymański *et al.*, 2003). The other part of the 5S rRNA present in the cytosol is directed into the mitochondria through some other proteins, among which two have been already identified (Smirnov *et al.*, 2010, 2011). The first one is the mitochondrial enzyme rhodanese (thiosulfate sulfur-transferase) (Smirnov *et al.*, 2010) and the second is the precursor of the mitochondrial ribosomal protein L18 (preMRP-L18) (Smirnov *et al.*, 2011). According to the current model, preMRP-L18 efficiently competes with ribosomal protein L5 to redirect part of the 5S rRNA from its major pathway to mitochondria. Then, preMRP-L18 hands over the 5S rRNA to rhodanese, to take it inside mitochondria. Thus, these two protein factors together would guide the efficient uptake of cytosolic 5S rRNA into mitochondria.

However, the exact mechanism for translocation of 5S rRNA through the mitochondrial membranes is still not clear (Smirnov *et al.*, 2010, 2011).

The structure of the 5S rRNA consists of five loops (A to E), alternating with five helices (I to V). Determinants of import located in the RNA itself were characterized by importing human 5S rRNA variants obtained after site-directed mutagenesis of the gene into isolated human mitochondria (Smirnov *et al.*, 2008). Two regions are required for mitochondrial uptake: the first is located in the proximal portion of helix I in domain a, the second is associated with the region composed of loop D and helix IV in domain g. Domain ß is not essential for import, hence the idea that inserting sequences of interest or replacing this domain would not impair mitochondrial uptake. In that way, the 5S rRNA would serve as an import shuttle (Smirnov *et al.*, 2008). The ability of the 5S rRNA to translocate customized sequences into mitochondria in human cells and to rescue pathogenic mutations is discussed in section 7 of the review paper (Niazi *et al.*, 2012) mentioned earlier.

III.2. PNPase-facilitated RNA targeting into mitochondria

Besides the 5S rRNA, mammalian mitochondria also import the RNA components of RNase MRP (Mitochondrial RNA Processing), a complex found in the nucleus and in the mitochondria (Chang and Clayton, 1987) and of RNase P, the enzyme responsible for the maturation of the 5' end of tRNAs (Doersen *et al.*, 1985). Altogether, this import is essential for mitochondrial DNA replication, transcription and translation (Doersen *et al.*, 1985; Duchêne *et al.*, 2009; Tarassov *et al.*, 2007). However, import of the RNase P RNA has been debated for long time. Although confirmed again recently (Wang *et al.*, 2010), its meaning remains puzzling, as mammalian mitochondrial RNase P does not seem to have an RNA component (Holzmann *et al.*, 2008).

The localization of these non-coding RNAs in the mitochondrial matrix seems to rely on an RNA import regulator, polynucleotide phosphorylase (PNPASE) (Wang *et al.*, 2010). PNPASE is a highly conserved 3′–5′ exoribonuclease, localized in the mammalian mitochondrial intermembrane space (IMS). It functions in RNA quality control through its RNA polymerase and degradation activities (Wang *et al.*, 2012a and references therein). PNPASE is encoded by only one gene (PNPT1) in humans (Leszczyniecka *et al.*, 2002), but there are two distinct genes in plants, one gene with a chloroplast transit peptide and the other having a mitochondrial targeting sequence (MTS) (Walter *et al.*, 2002; Yehudai-Resheff *et al.*, 2003). In mice, a whole-animal knockout mutant for PNPASE was

embryonic lethal, indicating its essential role for early mammalian development (Wang *et al.*, 2010). PNPASE has a role in mitochondrial homeostasis and its reduction results in reduced respiratory complex activities and reduced oxygen consumption (Chen *et al.*, 2006; Wang *et al.*, 2010).

PNPASE can regulate the import of selected nucleus-encoded small RNAs, including the RNase MRP/RNase P RNAs and the 5S rRNA, into mitochondria. However, the mechanism and pathway of PNPASE-regulated RNA import is still not clear (Wang *et al.*, 2010, 2012a, 2012b). The RNA import function of PNPASE is separable from its RNA-processing function and disturbing one of these has no effect on the other (Wang *et al.*, 2010). The molecular mechanism by which PNPASE distinguishes between RNAs for processing and RNAs for import remains unclear. It is hypothesized that PNPASE recognizes a specific stem-loop structure in some and perhaps in all of the RNAs where it helps to import (Wang *et al.*, 2010). That this stem-loop structure of about 20 nucleotides serves as a targeting signal for PNPASE-mediated mitochondrial translocation was highlighted through *in vitro* import of engineered RNase P and RNase MRP RNAs. It may have a role of protecting against degradation, similar to that of some RNA stem-loops in chloroplasts (Suay *et al.*, 2005). In further experiments with mitochondria isolated from yeast expressing human PNPASE, the import of RNase P and RNase MRP RNAs, as well as that of 5S rRNA, was augmented relative to control mitochondria, while GAPDH RNA levels remained unaffected. Similar results were obtained with *in vitro* import into mouse liver mitochondria, showing uptake of RNase P RNA, 5S rRNA and RNase MRP RNA but no import for GAPDH RNA (Wang *et al.*, 2010).

All these data indicate the role of PNPASE in translocation of RNAs into mitochondria, pointing towards the possibility of using the stem-loop structure from the RNase P or RNase MRP RNA as a new shuttle system to drive RNAs of interest into mitochondria. This aspect is discussed in more detail in section 6 of the review paper (Niazi *et al.*, 2012) mentioned earlier.

III.3. Import of tRNAs

Transfer RNA import was discovered in the 1970s through the study of tRNAs in the ciliated protozoan *Tetrahymena pyriformis* (Chiu *et al.*, 1974; Suyama, 1986). In most of the organisms, mitochondria do not encode a number of the tRNAs they need for protein synthesis and they import the missing tRNAs from the cytosol (Salinas *et al.*, 2008;

Schneider, 2011; Schneider and Maréchal-Drouard, 2000). The number and identity of the imported tRNAs and the import mechanisms are variable, depending on the organism and species. In *Trypanosoma* and *Leishmania*, the mtDNA does not code for any tRNA. Thus, mitochondrial tRNAs are all imported from the cytosol in these organisms (Mahapatra and Adhya, 1996; Simpson *et al.*, 1989). In contrast, it has long been considered that in mammals, including humans, the mtDNA encodes all the tRNAs involved in mitochondrial protein synthesis. This concept, already contradicted by the mitochondrial import of cytosolic $tRNA^{Lys}$ in marsupials (Dorner *et al.*, 2001), has been further questioned for rat and human mitochondria, which appear to import $tRNA^{Gln}$(CUG) and (UUG) encoded by the nuclear genome (Rubio *et al.*, 2008). In *S. cerevisiae*, $tRNA^{Lys}$(CUU) (Tarassov and Entelis, 1992) and two isoacceptors of cytosolic $tRNA^{Gln}$ were found in the organelles (Rinehart *et al.*, 2005). Finally, one third to one half of the mitochondrial tRNAs are imported from the cytosol in plants (Glover *et al.*, 2001; Kumar *et al.*, 1996; Marechal-Drouard *et al.*, 1990). The number and identity of imported tRNAs in plant mitochondria can vary even between very close species (Glover *et al.*, 2001; Kumar *et al.*, 1996).

The tRNAs imported from the cytosol complement those found in the mitochondria, thus raising the hypothesis of a possible influence of the population of tRNAs encoded by the mtDNA on the identity of the imported tRNAs. Also, in each case, the concerned tRNAs are shared between the cytosol and the mitochondria and are involved in protein synthesis in both compartments.

A number of import signals have been detected in tRNAs but, in view of the mechanisms described (Bouzaidi-Tiali *et al.*, 2007; Salinas *et al.*, 2008; Tarassov *et al.*, 2007), it turned out that these signals were often very different, depending on the organisms considered. Thus, the type of recognition is not general for all imported tRNAs. In protists, two types of imported tRNAs have been identified: type I tRNAs have import determinants located in the D loop or anticodon (Mahapatra *et al.*, 1998; Rusconi and Cech, 1996), whereas in type II tRNAs import determinants are in the T arm (Suyama *et al.*, 1998). Besides the determinants of import, there are anti-determinants that prevent the import of some cytosolic tRNAs (Bouzaidi-Tiali *et al.*, 2007). In plants, various determinants of import have been identified. The study of $tRNA^{Val}$ (Delage *et al.*, 2003b) and $tRNA^{Gly}$ (Salinas *et al.*, 2005) showed the involvement of the D arm and the anticodon, while analysis of $tRNA^{Ala}$ uptake highlighted the role of the G3:U70 base-pair in the acceptor arm (Dietrich *et al.*, 1996b).

It is commonly accepted that tRNAs are not free in the cytosol, but they are at all stages in association with proteins that essentially channel them to cytosolic translation. Hence the need for import factors that would allow a selected set of tRNAs to escape from the cytosolic protein synthesis pathway and to become directed towards the organelles. To date, two such protein factors have been identified in *S. cerevisiae* and *Trypanosoma brucei* (Brandina *et al*., 2006; Bouzaidi-Tiali *et al*., 2007). In plants, *in vivo* mitochondrial import does not occur if recognition of the concerned tRNA by the corresponding aminoacyl-tRNA synthetase (aaRS) is abolished (Dietrich *et al.*, 1996b; Delage, *et al*., 2003b). Therefore, it is tempting to think that in plants aaRSs could allow targeting of tRNAs to mitochondria. However, it has been shown that there are import determinants that are not determinants of recognition by the aaRS (Delage *et al.*, 2003b; Laforest *et al.*, 2005). On the other hand, recognition by an imported aaRS is not sufficient to promote import of a tRNA, suggesting the involvement of other factors in this process (Dietrich *et al.*, 1996a).

Altogether, the results suggest that, in all organisms, components of the mitochondrial protein translocation pathway are required for tRNA uptake *in vivo* (Schneider, 2011; Tschopp *et al.*, 2011). In yeast, after trafficking towards mitochondria as a complex with the enolase, cytosolic $tRNA^{Lys}$(CUU) seems to be handed over to the precursor of the mitochondrial lysyl-tRNA synthetase and to be driven by the protein import system (including TOM20 and TIM44) to pass through the mitochondrial membranes (Brandina *et al.*, 2006; Entelis *et al.*, 2006; Tarassov *et al.*, 1995). Mitochondria isolated from human HepG2 cells are able to import yeast $tRNA^{Lys}$CUU and several mutated forms of this tRNA (Kolesnikova *et al.*, 2000). The process requires ATP, protein receptors on the outer membrane, and a membrane potential. The precursor of yeast mitochondrial lysyl-tRNA synthetase, and other unidentified cytosolic factors, are able to direct $tRNA^{Lys}$ import into human mitochondria (Entelis *et al.*, 2002). The import of $tRNA^{Gln}$ into isolated yeast mitochondria requires no prior aminoacylation or addition of cytosolic factors, suggesting that in this case the mechanism is different from that followed by $tRNA^{Lys}$(CUU) (Rinehart *et al.*, 2005). In plants, the voltage-dependent anion channel (VDAC), together with subunits of the TOM translocase of the protein import pathway seem to be involved in tRNA translocation through the outer membrane (Salinas *et al.*, 2006). Studies are underway in the different organisms to identify the proteins of the inner membrane that play a role in the translocation of tRNAs.

Studies on *Leishmania tropica* describe a very different mechanism for mitochondrial tRNA import, not depending on the protein import pathway. The proposed mechanism involves a specific receptor (called "TAB") in the outer membrane (Adhya *et al.*, 1997) for tRNAs to be imported and an RNA import complex (called "RIC") in the inner membrane (Bhattacharyya *et al*., 2003). It was shown that, of the 11 subunits of the RIC complex, 6 are essential for the import of tRNAs (Mukherjee *et al.*, 2007). Based on the import of $tRNA^{Tyr}$ (type I) and $tRNA^{Ile}$(UAU) (type II), a model of allosteric control of tRNA translocation has been proposed for *L. tropica* (Bhattacharyya *et al.*, 2003; Chatterjee *et al.*, 2006; Goswami and Adhya, 2006). According to this model, $tRNA^{Tyr}$ binds to the receptor RIC1, subunit of the RIC complex, while $tRNA^{Ile}$ is recognized by RIC8A. Type I tRNAs can be imported directly and stimulate the import of type II tRNAs. Conversely, type II tRNAs inhibit the import of type I tRNAs. It must however be noted that the existence of the RIC complex is still a matter of controversy in the field and that part of these studies raised an expression of concern (Schekman, 2010).

The regulation of tRNA import into mitochondria is still poorly understood. In *S. cerevisiae*, studies showed the involvement of the temperature (Kamenski *et al.*, 2007) and of three components (Rpn8p, Rpn13p and Doa1p) of the ubiquitin proteasome system (UPS) in the regulation of $tRNA^{Lys}$(CUU) import (Brandina *et al.*, 2007).

All these data have opened up exciting therapeutic prospects. A large number of mitochondrial diseases in humans are caused by mutations in mitochondrial tRNA genes (Greaves *et al.*, 2012; Tuppen *et al.*, 2010). Therefore, strategies are being developed to manipulate the mammalian mitochondrial genetic system by exploiting the tRNA import mechanism, thanks especially to the robust knowledge gained on the import of $tRNA^{Lys}$(CUU) into yeast and human mitochondria. The relevance and the potential of customized tRNA targeting for the development of therapeutic strategies to cure mitochondrial disorders is discussed in section 5.2 and section 9 of the review paper (Niazi *et al.*, 2012) mentioned earlier.

III.4. A tRNA-derived mitochondrial shuttling system in plants

To date, the strategies developed to manipulate the mitochondrial genetic system are designed mainly in mammalian cells, expecting on the longer term to complement mtDNA mutations in humans rather than to analyze mitochondrial genetic mechanisms. Some are still in the *in vitro* or *ex-vivo* stage and *in vivo* potentialities are still uncertain (Karicheva *et*

al., 2011; Kolesnikova *et al.*, 2000, 2004; Mahata *et al.*, 2006; Smirnov *et al.*, 2008). Approaches based on the use of the RIC complex in mammals are apparently restricted to their home laboratory (Mahata *et al.*, 2006; Paris *et al.*, 2009). Conversely, two strategies dedicated to plant mitochondria have been developed in our department. The first one is still at the stage of isolated mitochondria and relies on an RNA-binding protein to promote the import of different RNA substrates (Sieber *et al.*, 2011). The second started from the idea to use a naturally imported tRNA as a shuttle to take RNA sequences of interest into the organelles *in vivo* (Val *et al.*, 2011).

In plants, $tRNA^{Leu}$ is among those tRNAs that are expressed by the nuclear genome and imported naturally into the mitochondria (Kumar *et al.*, 1996; Marechal-Drouard *et al.*, 1990). Small *et al*. (1992) used $tRNA^{Leu}$ from *Phaseolus vulgaris* as an *in vivo* import shuttle in potato (*Solanum tuberosum*) by inserting four extra nucleotides into the anticodon loop. The extended tRNA was expressed from a nuclear transgene and successfully recovered in mitochondria. However, it turned out to be difficult to introduce further additional sequences into the tRNA without losing functionality and *in vivo* stable expression, so that the strategy did not work as such for longer sequences or for insertions into other regions of the tRNA (Small *et al.*, 1992). In other attempts developed by our laboratory, foreign sequences were attached at the 5'-end of $tRNA^{Leu}$ but were removed by RNase P, which only recognizes the structure of the tRNA and cleaves any upstream sequence (Schon, 1996). Relying on natural nuclear tRNA gene expression was also a problem, as the transcription start site for polymerase III is located only a few nucleotides upstream of the tRNA gene (Choisne *et al*., 1998). These findings led to an RNA polymerase II-based approach using as a mitochondrial import shuttle, instead of an authentic tRNA, a structure that mimics a tRNA but that is not recognized *in vivo* by RNase P.

The genomic RNAs of many plant viruses have at their 3'-end a tRNA-like structure (TLS) that can be aminoacylated but is not a regular substrate for RNase P (Dreher, 2009; Fechter *et al.*, 2001). The choice pointed towards the TLS of the *Turnip yellow mosaic virus* (TYMV) (Matsuda and Dreher, 2004). The TYMV genome consists of a positive RNA strand with a length of 6318 nucleotides (Blok *et al.*, 1987). This RNA is capped at the 5' end and has a TLS at the 3'-end (Giegé *et al.*, 1993). The TYMV TLS consists of the last 82 nucleotides of the genomic RNA of the virus and functionally mimics $tRNA^{Val}$, sharing many properties with this tRNA (Filichkin *et al*., 2000; Matsuda and Dreher, 2004;

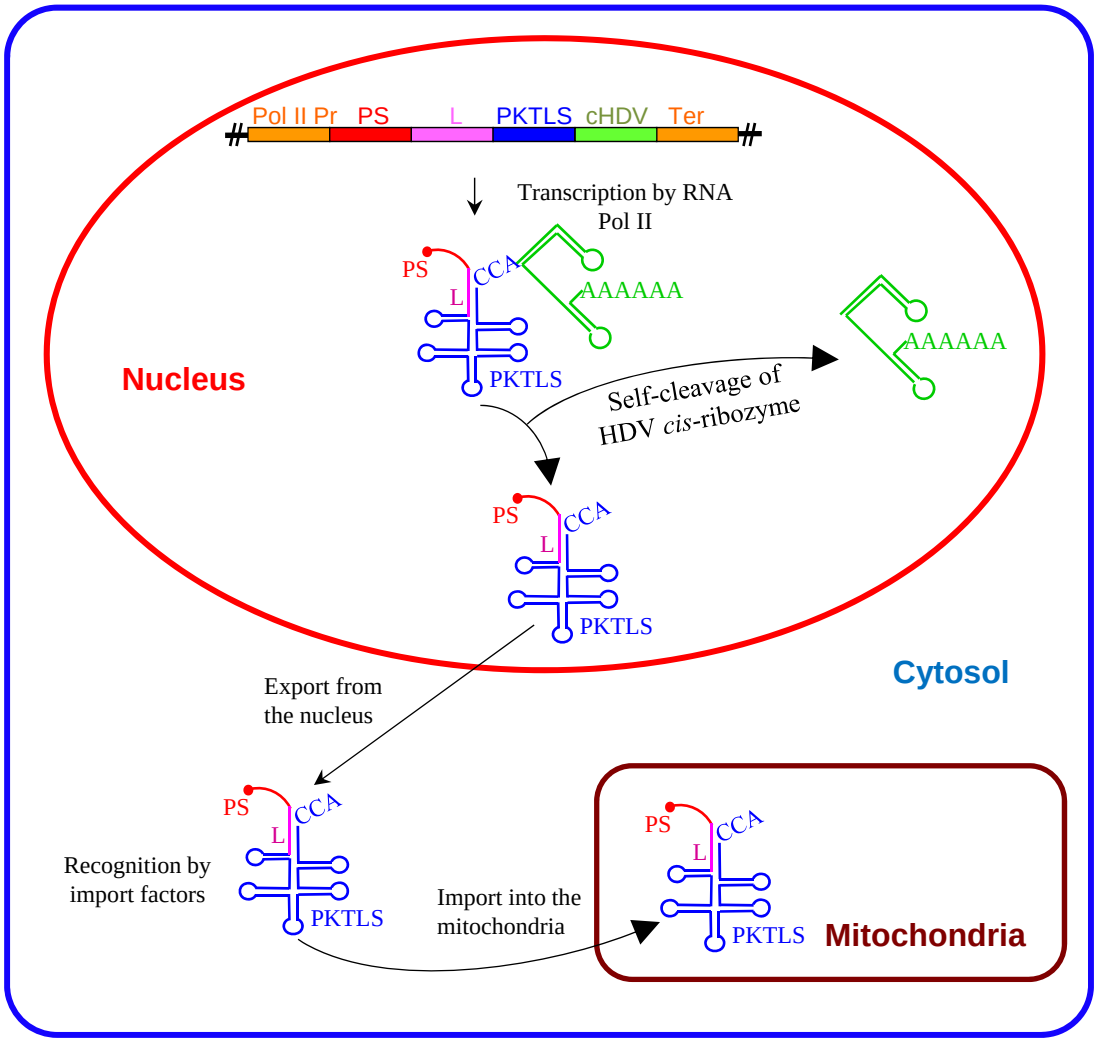

Figure 4: The tRNA-derived mitochondrial shuttling system
The passenger sequence along with the PKTLS is transcribed in the nucleus and transported into the mitochondria through the cytosol with the help of the natural tRNA import pathway. Pol II Pr: RNA polymerase II promoter, PS: Passenger sequence, L: linker sequence, PKTLS: pseudoknot/tRNA-like structure, cHDV: HDV *cis*-ribozyme, Ter: terminator.

Hammond *et al*., 2009; Dreher, 2010). The sequence between nucleotides 83 and 109 upstream of the TLS in the TYMV RNA adopts a pseudoknot structure (UPSK) that helps the TLS to be recognized by valyl-tRNA synthetase (Matsuda and Dreher, 2004). Recognition by the cognate aaRS is essential for *in vivo* tRNA import into mitochondria in plant cells (Delage *et al.*, 2003a; Dietrich *et al.*, 1996b). As it is located at the 3'-end of the viral genome, the TYMV TLS appears to be very stable even when it is associated with a long upstream sequence. It is not matured by RNase P *in vivo*, although *in vitro* experiments showed that, under certain conditions, it can be cleaved in the acceptor region by the RNase P of *E. coli* (Guerrier-Takada *et al.*, 1988; Mans *et al.*, 1990). The TYMV TLS mimics $tRNA^{val}$ and $tRNA^{Val}$ is naturally imported into the mitochondria of all higher plants studied to date. Altogether, these properties provided the basis for a shuttle system supporting mitochondrial targeting of customized RNAs expressed from nuclear transgenes in plant cells and whole plants. The ultimately selected mitochondrial import shuttle, called PKTLS, consists of the last 120 nucleotides (6199-6318) of the 3'-end of the TYMV genomic RNA, including the TLS and the UPSK that optimizes the interaction with the aaRS (Val *et al.*, 2011).

In this strategy (Figure 4), the "passenger" sequence of interest is attached to the TYMV PKTLS as a 5' trailor sequence. For expression, so as to account for all the constraints, the sequence coding for the chimeric RNA (passenger RNA fused to the 5'-end of the PKTLS through a linker) is followed by the sequence encoding the *cis*-cleaving ribozyme of the *Hepatitis delta virus* (HDV) and placed under the control of an RNA polymerase II promoter (constitutive or inducible) and terminator. These sequences are introduced as a transgene into the nuclear genome. A transcript including the passenger sequence, the linker, the TYMV-PKTLS, the HDV-*cis*-ribozyme and the termination sequence is first synthesized by RNA polymerase II. This RNA is capped at the 5'-end and polyadenylated at the 3'-end. The autocatalytic cleavage of the HDV *cis*-ribozyme subsequently eliminates the termination sequence and poly(A) tail, unmasking the 3'-CCA-end of the TLS. The resulting [passenger sequence/linker/TYMV-PKTLS] chimeric RNA is then exported out of the nucleus and imported into mitochondria (Val *et al.*, 2011). There is also a mutated form of the TYMV TLS that is aminoacylable with methionine (TLS^{met}) (Dreher *et al.*, 1996). But $tRNA^{Met}$ is not imported into the mitochondria of plants. TLS^{met} thus served as a negative control for the import of passenger sequence/PKTLS transcripts. A passenger

sequence/PKTLS^{met} RNA indeed was not recovered in mitochondria, which means that the process follows the natural pathway of tRNA uptake (Val *et al.*, 2011).

The passenger sequence (PS) may be of several types: a hammerhead ribozyme designed to cleave in *trans* a target RNA in mitochondria, an antisense RNA able to functionally block a mitochondrial target RNA, an RNA normally present in mitochondria (mRNA, non-coding RNA, CMS-associated RNA), an RNA expressing a novel protein or adding a new function. The linker, adapted from a random unstructured sequence of forty nucleotides, is introduced to prevent interactions and alternative structures between the passenger sequence and the PKTLS.

The strategy was functionally validated in stably transformed cell suspensions of *Nicotiana tabacum* and whole plants of *A. thaliana* through the import of a *trans*-cleaving hammerhead ribozyme directed against a mitochondrial mRNA. The approach was efficient and the chimeric catalytic RNA caused a strong knockdown of the target mRNA in mitochondria (Val *et al.*, 2011). For the first time, it became possible to act directly on the mitochondrial genetic system in plant cells, opening the way for the analysis of the molecular and physiological effects resulting from the manipulation of mitochondrial RNA levels.

IV. Coordination between the nucleus and the organelles

Plant cells contain two types of energy producing organelles: chloroplasts and mitochondria. Chloroplasts convert solar energy into chemical energy by photosynthesis, while mitochondria generate ATP by oxidative phosphorylation (OXPHOS). Besides their central functions, these organelles play important roles in diverse processes such as redox homeostasis, provision of precursor molecules for essential biosynthetic pathways, and programmed cell death. These different functions require the organelles to communicate with the rest of the cell by perceiving, transducing, and emitting signals. Moreover, chloroplasts and mitochondria contain thousands of proteins, most of which are encoded by nuclear genes. Only few genes, which encode mainly components of the OXPHOS complexes (in mitochondria) or the photosynthetic apparatus (in chloroplasts), are localized in the organellar genomes. Chloroplastic and mitochondrial complexes are thus assembled from both nuclear-encoded and organelle-encoded subunits, hence the need for coordinated genome expression to produce the correct protein ratios. Such coordinating mechanisms include both anterograde (nucleus to organelle) and retrograde (organelle to nucleus) signals

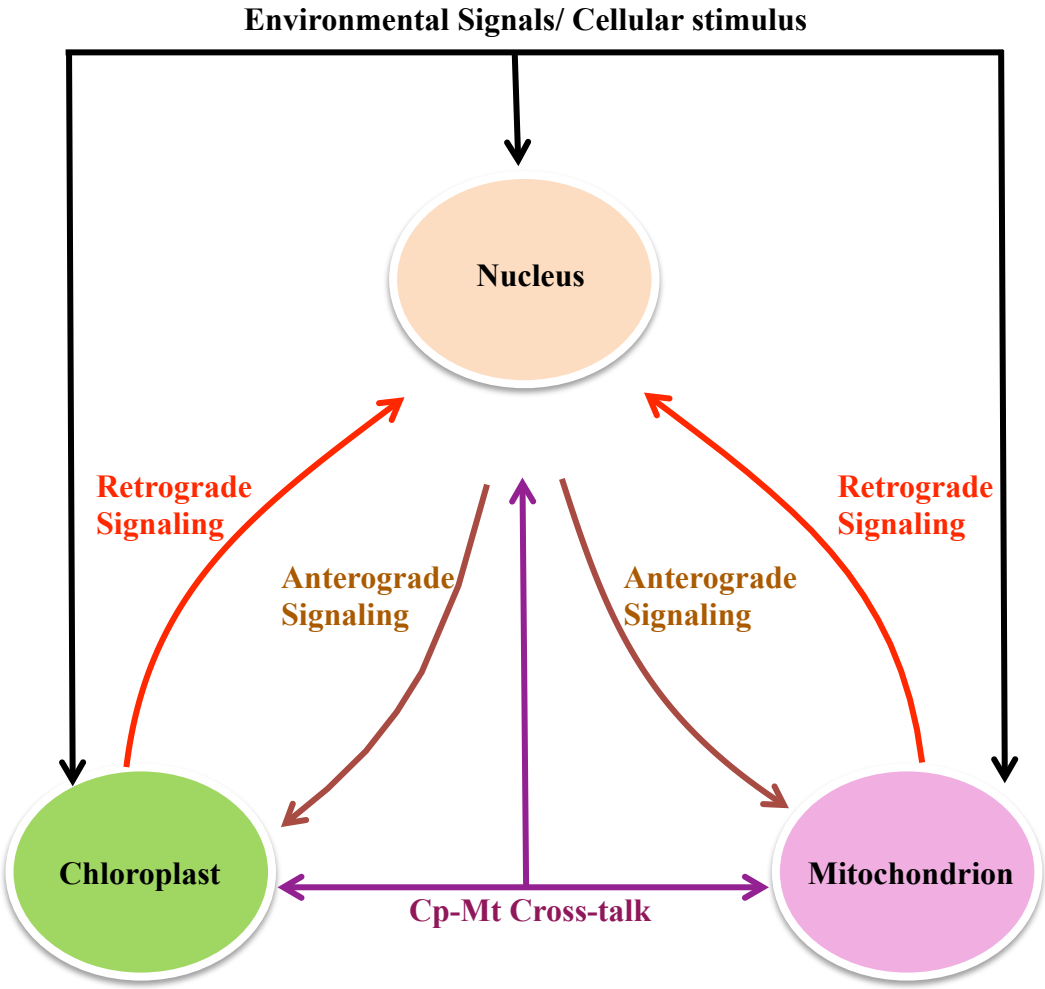

Figure 5: Genome coordination between the nucleus and plant organelles
Anterograde signaling: nuclear-coded proteins are the vehicles of communication from the nucleus to the mitochondrion or the chloroplast. Retrograde signaling: the metabolic signals released from the organelles play the role for communication from the organelles to the nucleus. Adapted from Woodson and Chory, 2008. Cp: Chloroplast, Mt: Mitochondrion.

(Figure 5). Anterograde mechanisms control organelle gene expression in response to endogenous or exogenous (environmental) signals that are perceived by the nucleus. Retrograde regulation refers to signals sent by the organelles to communicate their functional and developmental state to the nucleus, which can then modulate anterograde control and cellular metabolism accordingly (Liu and Butow, 2006; Woodson and Chory, 2008). Mitochondrial retrograde regulation (MRR) is an important mechanism of communication between mitochondria and the nucleus in both unicellular eukaryotes and metazoan cells. Mitochondrial signaling pathways impact a wide spectrum of cellular activities under both normal and pathophysiological conditions. The existence of MRR is thus conserved among yeast, mammals, and plants. However, the signaling molecules and the signal transduction mechanisms are diversed among species (Liu and Butow, 2006). MRR is documented in yeast and in animals (Liu and Butow, 2006), whereas the nature of these signaling pathways and their underlying signaling molecules are mostly unknown in plants. Conversely, chloroplast retrograde regulation (CRR) is well described (Nott *et al.*, 2006; Zhang, 2007).

In animals, MRR has been explored in particular due to its connection with mitochondrial dysfunction and it is now thought to be an important factor in many human diseases. Mitochondrial dysfunction, and in some cases MRR, has been connected to Alzheimer's disease, Huntington's disease, schizophrenia, bipolar disorder, Parkinson's disease, cancer, type 2 diabetes, and aging. MRR is a relatively new view angle on the importance of mitochondria in diseases (Rhoads and Subbaiah, 2007 and reference therein). There are multiple signaling pathways from mitochondria to the nucleus in mammals, although some target genes are common between the MRR pathways (Butow and Avadhani, 2004). In both mammalian cells and yeast, retrograde signaling is also linked to TOR signaling, but the precise signals and connections that interlink these pathways are unclear (Liu and Butow, 2006). In plants, some signaling components that may function in MRR pathways have been identified, but much more about MRR mechanisms and components is yet to be discovered. MRR plays an important role in cytoplasmic male sterility, biotic and abiotic stress (Yang *et al.*, 2008).

In this section, I will first give a brief description of MRR pathways in yeast, where they are best described, and I will explain the RTG pathway. Then, I will discuss CRR, MRR and cross-talk between mitochondria and chloroplasts in plants.

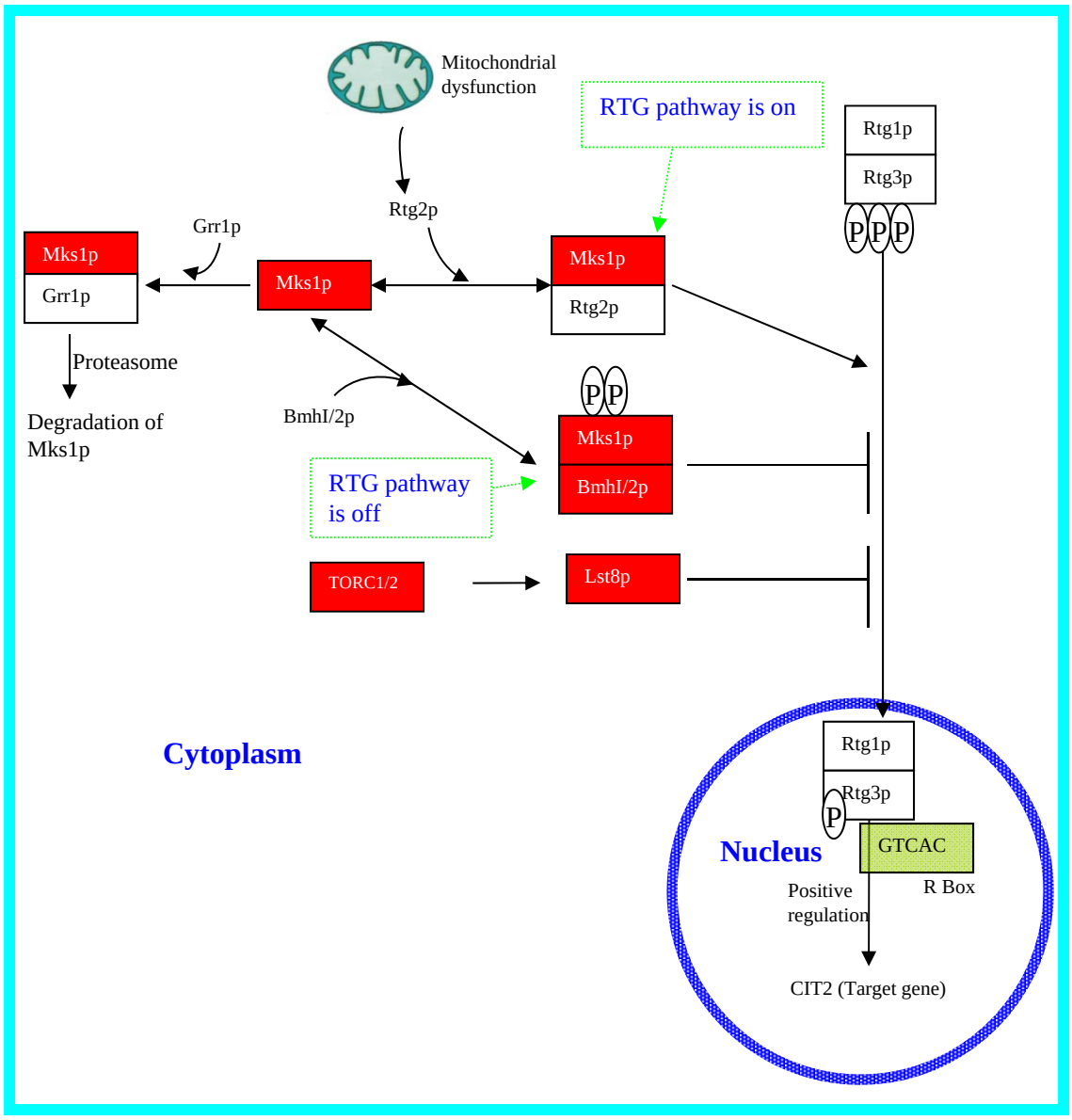

Figure 6: The mitochondrial retrograde signaling pathway

The mitochondrial retrograde signaling pathway is mediated by four positive regulatory factors, Rtg1p, Rtg2p, Rtg3p, Grr1p, and four negative regulatory factors, Mks1p, Lst8p, and two 14-3-3 proteins, Bmh1p and Bmh2p. Rtg2p-dependent dephosphorylation of Rtg3p correlates with Rtg3p's nuclear translocation and activation of the RTG pathway. The critical regulatory step of the RTG pathway is the dynamic interaction between Rtg2p and Mks1p. Binding of Rtg2p to Mks1p leads to activation of the RTG pathway. Interaction of Mks1p with Bmh1/2p after its dissociation from Rtg2p leads to inhibition of nuclear translocation of Rtg1/3p. Grr1p-dependent degradation of free Mks1p ensures an efficient Mks1p switch between the Rtg2p-Mks1p complex and the Mks1p-Bmh1/2p complex and is required for induced expression of *CIT2*. Lst8p is an integral component of TOR kinase complexes. It negatively regulates the RTG pathway. In figure, the negative regulators are shown by red boxes and positive regulators are shown by white boxes. (Modified from Butow and Avadhani, 2004; Liu and Butow, 2006)

IV.1. Mitochondrial retrograde regulation (MRR) in Yeast

The first work on mitochondrial retrograde signaling was performed in yeast (Parikh *et al.*, 1987). The most detailed information on the scope and regulation of the retrograde response has been obtained with *S. cerevisiae* (Liu and Butow, 2006; Zdralević *et al.*, 2012). In yeast, retrograde signaling senses mitochondrial activities, allowing cells to adjust their metabolism appropriately. These adjustments involve the integration of carbohydrate and nitrogen metabolisms. Signal transduction from mitochondria to the nucleus in yeast involves several MRR pathways with different types of signaling molecules. The RTG and mTOR pathways, hypoxic signaling, and intergenomic signaling are currently documented in yeast (Liu and Butow, 2006; Woo *et al.*, 2009).

The most extensive studies on retrograde regulation have been done using ClT2 (coding for citrate synthase) as a nuclear target gene. The best studied MRR pathway in yeast is the so-called RTG-dependent pathway (also called RTG pathway, Figure 6) that involves the activation of genes directed at restoring TCA cycle intermediates through RTG transcription factors (Butow and Avadhani, 2004). I will explain this pathway later. However, some other pathways have been described that appear to be independent from the RTG pathway. Mitochondrial dysfunction in *rho*^{0} cells can cause increased expression of PDR5, a nuclear gene encoding an ABC (ATP-binding cassette) membrane transporter involved in multiple or pleitropic-drug resistance. Induction is *via* transcription factors Pdr1p and Pdr3p (Devaux *et al.*, 2002; Moye-Rowley, 2005). Similarly, mitochondrial dysfunction also induces ATO3, which encodes a plasma membrane ammonium transporter, through transcription factors Gcn4p and Ssy1-Ptr3-Ssy5, which are involved in amino acid control (Guaragnella and Butow, 2003). Another pathway acting *via* the Abf1p transcription factor, called intergenomic signaling, alters nuclear gene expression in response to the lack of mtDNA and also appears to be independent from the RTG pathway (Woo *et al.*, 2009). Further, there is a separate hypoxic signaling pathway between mitochondria and the nucleus, where cells respond to low oxygen concentrations by upregulating hypoxic nuclear genes. Mitochondrially produced nitric oxide (NO) is involved in this kind of signaling, possibly *via* a pathway that involves protein tyrosine nitration (Castello *et al.*, 2006; Kwast *et al.*, 1998).

As said earlier, the most elaborated MRR pathway in yeast is the RTG pathway (reviewed in Butow and Avadhani, 2004; Liu and Butow, 2006). There are four positive regulators (*i.e.* Rtg1p, Rtg2p, Rtg3p and Grr1p) and four negative regulators (*i.e.* Mks1p, Lst8p and two

14-3-3 proteins, Bmh1p and Bmh2p) in the RTG pathway. The *RTG1* and *RTG3* genes encode basic helix-loop-helix leucine zipper-type (bHLH/Zip) transcription factors (Jia *et al.*, 1997). These factors bind to the promoter region of target genes by forming a heterodimer. The transcriptional activation domain of the Rtg1/3p complex is contained within Rtg3p (Sekito *et al.*, 2000). Unlike most bHLH-type transcription factors, which bind to the consensus sequence CANNTG called the E box (Massari and Murre, 2000), Rtg1/3p binds to a different site, GTCAC, termed as R box (Jia *et al.*, 1997). Rtg1p helps Rtg3p to bind to the R box. Rtg3p is a phosphoprotein, whereas Rtg1p is not. Activation of Rtg3p correlates with its partial dephosphorylation and its nuclear translocation with Rtg1p (Sekito *et al.*, 2000). Rtg2p is a novel cytosolic protein with an N-terminal ATP binding domain belonging to the actin/Hsp70/sugar kinase superfamily (Bork *et al.*, 1992). Rtg2p acts upstream of the Rtg1/3p complex while regulating CIT2 expression (Rothermel *et al.*, 1997). Rtg2p is essential for Rtg3p dephosphorylation and nuclear translocation of Rtg1/3p. Integrity of the ATP binding domain of Rtg2p is essential for its function (Liu *et al.*, 2003). Grr1p functions as a positive regulator of the retrograde pathway by mediating ubiquitination of the cytosolic negative regulator Mks1p, resulting in degradation of Mks1p (Liu *et al.*, 2005).

As to the negative regulators, Mks1p has been implicated in several pathways. It functions downstream of Rtg2p but upstream of Rtg1/3p. Mks1p negatively regulates the RTG pathway by promoting the phosphorylation of Rtg3p and inhibiting the nuclear translocation of Rtg1/3p (Sekito *et al.*, 2002). Mks1p is regulated through reversible binding with Rtg2p and through SCF Grr1-dependent ubiquitination and subsequent degradation (Liu *et al.*, 2003, 2005). Lst8p is an essential protein consisting of seven WD-repeats (tryptophan-aspartate repeats) (Liu *et al.*, 2001). A WD-repeat is a sequence motif consisting of about 40 amino acids, multiples of which are predicted to form a ß-propeller structure, as exemplified in the G protein ß subunit (Smith *et al.*, 1999). Lst8p is an integral component of two TOR (target of rapamycin) kinase complexes, TORC1 and TORC2 (Wedaman *et al.*, 2003). Genetic data suggest that Lst8p negatively regulates the RTG pathway (Liu *et al.*, 2001). One mechanism for 14-3-3 proteins to function as negative regulators in the RTG pathway is by preventing Mks1p from SCF Grr1-dependent ubiquitination and degradation (Liu *et al.*, 2003, 2005). There are two functionally redundant 14-3-3 proteins in yeast, encoded by *BMH1* and *BMH2*. Bmh1p is more abundant than Bmh2p (Gelperin *et al.*, 1995). Deletion of both genes is lethal in most strain backgrounds. Another possible mechanism for

Bmh1/2p to regulate the RTG pathway is to bind to Rtg3p and keep it in an inactive state (Van Heusden and Steensma, 2001).

In short, it is clear that altered nuclear gene expression in yeast is important for the response of cells to various environmental and developmental cues and that several distinct MRR pathways are involved in promoting this altered expression. It will be very interesting to determine if there are any overlaps in plants.

IV.2. Chloroplast retrograde regulation (CRR)

CRR pathways in plants have been relatively well studied, due to their significance and to the readily observable phenotypes of CRR mutants affected in the greening process (Woodson and Chory, 2008). From studies on *albostrians* and *Saskatoon* mutants of barley, which contain undifferentiated plastids that lack ribosomes, Bradbeer *et al*. (1979) provided the first evidence some 30 years ago that signals from chloroplasts can regulate nuclear gene expression. These mutants did not accumulate nuclear-encoded photosynthetic mRNA transcripts (Emanuel *et al.*, 2004; Nott *et al.*, 2006). This retrograde signal from chloroplasts, originally termed ‘‘plastid signal’’ or ‘‘plastid factor’’, alters the transcriptional regulation of nuclear genes and is quite complex (Oelmüller and Mohr, 1986; Oelmuller, 1989). During the past decade, genetic and biochemical studies in *Chlamydomonas reinhardtii* and *A. thaliana* have helped to characterize CRR pathways. It looks like distinct plastid signals are produced from different sources and that multiple, partially redundant CRR pathways exist (Pesaresi *et al.*, 2007; Woodson and Chory, 2008). No CRR pathway is well deciphered mechanistically but, still based on the sources of the signals, four distinct putative CRR pathways have been traditionally recognized: tetrapyrrole signaling, PGE (plastid gene expression) signaling, redox and reactive oxygen species (ROS) signaling. However, in addition to these classical pathways, several novel CRR pathways have also been proposed. For example, PAP (3’-phosphoadenosine 5’-phosphate) and MEcPP (methylerythritol cyclodiphosphate) are two novel possible retrograde signals involved in stress responses (Chi *et al.*, 2013).

The tetrapyrrole signaling pathway is the best characterized CRR pathway and involves tetrapyrrole intermediates of the chlorophyll biosynthetic pathway. Four classes of tetrapyrroles— chlorophyll, heme, siroheme, and phytochromobilin—are derived from a common biosynthetic pathway that resides in plastids. Chlorophyll, heme, and phytochromobilin synthesis follow a common pathway till proto IX but, at the point of iron

insertion, chlorophyll synthesis diverges from others (Mochizuki *et al.*, 2010; Tanaka and Tanaka, 2007). Two sets of evidence support the involvement of tetrapyrrole intermediates in CRR. First, the normal expression profile of nuclear genes is impaired by applying inhibitors at several steps of the tetrapyrrole biosynthesis pathway, whereas a normal profile can be restored by feeding back specific tetrapyrrole intermediates. Second, mutants defective in tetrapyrrole biosynthesis show disrupted retrograde signaling (Chi *et al.*, 2013). Both Mg–proto IX and heme may be involved in this pathway in *C. reinhardtii.* Mg–proto IX, its methyl ester (Mg–proto–Me) and its immediate precursors in chlorophyll synthesis may act as negative factors required for the repression of LHCB gene transcription in the nucleus (Beck and Grimm, 2006). CRR signaling was further supported by the observation that Mg–proto IX and Mg–proto–Me can also substitute for light to induce the transcription of at least two heat shock proteins, HSP70A and HSP70B, that can be induced by light *via* a heat-independent pathway (Kropat *et al.*, 1997, 2000). GUN5/CHLH and GUN4 are involved in the biosynthesis of Mg–proto IX, which is suggested to be exported from plastids *via* an unknown mechanism, then to bind to the HSP90 protein and trigger further signaling (Chi *et al.*, 2013). In contrast to Mg– proto IX, heme may be a positive retrograde signal in plants, as in the *gun6* mutant increased flux through the heme branch of the tetrapyrrole biosynthesis pathway increases the expression of photosynthesis-associated nuclear genes (Woodson *et al.*, 2011). GUN6 (ferrochelatase), GUN2 (heme oxygenase), and GUN3 (phytochromobilin synthase) are involved in heme signaling but the nuclear components and the exact mechanism by which heme reaches the nucleus are unknown (Chi *et al.*, 2013).

A second CRR pathway, the PGE pathway (plastid gene expression), was discovered by using inhibitors of chloroplast translation such as lincomycin (LIN), which resulted in the repression of nuclear photosynthesis genes (Gray *et al.*, 2003). Initially, it was proposed that the PGE signaling pathway works only during organelle development, as it was thought that in mustard it is active only in young seedlings (Nott *et al.*, 2006). Later characterization of the *A. thaliana prors1* mutant, which has mutations in the PRORS1 nuclear gene encoding a plastidial and mitochondrial prolyl-tRNA synthetase, indicated that PGE signaling may also persist in mature leaf tissues (Pesaresi *et al.*, 2006). It appears that the PGE pathway is light-independent, since treatment with LIN in the dark can still repress LHCB expression (Sullivan and Gray, 1999). The signals arising from PGE are largely unknown. Those signals and tetrapyrrole signals may converge on GUN1, which either generates or transmits

a second signal to the nucleus and modulates nuclear gene expression by inducing the ABI4 transcription factor (Chi *et al.*, 2013).

Redox signaling is another important CRR pathway. Three main sources of retrograde signals have been proposed that involve the assembled photosynthetic electron transport chain in developed chloroplasts: the redox state of the plastoquinone pool, the photosystem I (PSI) acceptor site and the thioredoxin system. Multiple nuclear genes are under the control of plastid redox signals, but different genes respond to signals originating from different sources (Baier and Dietz, 2005; Pesaresi *et al.*, 2007). For example, the expression of the LHCB, PETE, and APX genes is regulated by the redox state of the plastoquinone pool, whereas the expression of the PSAF, PSAD, nitrate reductase and ferredoxin genes is regulated by components in the electron transport chain distinct from the plastoquinone pool (Chi *et al.*, 2013). Little is known about the detailed mechanisms of chloroplast redox retrograde signaling. However, genetic analyses in *A. thaliana* have identified some components, including STN7, a thylakoid light-harvesting complex II (LHCII) membrane protein kinase involved in state transition and photosynthetic acclimation. STN7 (known as STT7 in *C. reinhardtii*) may participate in transmitting the changes in chloroplast redox status to the nucleus (Pesaresi *et al.*, 2009; Puthiyaveetil *et al.*, 2012). In a recent study, another component, PLASTID REDOX INSENSITIVE 2 (PRIN2), which is required for expression of the genes transcribed by the plastid-encoded RNA polymerase (PEP), was identified as participating in redox signaling. The *A. thaliana prin2* mutants show misregulation of photosynthesis-associated nuclear genes in response to excess light and inhibition of photosynthetic electron transport, suggesting a direct role for PEP activity in redox signaling (Kindgren *et al.*, 2012).

ROS molecules derived from plastids are involved primarily in stress signaling rather than in genome coordination. Under stress, several chemically distinct ROS molecules (superoxide ion; hydrogen peroxide; hydroxyl radical and hydroxyl ion) are simultaneously generated in plastids and it is difficult to link a particular stress response to a specific ROS. H_2O_2 and $^1O_2$ accumulate and activate distinct signaling pathways during exposure to excess light. Accumulation of $^1O_2$ is sensed or transmitted to the nucleus through two chloroplast proteins, EXECUTER 1 (EX1) and EXECUTER 2 (EX2). ROS and redox signaling may also converge on the GUN1-ABI4 pathway (Chi *et al.*, 2013; Woodson and Chory, 2008).

The nature of the plastid signaling molecules remains a mystery, although Mg–proto IX, heme, ROS and metabolites are among the top candidates to serve as retrograde signaling molecules (Chi *et al.*, 2013; Woodson and Chory, 2008). Nuclear responses to plastid retrograde signals involve several layers of transcriptional controls mediated by key transcription factors and *cis*-elements of nuclear gene promoters. Two distinct mechanisms are probably involved in this control. One is a proposed "master switch" (GUN1 being the top candidate) that induces or represses the same large set of nuclear genes, while the other is co-regulation of nuclear and plastid genes, which seems to be widely conserved in plants (Chi *et al.*, 2013; Leister, 2012). Retrograde signals are produced in plastids, but how they are perceived in the cytosol and communicated to the nucleus remains largely unknown.

IV.3. Mitochondrial retrograde regulation (MRR) in plants

The study of plant MRR is a young field, as compared to yeast and animals, and the mechanisms and components are just beginning to be discovered. So far, MRR targets and mechanisms appear to differ between plants and yeast. Like in mammals, there are no homologs of the yeast RTG pathway in plants. Mitochondrial retrograde signaling in plants has been implicated in ROS signaling, O_2 sensing, heat shock, pathogen sensing and programmed cell death. Retrograde signals from dysfunctional mitochondria might also be the cause of at least some types of CMS in flowering plants (Rhoads and Subbaiah, 2007). Evidence suggests that ROS can be involved in plant MRR pathways but not in all cases. For example, TCA cycle inhibition strongly induces alternative oxidase (AOX) gene expression but does not lead to a dramatic increase in cellular ROS, while for most of the biotic and abiotic stresses production of ROS is strongly correlated with expression of AOX genes (Dojcinovic *et al.*, 2005; Rhoads, 2011).

What is known about plant MRR mostly comes from systems with disturbed mitochondrial functions induced by mutations, chemical agents, transgenes or biotic and abiotic stresses. Mitochondrial dysfunction ultimately causes changes in nuclear gene expression and impairment of the mitochondrial electron transport chain. This causes subsequent induction of genes coding for proteins that are involved in recovery of mitochondrial functions, such as alternative NAD(P)H dehydrogenases and AOX, and genes coding for antioxidant enzymes aimed at normalizing ROS levels, such as glutathione transferases, catalases, superoxide dismutases and ascorbate peroxidases (Rhoads, 2011; Rhoads and Subbaiah, 2007). However, with the emerging evidence of new and interesting targets of MRR, this picture is likely to change. The complexity and importance of MRR in plant responses to

stresses and the decision for recovery or death will become more apparent (Rhoads and Subbaiah, 2007).

As to signaling components, the participation of the ABSCISIC ACID INSENSITIVE 4 (ABI4) transcription factor in repression of AOX gene expression is the only established evidence for the involvement of a specific protein in plant MRR pathways (Giraud *et al.*, 2009). There are several other candidates, including signal transduction proteins like kinases and transcription factors, whose genes are induced following inhibition of the cytochrome respiratory pathway and/or the TCA cycle in *A. thaliana* plants (Rhoads, 2011). The leading candidates for non-protein signaling components of MRR in plants are calcium level changes, redox changes, and changes in metabolite levels. Growing evidence indicates that MRR may overlap with other signaling pathways, including those from chloroplasts. MRR could be linked to metabolic signaling and/or ROS production, but these would still originate in mitochondria and be components of MRR. This is an emerging view that plant mitochondria sense stress and contribute to decisions regarding cell fate in a way that differs from yeast and animal systems.

At present, the best studied plant MRR system is the response of the nuclear gene encoding AOX to mitochondrial dysfunction (Mackenzie and McIntosh, 1999). AOX serves as an adaptative response to recover from mitochondrial electron transport chain inhibition and to reduce the production of ROS. It is believed that AOX expression responds to two pathways of signal transduction to the nucleus: one with the involvement of organic acids and the other mediated by ROS (Gray *et al.*, 2004).

Transcription in plant mitochondria is relaxed, as almost the entire mtDNA is transcribed, including the large non-coding regions (Holec *et al.*, 2006). It is commonly thought that the regulation of organelle gene expression mostly occurs through multiple post-transcriptional mechanisms and involves nuclear-encoded protein factors (Leon *et al.*, 1998; Woodson and Chory, 2008). These processes, such as 5'- and 3'-end maturation, intron splicing, and RNA editing, are all potential sites of regulation (Woodson and Chory, 2008). Giegé *et al*. (2005) found that, in response to sugar starvation, mitochondrial gene expression remained more or less unaffected at the transcriptional, post-transcriptional, and translational levels. The observed reduction of mitochondrial inner membrane multisubunit complexes resulted from down-regulation of the nuclear-encoded subunits of these complexes. The mitochondrially-encoded components remained unchanged, while nuclear-encoded components served as

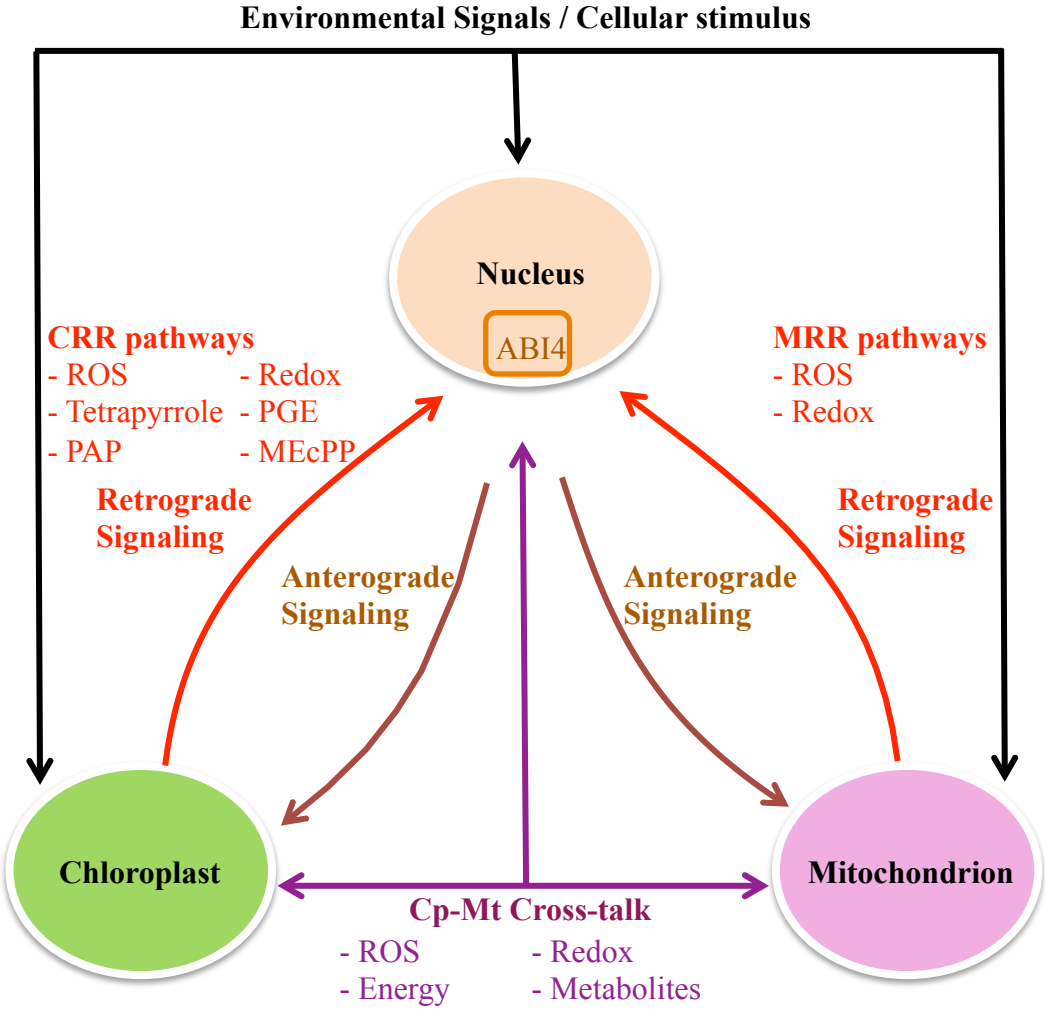

Figure 7: Communication between organelles and with the nucleus
Environmental stress or cellular stimulus can induce anterograde signaling and retrograde signaling. In plants, chloroplast retrograde signaling (CRR) uses ROS, Redox, Tetrapyrrole, PGE, PAP and MEcPP pathways, while mitochondrial retrograde signaling (MRR) uses ROS and Redox pathways. Retrograde signals coming from both mitochondria and chloroplasts can act on the ABI4 nuclear factor. Mitochondria and chloroplasts have interactions with each other through metabolism, ROS, energy status, and redox status. Cp: chloroplast, Mt: mitochondrion, ROS: reactive oxygen species, Redox: reduction oxidation status, PGE: plastid gene expression, PAP: 3'-phosphoadenosine 5'-phosphate, MEcPP: methylerythritol cyclodiphosphate, ABI4: abscisic acid insensitive 4. Adapted from Woodson and Chory, 2008; Rhoads, 2011.

rate-liming factor in the assembly of new complexes. The correct stoichiometric proportions seemed to be achieved post-translationally at the level of protein complex assembly. It is believed that under sugar starvation conditions, excess unassembled mitochondrial subunits are degraded by specific nuclear-encoded proteases (Giegé *et al.*, 2005).

IV.4. Cross-talk between mitochondria and chloroplasts

In plants, mitochondria and plastids have interactions not only with the nucleus, but also with each other (Figure 7). They are inter-dependent at multiple levels and intimately connected through metabolism, ROS, energy status, and redox status (Figure 7). For example, chloroplasts use CO_2 and ATP (mitochondrial products) during photosynthesis. On the other hand, O_2 and metabolic compounds are provided by chloroplasts for mitochondrial respiration. The balance between respiration and photosynthesis determines the rate of plant biomass accumulation. The transport of reducing equivalents from chloroplasts to mitochondria by redox-shuttling machineries and the supply of carbon skeletons for nitrogen assimilation in chloroplasts are some other examples of metabolic interactions between organelles (Woodson and Chory, 2008; Yoshida and Noguchi, 2011; Yurina and Odintsova, 2011). Due to this close inter-dependence, it is logical that overlapping/interacting mechanisms of retrograde regulation exist but these are only beginning to be identified (Raghavendra and Padmasree, 2003; Rhoads, 2011). Little is known about direct signaling between chloroplasts and mitochondria or coordination of the activities of the two organelles by signals other than carbon metabolites. To date, no solid experimental evidence has been provided and the existence of mitochondrion-chloroplast cross-talk signaling pathways has mostly been surmised from genetic studies.

Nicotiana sylvestris CMS mutants deficient for NADH dehydrogenase activity (Dutilleul *et al.*, 2003; Sabar *et al.*, 2000) and barley or potato mutants deficient for glycine decarboxylase activity (Heineke *et al.*, 2001; Raghavendra and Padmasree, 2003) in mitochondria showed reduced photosynthesis and impaired photorespiration, respectively. On the other hand, some chloroplast genes in *C. reinhardtii* act as mitochondrial mutant suppressors, which led to the suggestion that tRNA exchange occurs between the organelles and strengthened the hypothesis that mitochondrial protein synthesis depends on chloroplast protein synthesis in *C. reinhardtii* (Bennoun and Delosme, 1999). Other studies show that mitochondrion-chloroplast cross-talk involves a retrograde signal from one of the organelles that affects the anterograde control of the other. In *C. reinhardtii,* remote control of photosynthetic genes by the mitochondrial respiratory chain was shown, where

photosynthesis-related genes in the nucleus are induced in response to activation of the cytochrome pathway in mitochondria (Matsuo and Obokata, 2006). In plastid ribosome-deficient cells from the *albostrians* barley mutant, RpoTm (the nuclear-encoded mitochondrial RNA polymerase) is upregulated, resulting in increased mitochondrial transcription (Emanuel *et al.*, 2004).

Finally, an overlap in MRR and CRR signal transmission has recently been revealed. The ABI4 transcription factor is a common component of at least three CRR pathways (Chi *et al.*, 2013; Woodson and Chory, 2008), of abscisic acid, sugar, and developmental signaling (Rook *et al.*, 2006) and of numerous other pathways (reviewed recently in León *et al*., 2012). While working with *A. thaliana abi4* mutants, Giraud *et al*. (2009) showed that ABI4 is also involved in MRR. It acts as a promoter-binding, negative regulator of gene expression that allows repression of the AOX1a nuclear gene during MRR. ABI4 seems to play a central role in mediating MRR signals to induce the expression of AOX1a. Regulation of AOX1a by ABI4 thus provides a point of convergence for MRR and CRR pathways (Giraud *et al.*, 2009).

There are several other recently published reports that address the relationship between mitochondrial respiration, photosynthesis and chloroplast functions (Busi *et al.*, 2011; Leister, 2012; Woodson and Chory, 2012). But still it is not clear whether mitochondria are signaling directly to the chloroplast or indirectly to the nucleus, to affect anterograde mechanisms. We cannot exclude the possibility that simultaneous retrograde signals from chloroplasts and mitochondria might also interact to affect nuclear gene transcription. How such signals would be perceived and integrated remains to be determined (Pesaresi *et al.*, 2007; Woodson and Chory, 2008, 2012).

V. Framework and objectives of the thesis

As it appears from the previous sections, the data are fragmentary on mitochondria-to-nucleus retrograde signaling in plants. The molecules that induce the signal, the mechanisms of signal transduction and the components of the signaling cascade are still unknown. No real information is available on the specificity and cross-talk between different signaling pathways. Moreover, along with MRR, regulation, expression, and processing of mitochondrial RNAs appear to be complex after the analysis of the whole plant or human organelle transcriptome (Fujii *et al.*, 2011; Mercer *et al.*, 2011; Rackham *et al.*, 2012). Whether mitochondria in these organisms possess an MRR pathway similar to intergenomic

signaling in yeast or PGE in chloroplasts remains an open question. So far, only a limited amount of published data supports the view that coordination exists between the expression of mitochondrial genes and that of nuclear genes encoding mitochondrial proteins. Nevertheless, organelle RNA levels do vary in different conditions, raising the idea that there exists some transcriptional regulation in both mitochondria and chloroplasts. All known organelle transcription factors are nuclear-encoded proteins in yeast, mammals and plants, with the exception of the PEP in chloroplasts. Kühn *et al*. (2009) found a positive correlation between decreased levels of specific mitochondrial RNAs and reduced abundance of the corresponding protein complexes in an organelle RNA polymerase mutant. Furthermore, in *A. thaliana*, the activity of RpoTmp (the organelle dual-targeted nuclear-encoded RNA polymerase) is gene-specific rather than promoter-specific, which suggests that RpoTmp-dependent RNA synthesis may represent a transcriptional mechanism allowing plant mitochondria to control the expression of certain genes in the mtDNA (Kühn *et al.*, 2009). In mammals also, Asin-Cayuela and Gustafsson (2007) proposed that there must be other yet-to-be-identified factors that might influence organelle gene transcription. All the complexes of the mitochondrial oxidative phosphorylation chain consist of subunits encoded by the nuclear genome and subunits expressed from mitochondrial genes. Integrated genetic mechanisms thus seem necessary. That genetic control in mitochondria of plants would mainly occur at the post-transcriptional level is currently the most popular view but the field remains open and the idea of RNA-mediated regulation mechanisms in mitochondria connected with MRR pathways has to be considered.

The study of such regulation mechanisms still cannot benefit from organellar transgenic approaches, but the RNA shuttling strategy developed in our laboratory (section III.4.) opened the unprecedented possibility to introduce customized RNAs into mitochondria in plant cells and to manipulate the levels of specific organellar RNAs, positively or negatively. Based on this approach, my doctoral thesis project was to manipulate the transcriptome in plant mitochondria, aiming to highlight mechanisms of mitochondrial gene regulation and coordination with the nucleus and the plastids. As described, the strategy was to express from nuclear transgenes passenger sequences linked to the PKTLS tRNA mimic, so as to obtain import into mitochondria. Taking as a passenger sequence a *trans*-cleaving ribozyme directed against the *atp9* mRNA, encoding subunit 9 of ATP synthase, previously allowed to obtain the specific knockdown of this major mitochondrial RNA in the mitochondria of transformed plants (Val *et al.*, 2011). I extensively exploited this system

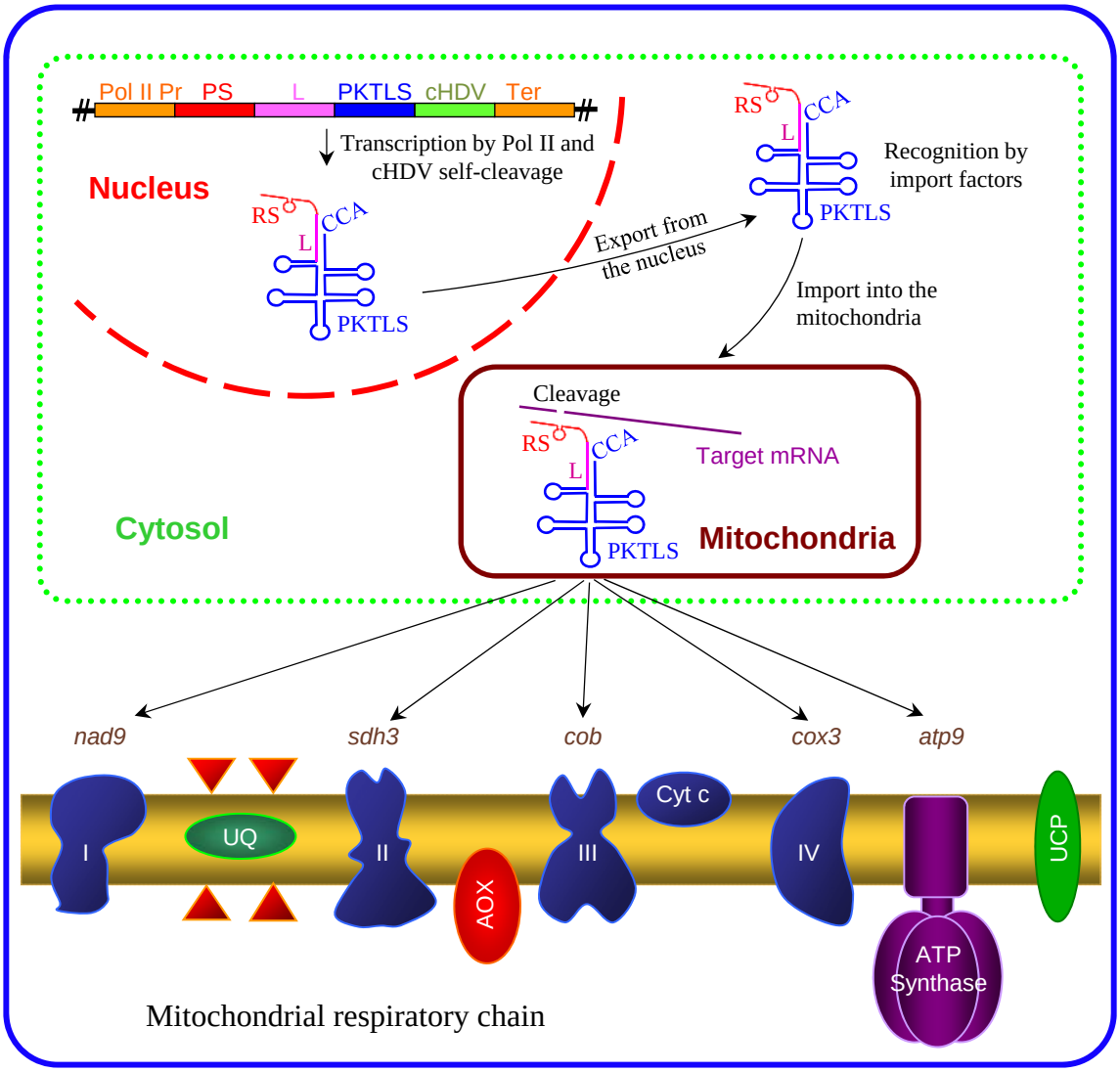

Figure 8: Experimental scheme of the project

The ribozyme-PKTLS RNA is synthesized in the nucleus and transported into the mitochondria through the cytosol with the help of the natural tRNA import pathway. Five mitochondrial mRNAs were chosen as targets: *nad9* (subunit 9 of complex I), *sdh3* (subunit 3 of complex II), *cob* (cytochrome b, complex III), *cox3* (subunit 3 of complex IV) and *atp9* (subunit 9 of ATP synthase). Pol II Pr: RNA polymerase II promoter, RS: ribozyme sequence, L: linker sequence, PKTLS: pseudoknot/tRNA-like structure, cHDV: HDV *cis*-ribozyme, Ter: terminator.

and developed *in vivo trans*-ribozyme strategies (Figure 8) against four further mitochondrial mRNAs coding for oxidative phosphorylation chain proteins: *nad9* (subunit 9 of complex I), *sdh3* (subunit 3 of complex II), *cob* (cytochrome b, complex III) and *cox3* (subunit 3 of complex IV). The mitochondrial, nuclear and plastidial response to the knockdown of a single mitochondrial mRNA was analyzed by high throughput transcriptomic approaches. Establishing *in vivo* directed knockdown of specific organelle RNAs brought strong evidence that mitochondrial RNA levels are coordinated in plants and that changes in mitochondrial RNA levels cause a retrograde response that affects the nuclear and chloroplast transcriptomes.

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Targeting nucleic acids into mitochondria: Progress and prospects

Adnan Khan Niazi, Daria Mileshina, Anne Cosset, Romain Val, Frédérique Weber-Lotfi, André Dietrich *

Institut de Biologie Moléculaire des Plantes, CNRS and Université de Strasbourg, 12 rue du Général Zimmer, 67084 Strasbourg, France

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ABSTRACT

Given the essential functions of these organelles in cell homeostasis, their involvement in incurable diseases and their potential in biotechnological applications, genetic transformation of mitochondria has been a long pursued goal that has only been reached in a couple of unicellular organisms. The challenge led scientists to explore a wealth of different strategies for mitochondrial delivery of DNA or RNA in living cells. These are the subject of the present review. Targeting DNA into the organelles currently shows promise but remarkably a number of alternative approaches based on RNA trafficking were also established and will bring as well major contributions.

1. Introduction

The vast majority of mitochondrial proteins are encoded by nuclear genes and imported into the organelles upon cytosolic translation. Nevertheless, mitochondria also have their own complete genetic system that provides a set of essential polypeptides involved in the structure or biogenesis of the oxidative phosphorylation complexes. The mammalian mitochondrial DNA (mtDNA) is a compact entity of 16.5 kb that encodes 13 polypeptides of the oxidative phosphorylation chain, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) (Saccone et al., 1999). It is deprived of intergenic regions and introns. Conversely, higher plant mitochondria possess large size genomes that can exceed 1500 kb (Alverson et al., 2011; Kubo and Newton, 2008). Plant mtDNAs contain long intergenic regions, multiple promoters and introns but still contribute less than 60 identified genes, coding for 3 rRNAs, 15–20 tRNAs and a set of 30–35 known proteins. More than half of the sequences in these genomes are of unknown function and have no homologs in databases (Unseld et al., 1997).

Given the essential role of organelles in cell homeostasis and response to the environment, deciphering the complex mitochondrial genetic processes is of fundamental interest and relevance. Mutations in the organelle genome cause severe neurodegenerative diseases that are currently incurable and await the development of gene therapy strategies (Greaves et al., 2012; Tuppen et al., 2010). Over 300 pathogenic mutations have been identified in the human mtDNA (http://www.mitomap.org), referring to missense mutations in protein-coding genes, point mutations in tRNA or rRNA genes and duplications or deletions. Mutations can affect all mtDNA copies in the cell (homoplasmic state) or only some copies (heteroplasmic state). Heteroplasmy is the most general situation and the onset of clinical symptoms is determined by the ratio of wild-type to mutant mtDNA, with a typical threshold effect (Wong, 2007). In plants, mitochondrial genetics influences a number of agronomically relevant traits, including cytoplasmic male sterility (CMS) (Chase, 2007; Frei et al., 2004). CMS lines are unable to generate functional pollen, which is of particular interest for breeders to create and maintain hybrids. The CMS trait is often associated with the presence of chimeric open reading frames in the mtDNA, presumably resulting from aberrant recombination. These give rise to abnormal protein products that have been identified in CMS lines from a number of species (Schnable and Wise, 1998).

Manipulating the mtDNA seems the obvious way to fill the gaps in the understanding of organelle genetic processes, complement mitochondrial mutations, develop gene therapy or direct male sterility. Unfortunately, conventional transfection methodologies remained unable to promote mitochondrial transformation in mammalian or plant cells, so that only the unicellular organisms yeast and *Chlamydomonas reinhardtii* are currently amenable to mtDNA manipulation (Bonnefoy et al., 2007; Zhou et al., 2010). To by-pass the absence of a mitochondrial transformation methodology, approaches based on protein transport pathways have been explored. Most of the mitochondrial proteins are synthesized in the cytosol from nuclear mRNAs and translocated into the organelles. Assays were thus designed to complement pathogenic mutations in mtDNA protein genes through nuclear expression and mitochondrial import of the corresponding polypeptides combined with an organelle targeting sequence. Upon proof of principle in the yeast *Saccharomyces cerevisiae* (Nagley et al., 1988), such allotopic expression of proteins that are normally gene products of the mtDNA was developed in mammalian model systems (Guy et al., 2002; Ojaimi et al., 2002). In further studies based on subcellular mRNA localization data (Sylvestre et al., 2003), the allotopically expressed transcripts were

* Corresponding author. Tel.: +33 3 88 41 72 41; fax: +33 3 88 61 44 42.
E-mail address: andre.dietrich@ibmp-cnrs.unistra.fr (A. Dietrich).

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targeted to the mitochondrial surface, so as to promote co-translational import of the corresponding proteins into the organelles (Bonnet et al., 2008; Ellouze et al., 2008; Kaltimbacher et al., 2006). As a whole, rescue of mtDNA mutation-triggered deficient phenotype was both claimed and contradicted, leaving the issue open (Bokori-Brown and Holt, 2006; Oca-Cossio et al., 2003; Perales-Clemente et al., 2010). Other assays exploited genes normally absent in human but able to by-pass non-functional steps in the respiratory chain (Dassa et al., 2009; Seo et al., 2006; Yagi et al., 2006). Finally, allotopic expression and organelle import of restriction enzymes or zinc finger-coupled nucleases directed against mutated mtDNA were investigated (Alexeyev et al., 2008; Bacman et al., 2010; Minczuk et al., 2008). In plants, nuclear expression and mitochondrial import of specific CMS polypeptides were tentatively used to generate directed male sterility but yielded variable results, depending on the system considered (Chaumont et al., 1995; Duroc et al., 2006; He et al., 1996; Kumar et al., 2012; Wintz et al., 1995; Yang et al., 2010). Although these protein delivery approaches altogether generated some encouraging observations, direct manipulation of the mitochondrial genetic system through nucleic acid targeting has remained the subject of constant interest and cutting edge research. A series of very different strategies have been imagined and will be described in this review.

2. Initial cell biology and biological ballistics strategies

2.1. Transfer of whole mitochondria

In early experiments, Clark and Shay co-incubated mammalian cells with purified mitochondria carrying an mtDNA mutation that provides antibiotic resistance. The isolated organelles were taken up by endocytosis, transferring the antibiotic resistance to the sensitive cells (Clark and Shay, 1982). The strategy however had no real follow up for two and a half decades, until Katrangi et al. reported uptake of isolated murine mitochondria and respiration rescue in human A549 lung carcinoma cells devoid of mtDNA (Katrangi et al., 2007). In the mean time, it had been established that isolated mitochondria are actually capable to import linear DNA (Koulintchenko et al., 2003, 2006). In further assays, the authors thus pre-loaded the isolated organelles with a minicircle DNA construct prior to their cellular uptake. The construct was recovered in the cells, which supports the feasibility of introducing exogenous DNA into the mitochondrial population by combining organelle competence with the ability of mammalian cells to internalize isolated mitochondria (Katrangi et al., 2007) (Fig. 1). Following a more directed strategy, foreign mitochondria with a selectable phenotype were successfully microinjected into human somatic cells, leading to a rapid replacement of the endogenous mtDNA (King and Attardi, 1988). Similarly, ρ^0 human cells, i.e. cells with mitochondria deprived of DNA, were repopulated upon microinjection of exogenous organelles (King and Attardi, 1989) (Fig. 1). Also, mitochondria isolated from *Mus spretus* liver were microinjected into fertilized ova from *Mus musculus* females. The foreign organelles and mtDNA could be detected through the first stages of embryonic development but their level subsequently decreased to a large extent (Pinkert and Trounce, 2002; Pinkert et al., 1997). Finally, different studies developed the use of mammalian ρ^0 cells as recipients for cytoplasts, i.e. enucleated cells, to generate mtDNA-repopulated cybrids (Kagawa and Hayashi, 1997; King and Attardi, 1989; King et al., 1992) (Fig. 2). Although such prospects have been put forward, the application of approaches based on the transfer of whole mitochondria to gene therapy for mtDNA alterations would still be extremely complex.

As to preloading of isolated mitochondria with the desired DNA construct, several approaches can be exploited (Fig. 1). The prokaryotic origin of mitochondria brought to the idea of exploring DNA acquisition through conjugation. Bacterial conjugation involves specific protein factors and an origin of transfer (*OriT*) in the mobilizable or conjugative plasmid (e.g. Smillie et al., 2010). Yoon and Koob added an *OriT* sequence to a DNA construct that they introduced into *E. coli*. In a second step, the mobilizable DNA was successfully transferred into isolated mammalian mitochondria through conjugation with the transformed *E. coli* strain and was transcribed in organello (Yoon and Koob, 2005). As a physical approach, electroporation was shown to trigger the uptake of DNA constructs into isolated mitochondria from mammals, protists or plants (Collombet et al., 1997; Estevez et al., 1999; Farré and Araya, 2001; Staudinger and Kempken, 2003; Yoon and Koob, 2003). Regular expression of the electroporated DNA was obtained in plant mitochondria, which enabled detailed investigations on the mechanisms of RNA splicing and editing in these organelles (Bolle et al., 2007; Castandet and Araya, 2011 and references therein). Interesting to note, recent mathematical cell models predict that conventional electroporation might create intracellular fields sufficient to porate organelle membranes (Esser et al., 2010), asking the question of mitochondrial electroporation within the cells. Conversely, developing physiological assays, Koulintchenko et al. established that isolated plant, mammalian and fungal mitochondria are actually competent and, as mentioned above, they take up naked linear DNA along an active pathway in the absence of any artificial procedure (Koulintchenko et al., 2003, 2006; Weber-Lotfi et al., 2009). These data highlighted a novel process of macromolecule transport into organelles. The imported DNA can be transcribed in organello and the transcripts are accurately processed (Koulintchenko et al., 2003, 2006; Placido et al., 2005). The exogenous DNA can also be repaired upon uptake into plant or mammalian mitochondria (Boesch et al., 2009, 2010). Moreover, a construct comprising a marker gene flanked upstream and downstream with sequences homologous to the mtDNA recombines in organello with the resident DNA in plant mitochondria, leading to the integration of the marker gene (Mileshina et al., 2011). The uptake is sequence-dependent for large DNA substrates but isolated human mitochondria seem to be less selective (Ibrahim et al., 2011). As to the mechanism, translocation through the outer membrane involves the voltage-dependent anion channel (VDAC) (Koulintchenko et al., 2003, 2006; Weber-Lotfi et al., 2009). How the DNA crosses the inner membrane remains to be clarified.

2.2. Biolistics

Biological ballistics or "biolistics" (e.g. Villemejane and Mir, 2009) has been another strategy in pioneering experiments aiming at genetic transformation of mitochondria in living cells (Fig. 3). Widely used for nuclear transformation, especially in plants, the methodology consists of bombarding cells or tissues with DNA-coated high-velocity tungsten or gold particles. In the mitochondrial field, this approach was first successful in transforming the organelles in yeast. Fox et al. (1988) transferred a mitochondrial gene carried on a bacterial plasmid into the organelles of *S. cerevisiae* ρ^0 cells, whereas Johnston et al. (1988) corrected a mitochondrial mutation by integrating a functional gene copy into the mtDNA through homologous recombination. Biolistics was subsequently applied to generate various alterations in the mtDNA and was recently extended to the haploid yeast *Candida glabrata* (Zhou et al., 2010). Transformation frequencies are relatively low but strong mitochondrial selectable markers are available for yeast (Bonnefoy and Fox, 2007).

It is noteworthy that, based on biolistic-mediated transfection and homologous recombination of the transfected DNA with the resident organelle DNA, chloroplast transformation was established at the same period, first in the unicellular alga *C. reinhardtii* (Boynton et al., 1988) and subsequently in tobacco (*Nicotiana tabacum*) (Svab et al., 1990). Plastid transgenesis has significantly contributed to the understanding of chloroplast genetic processes and is now at the stage of biotechnological application (Maliga, 2004; Scotti et al., 2012). Mitochondrial transformation in *C. reinhardtii* came later, when Randolph-Anderson et al. complemented a respiratory-deficient mutant upon bombardment with the cognate mtDNA (Randolph-Anderson et al., 1993). The approach was later revived with cloned mtDNA and PCR fragments, introducing both non-deleterious and deleterious mutations on the basis of homologous recombination (Remacle et al., 2006). High transformation efficiency was obtained and the best results were observed with linearized plasmid

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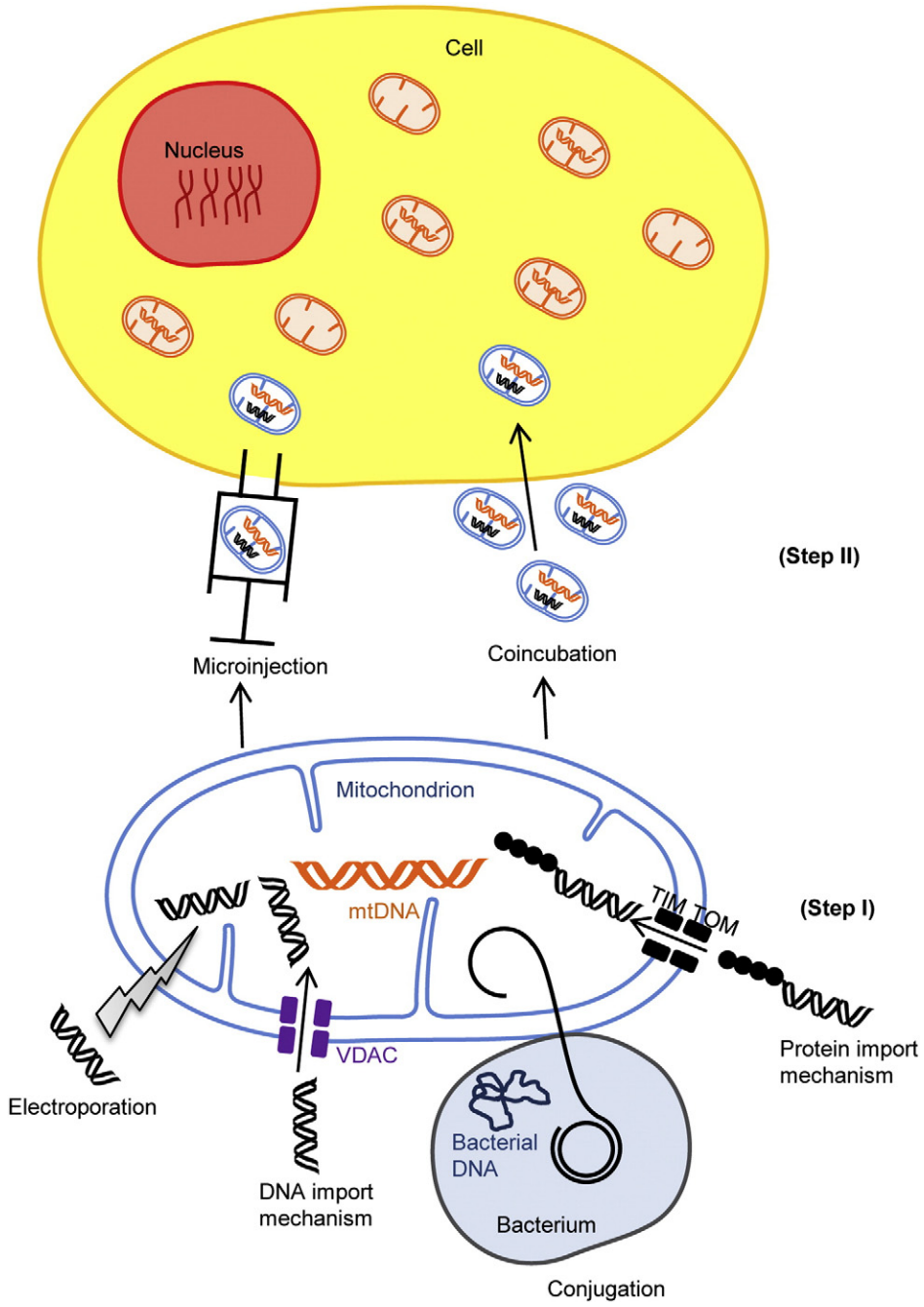

Fig. 1. Schematic view of strategies to preload DNA into mitochondria that will be introduced into cells. Step I: DNA can enter mitochondria by the natural import pathway, by electroporation or by conjugation with a bacterium containing a mobilizable plasmid. DNA conjugated with a mitochondrial targeting peptide enters mitochondria via the protein import pathway. Step II: preloaded mitochondria can be introduced into normal cells or cells with mitochondria deprived of DNA (ρ^0 cells) by microinjection or by coincubation. VDAC: Voltage Dependent Anion Channel, TOM: Translocase of the Outer Membrane, TIM: Translocase of the Inner Membrane.

DNA (Bonnefoy et al., 2007; Remacle et al., 2006). Finally, the heterologous marker gene *egfp* could be integrated into the mtDNA and expressed in the organelles of *C. reinhardtii* transformants (Hu et al., 2011). Despite success in yeast and *C. reinhardtii*, biolistics failed so far to transform mitochondria in animal and higher plant cells.

3. Targeting DNA into mitochondria through the protein import pathway

In line with allotopic expression of polypeptides, the idea of exploiting the best known mitochondrial macromolecule uptake mechanism, i.e. the protein import pathway (Becker et al., 2012; Schleiff and Becker, 2011), to drive DNA into the organelles also emerged early and is still investigated.

3.1. Mitochondrial targeting sequence fusions

In initial experiments, a single-stranded or double-stranded 24-base-pair DNA oligonucleotide was covalently linked to the C-terminus of a mitochondrial precursor protein and the conjugate was introduced into isolated *S. cerevisiae* mitochondria (Vestweber and Schatz, 1989) (Fig. 1). Later, chimeras composed of an N-terminal mitochondrial targeting peptide and a specially designed palindromic DNA moiety of 17 or 322 base-pairs were reportedly imported into isolated rat (*Rattus norvegicus*) liver mitochondria through the protein import pathway (Seibel et al., 1995).

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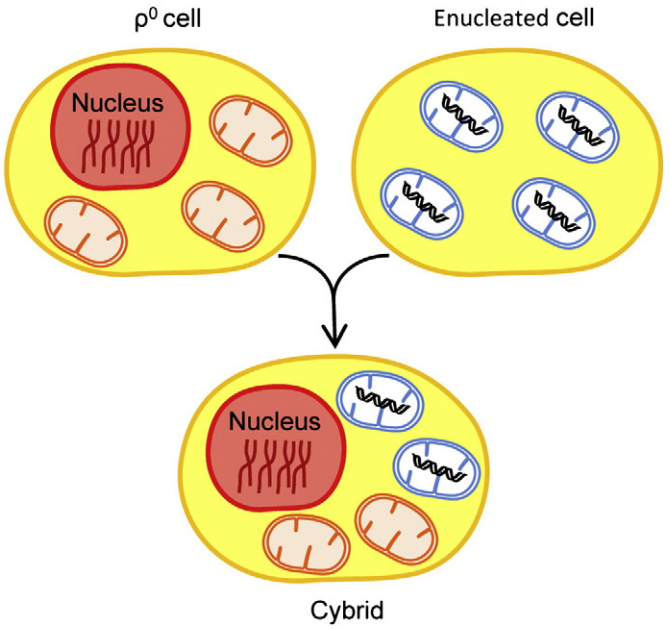

Fig. 2. Schematic view of a cellular strategy to transfer mtDNA. ρ^0 cells (cells with mitochondria deprived of DNA) are fused with enucleated cells.

Special attention was paid to the cleavage of the targeting peptide upon import, so as to avoid an interference with the functionality of the DNA in the organelles (Seibel et al., 1999). However, DNA–peptide conjugates are not easy to synthesize and are sensitive to hydrolysis. In more complex assemblies (Flierl et al., 2003), the mitochondrial targeting peptide was then conjugated to a fragment of PNA (peptide nucleic acid), i.e. a synthetic polymer made of a peptide bond backbone carrying regular nucleobases. PNAs base-pair with DNA or RNA, their absence of charge providing a high affinity. In a second step, a DNA oligonucleotide of interest can thus be annealed to the PNA moiety of the conjugate. Such complexes were reported to be imported into isolated mammalian mitochondria and into the mitochondria of cultured mammalian cells permeabilized to allow cytosolic uptake (Flierl et al., 2003). In the most recent follow up, the mitochondrial targeting peptide was conjugated to a polyethylenimine (PEI) moiety that binds the DNA (Lee et al., 2007). The resulting peptide/PEI/DNA complexes were shown to co-localize with the mitochondria in living cells.

3.2. Protofection

The above approaches remain restricted by the possibility for the mitochondriotropic complexes to enter the cells. This step is taken into account in a further strategy called "protofection". In that case, the DNA "vehicle" combines a protein transduction domain (e.g. Suzuki, 2012 and references therein) and a mitochondrial targeting peptide with the TFAM mitochondrial transcription/mtDNA-binding factor (Iyer et al., 2009; Thomas et al., 2011). In such a design named MTD-TFAM, the protein transduction domain enables translocation through the plasma membrane, the mitochondrial targeting sequence promotes organelle targeting and uptake, whereas TFAM carries the DNA (Fig. 3). The proof of principle was tested in Parkinson's disease cybrid model cells impaired in mitochondrial functions. The MTD-TFAM protein loaded with human mtDNA co-localized with mitochondria, whereas the mitochondrially deficient phenotype was rescued (Keeney et al., 2009). Also, treating human

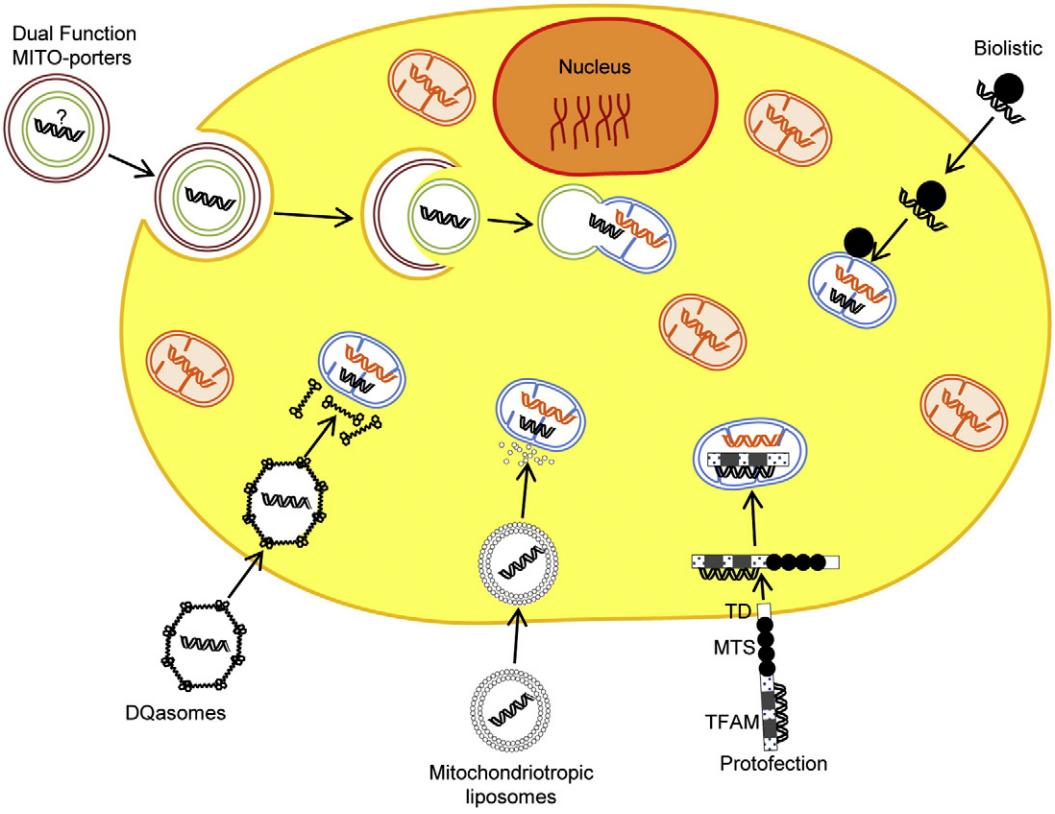

Fig. 3. Schematic view of strategies used to transfect mitochondria with DNA in intact cells. By biolistics, the bombardment of cells with DNA-coated tungsten or gold particles leads to the entry of DNA into the cell and finally into the mitochondria. By protofection, the DNA is loaded on TFAM combined with a mitochondrial targeting sequence (MTS) and a protein transduction domain (TD). The TD permits the entry into the cell through the plasma membrane and the MTS leads to translocation into mitochondria. DNA can be loaded into mitochondriotropic liposomes or DQAsomes that permit the entry into the cell and the localization to the vicinity of mitochondria. Macromolecules can be loaded into Dual Function MITO-porters that contain two envelopes, the outer one able to fuse with the endosomal membranes and the inner one able to fuse with the mitochondrial membranes, allowing the cargo macromolecule to enter the mitochondria. Whether DNA can be delivered through MITO-porters remains to be established.

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cells carrying mutant mtDNA with healthy donor mtDNA loaded on MTD-TFAM partially restored mitochondrial functions (Iyer et al., 2012). A puzzling aspect of these data is however that the MTD-TFAM protein without a DNA load triggered a similar genetic and functional boosting of mitochondria both in cultured cells and in mice (Iyer et al., 2009; Keeney et al., 2009; Thomas et al., 2011). In reciprocal assays, the MTD-TFAM carrier was used to introduce a pathogenic mutated mtDNA into human neural progenitor cells (Iyer et al., 2011). Mitochondrial co-localization and expression of the exogenous pathogenic mtDNA were reported. As a whole, direct molecular evidence for MTD-TFAM-driven mitochondrial uptake of cargo DNA would much strengthen this strategy.

4. Nanocarrier-mediated targeting of DNA into mitochondria

4.1. Non-cationic liposome-based carriers

Association of conventional non-cationic liposomes with mitochondria and possible organelle delivery of their aqueous content were reported in very early experiments (Cudd and Nicolau, 1985, 1986; Cudd et al., 1984). The potential usefulness of proteoliposomes for mitochondrial gene transfer was suggested (Inoki et al., 2000) but the follow up switched to a further type of non-cationic, carrier liposomes called "MITO-Porters" (Yamada et al., 2008). The most recently designed, Dual Function MITO-Porters (DF-MITO-Porters) are nanoparticles that contain the compound to be delivered and are coated successively with a mitochondria-fusogenic envelope and an endosome-fusogenic envelope (Yamada et al., 2011). Following cell entry of the whole structure by endocytosis, the two envelopes allow translocation through the endosomal and the mitochondrial membranes thanks to step-wise membrane fusion and the "core" particle carrying the compound of interest is finally released into the mitochondrial matrix (Fig. 3). Cell uptake and mitochondrial targeting are promoted by stearyl-octaarginine moieties present in the envelopes. So far, mitochondrial delivery of MITO-Porter-encapsulated green fluorescent protein (GFP) or DNase I was reported in human cells (Yamada and Harashima, 2012; Yamada et al., 2008, 2011), although again further direct molecular analysis of the delivery process would be welcome. Whether MITO-Porters can accommodate and deliver DNA remains to be established.

4.2. Mitochondriotropic amphiphile carriers

Attracted by the potential that polarizes the organelle inner membrane, certain amphiphile compounds are able to cross both mitochondrial membranes and to accumulate in the inner compartment. This raised the idea that such compounds might provide carriers for organelle delivery of DNA. Mitochondriotropic cationic amphiphiles include phosphonium salts, such as triphenylphosphonium. The latter permeate lipid bilayers, which allow uptake into the cytosol and accumulation in mitochondria. Muratovska et al. conjugated a PNA oligomer (see above) to a triphenylphosphonium moiety and reported successful membrane potential-driven import of the conjugate into the mitochondria of cultured human cells (Muratovska et al., 2001). However, similar assays with DNA moieties remain to be developed.

4.3. Mitochondriotropic vesicles

Considering the abundant and robust data obtained with isolated mitochondria (see Section 2.1), one can speculate that organelle competence for DNA uptake is an intrinsic property and has the potential to support mitochondrial transfection in whole cells. In such a view, the vehicle does not need to cross the organelle membranes and the challenge gets restricted to carrying the substrate DNA into the cells and to the vicinity of the organelles in the right physiological context. A number of mitochondriotropic nanocarriers able to bind DNA and enter mammalian cells have been characterized (Horobin et al., 2007). Particular interest has been devoted to vesicles made of dequalinium, so-called DQAsomes (Weissig et al., 1998). These can bind and condense DNA. The resulting complexes enter mammalian cells by endocytosis, manage to escape from endosomes and migrate towards the mitochondria, where they release their load (D'Souza et al., 2003) (Fig. 3). The released DNA remains associated with the organelles, in agreement with the above hypothesis of a natural competence-mediated uptake. Also in line with the characteristics established for import into isolated mitochondria (Koulintchenko et al., 2003), organelle co-localization was more efficient with linear DNA than with a circular substrate (D'Souza et al., 2005). Further studies recently reported DQAsome-mediated mitochondrial delivery and expression of a DNA construct containing a recoded GFP gene in mammalian cell lines (Lyrawati et al., 2011). Although the efficiency was found to be low, the actual organelle delivery of the DNA was supported by a series of direct molecular, microscopic and immunological analyses.

Altogether, DQAsomes showed promise for mitochondrial transfection but turned out to be highly toxic for mammalian cells (Lyrawati et al., 2011). Other formulations have thus been developed. As a less toxic alternative, mitochondriotropic liposomes composed of phospholipids, amphiphilic cations exposed on the surface and DNA-binding lipids also proved capable to deliver DNA into mammalian cells and to drive their cargo towards mitochondria (Boddapati et al., 2005; D'Souza et al., 2007; Weissig et al., 2006) (Fig. 3). For further improvement, the liposomes were prepared in the presence of a mitochondrial fraction or mitochondrial lipids (Wagle et al., 2011). These were well tolerated by cell cultures, also ensured intracellular delivery of DNA and actually promoted enhanced co-localization of the delivered DNA with mitochondria, as compared to formulations not containing a mitochondrial fraction. Whether the DNA is incorporated into the organelles in such experiments remains to be firmly established.

5. Mitochondrial targeting of tRNAs and tRNA fusions

Although mitochondria are able to import DNA, there is usually no DNA to take up from the cytosol in the normal life of a cell. On the contrary, in most organisms mitochondrial import of specific RNAs is a regular process. The most widespread pathway in this respect is organelle import of transfer RNAs (tRNAs). Indeed, the mitochondrial genome of many lineages does not code for a complete set of tRNAs, a situation that requires sharing of nuclear-encoded tRNAs with the cytosolic translation system (reviewed for instance in Salinas et al. 2008; Schneider, 2011). The number of imported tRNAs varies from one in marsupials (Dorner et al., 2001) to all of them in trypanosomatids (Schneider and Maréchal-Drouard, 2000) and one third to one half in higher plants (Kumar et al., 1996; Maréchal-Drouard et al., 1990). Major aspects of the targeting and uptake mechanisms remain unknown, but most likely there is no unique pathway. Nevertheless, the existence of natural tRNA trafficking has been exploited in different ways for fundamental studies, mtDNA mutation rescue or manipulation of mitochondrial gene expression (Fig. 4).

5.1. Nuclear expression and mitochondrial import of customized tRNAs

Nuclear expression of relevant tRNA variants has been used in different organisms to investigate functional aspects of mitochondrial targeting and import (see in recent reviews Alfonzo and Soll, 2009; Salinas et al., 2008; Schneider, 2011; Sieber et al., 2011a; Tarassov et al., 2007 and references therein). These studies contributed to the identification of sequence determinants or import factors and to the modelization of trafficking pathways. In some of the most recently reported experiments, allotopic expression of a tagged tRNA was combined with RNA interference-mediated ablation of various protein factors in *Trypanosoma brucei* cell lines. Extending previous observations made in *S. cerevisiae* and in plants, the results strengthened the idea that, in all organisms, components of the mitochondrial protein import machinery are involved in tRNA uptake (Tschopp et al., 2011).

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5.2. Allotopic expression of therapeutic tRNAs

A large number of mitochondrial disorders in human are due to mtDNA mutations in tRNA-encoding genes (Greaves et al., 2012; Tuppen et al., 2010), which raised a special interest in rescuing the resulting dysfunctions through import of functional tRNAs from the cytosol. That an allotopically expressed importable tRNA can correct respiratory deficiencies due to a mutation in the mtDNA was originally documented in *S. cerevisae* (Kolesnikova et al., 2000). In this case, an engineered suppressor tRNA expressed from a plasmid was shown to suppress an mtDNA nonsense mutation upon mitochondrial delivery. The strategy was based on the extensively studied pathway that enables organelle uptake of one of the cytosolic $tRNAs^{Lys}$ in yeast (Brandina et al., 2007; Entelis et al., 2006). As to human mitochondria, these were considered to encode all their tRNAs, until they proved able to import the yeast cytosolic $tRNA^{Lys}$(CUU), synthetic variants of this tRNA, as well as human organelle $tRNA^{Lys}$, in vitro (Entelis et al., 2001; Kolesnikova et al., 2000). The latter data established that human mitochondria have at least kept the capability to take up tRNAs. Further evidence was subsequently reported supporting natural import of cytosolic $tRNAs^{Gln}$ (Rubio et al., 2008).

These pathways were exploited in human cellular models for the rescue of organelle deficiencies due to mitochondrial tRNA gene mutations. In this respect, *S. cerevisiae* $tRNA^{Lys}$ derivatives were expressed in human cybrid cells and in patient-derived fibroblasts carrying the $tRNA^{Lys}$ 8344A>G mutation. The latter is a major cause of the MERRF (myoclonic epilepsy with ragged-red fibers) syndrome (Greaves et al., 2012; Tuppen et al., 2010). The nuclear transgene-derived heterologous $tRNAs^{Lys}$ were delivered into mitochondria and proved able to participate in organelle translation, providing partial rescue of the functional deficiencies caused by the mtDNA mutation (Kolesnikova et al., 2004). A similar strategy was set up to counteract the deleterious effects of the mitochondrial $tRNA^{Leu}$ 3243A>G mutation, a major cause of the MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes) syndrome. For this, the aminoacylation identity of the recombinant tRNAs was engineered so as to switch from lysine to leucine. These tRNA variants possessing the identity elements for mammalian organelle leucyl-tRNA synthetase were subsequently expressed from nuclear transgenes in human cybrid cells carrying the 3243A>G mutation (Karicheva et al., 2011). Again, mitochondrial delivery was successful, leading to a significant functional rescue. As a whole, the data demonstrate the relevance and the potential of customized tRNA targeting for the development of therapeutic strategies to cure mitochondrial disorders (Karicheva et al., 2011; Kolesnikova et al., 2004).

5.3. Transfer RNA-driven targeting of cargo RNAs

The existence of a natural tRNA uptake pathway raised the idea of exploiting tRNAs for mitochondrial targeting of cargo sequences. As mentioned, all mitochondrial tRNAs are taken up from the cytosol in trypanosomatids (Schneider and Maréchal-Drouard, 2000). Among these, $tRNA^{Tyr}$ is synthesized as a precursor containing an 11-nucleotide intron. Replacing the latter by synthetic sequences in a splicing-deficient $tRNA^{Tyr}$ variant enabled to introduce foreign cargos up to 38 nucleotides into the mitochondria of transgenic *Leishmania tarentolae* cell lines (Sbicego et al., 1998). Splicing-deficient $tRNA^{Tyr}$ can thus be used as a vehicle for targeting short embedded sequences into trypanosomatid mitochondria in vivo. On the other hand, mitochondrially imported tRNAs in higher plants include $tRNAs^{Leu}$ (Kumar et al., 1996; Maréchal-Drouard et al., 1990). In early experiments, a bean (*Phaseolus vulgaris*) $tRNA^{Leu}$ carrying a four base-pair insertion in the anticodon loop was expressed in potato (*Solanum*

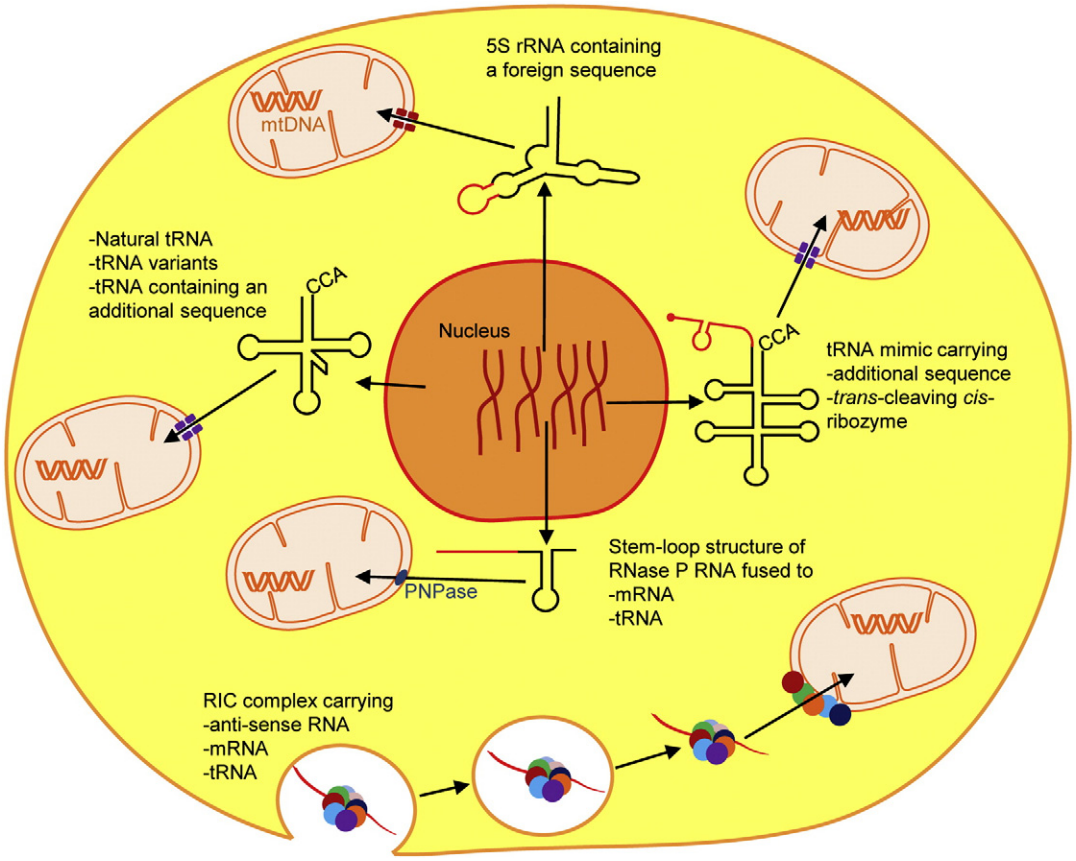

Fig. 4. Schematic view of strategies used to transfer customized RNAs into mitochondria in whole cells. The RNA of interest can be synthesized in the nucleus as a combination with an RNA shuttle (tRNA mimic or stem-loop structure of the RNase P RNA) or embedded in a regularly imported RNA (tRNA, 5S RNA). RNAs loaded on the RIC complex (RNA Import Complex) have been reported to be transferred into the cells and to the mitochondria with the help of RIC proteins.

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tuberosum) from a nuclear transgene and recovered in mitochondria (Small et al., 1992). However, in this case the strategy was not successful when inserting longer sequences or when the insertion was in other regions of the tRNA. Further attempts in plants thus explored the possibility to graft the cargo RNA to the 5′ end of the tRNA as a trailor sequence. To escape from the constraints of normal plant tRNA gene expression based on internal promoters (Choisne et al., 1998; Geiduschek and Tocchini-Valentini, 1988 and references therein), to allow for RNA polymerase II-driven transcription and to avoid cleavage by the 5-end processing enzyme RNase P, a tRNA mimic was substituted for the regular tRNA. The choice was made for the tRNA-like structure (TLS) that forms at the 3′-end of the *Turnip yellow mosaic virus* (TYMV) genomic RNA (Matsuda and Dreher, 2004). The TYMV TLS functionally mimics $tRNA^{Val}$, another tRNA naturally imported into plant mitochondria (Kumar et al., 1996; Maréchal-Drouard et al., 1990), but its sequence does not contain the consensus RNA polymerase III internal promoters and it is not a substrate for RNase P. Polymerase II-mediated nuclear expression of the TYMV TLS with a 72-nucleotide 5′ extension indeed led to mitochondrial import of the bipartite transcript in tobacco (*N. tabacum*) cell cultures (Val et al., 2011). Notably, engineering the aminoacylation specificity of the TYMV TLS from valine to methionine abolished organelle targeting of the combined RNA, in line with the fact that $tRNAs^{Met}$ are not taken up by plant mitochondria (Kumar et al., 1996; Maréchal-Drouard et al., 1990). These results altogether established that the regular tRNA import pathway can mediate mitochondrial targeting of customized sequences driven by a tRNA mimic (Val et al., 2011) (Fig. 4).

The strategy was subsequently used to initiate functional and regulatory studies. For that purpose, a *trans*-cleaving hammerhead ribozyme directed against a mitochondrial RNA was combined with the TYMV TLS as a 5′-cargo sequence. Upon expression in stably transformed *N. tabacum* cells and *Arabidopsis thaliana* plants, the combined RNA was efficiently imported into mitochondria. Cleavage mediated by the chimeric catalytic RNA was active in the organelles and triggered a strong knockdown of the target RNA, validating RNA-directed manipulation of the mitochondrial genetic system for both fundamental investigations and biotechnological applications in living plant cells (Val et al., 2011).

A randomized RNA library was also generated from yeast $tRNA^{Lys}$ and submitted to an in vitro selection approach (SELEX) based on uptake efficiency into isolated *S. cerevisiae* mitochondria and import factor binding. The sequences collected highlighted RNAs that had lost the classical tRNA conformation and brought evidence for RNA secondary structure rearrangement in the mechanism of uptake selectivity (Kolesnikova et al., 2010). The observations allowed to build a set of small tRNA-derived aptamers that showed an improved importability into yeast or human mitochondria. These data might in turn lead to a further vector system to target customized oligoribonucleotides embedded in specific small RNAs into mitochondria in human cells, in particular to inhibit the replication of mutated mtDNA copies (Kolesnikova et al., 2010).

6. PNPase-facilitated RNA targeting

Early studies in mammalian cells reported mitochondrial import of the nuclear-encoded catalytic RNA components of RNase P, the enzyme that processes the 5′-end of tRNAs (Doersen et al., 1985), and RNase MRP, an endoribonuclease involved in mtDNA replication (Chang and Clayton, 1989). These data became a subject of controversy (Kiss and Filipowicz, 1992), were subsequently supported by further observations (Li et al., 1994; Lu et al., 2010; Puranam and Attardi, 2001) but were contradicted when it turned out that human mitochondrial RNase P contains only protein components (Holzmann et al., 2008). Notably, the latter observation was also made for plant mitochondrial RNase P (Gobert et al., 2010). Whatever its role in the organelles, mitochondrial import of the RNase P/RNase MRP RNA in mammals was again put forward by functional analyses of the RNA uptake mechanism in vitro and in vivo. These established that polynucleotide phosphorylase (PNPASE) located in the mitochondrial intermembrane space mediates organelle import of small RNAs, including the RNase P/RNase MRP RNAs and the 5S rRNA (Wang et al., 2010, 2011). In vitro import of engineered RNase P and MRP RNAs identified two 20-nucleotide stem-loop structures that function as targeting signals for PNPASE-mediated mitochondrial translocation (Wang et al., 2010). In further assays, these stem-loop structures were shown to license the uptake of the GAPDH nuclear mRNA or the human mitochondrial $tRNA^{Trp}$ into isolated yeast or mouse mitochondria, respectively, thus assessing another mitochondrial shuttling system (Fig. 4). The data were extended to engineered precursors for mitochondrial $tRNA^{Lys}$ or $tRNA^{Leu}$ that were imported into isolated mammalian mitochondria when associated with the RNase P RNA-derived stem loop (Wang et al., 2012). Moreover, appropriate versions of these tRNA precursors were combined with both the RNase P RNA stem loop and a specific mRNA-derived 3′-UTR sequence that allows trafficking to the mitochondrial surface. Upon stable nuclear expression in human cybrid cells carrying the mtDNA $tRNA^{Lys}$ 8344A>G MERRF mutation or the $tRNA^{Leu}$ 3243A>G MELAS mutation, the composite chimeric RNAs were translocated into the mitochondria and rescued to a large extent the functional defects caused by the mutations (Wang et al., 2012). Finally, the RNase P RNA stem loop was appended to the mouse mitochondrial mRNA encoding the COX2 subunit of complex IV. When transiently expressed in HeLa cells, such an engineered RNA was recovered in mitochondria. In reciprocal assays, the human *COX2* mRNA combined with the RNase P RNA stem loop was stably expressed in mouse cells, imported into mitochondria and translated, whereas the resulting polypeptide assembled into the organelle inner membrane (Wang et al., 2012). The shuttling system developed in these studies can thus deliver into mammalian mitochondria large functional RNAs (over 680 nucleotides for *COX2*), providing a general approach for organelle genetic investigation and a broad potential for functional complementation of mitochondrial disorders.

7. Ribosomal RNA as a mitochondrial shuttle

A further organelle trafficking characterized in mammals is the mitochondrial import of nuclear-encoded 5S rRNA (Entelis et al., 2001; Magalhaes et al., 1998; Yoshionari et al., 1994). Although its meaning is still questioned, import of 5S rRNA seems critical for mitochondrial translation and there is evidence for an association of this RNA with the mammalian organelle ribosomes (Smirnov et al., 2010, 2011). The potential involvement of the different structural elements building up human 5S rRNA in the mitochondrial uptake mechanism was analyzed in vitro and in vivo. Extensive deletion in domain beta failed to significantly affect organelle import, opening the possibility to substitute this region for customized sequences of interest (Smirnov et al., 2008). On this way, a 5S rRNA derivative in which part of this domain had been replaced by a 13-nucleotide piece of foreign sequence was expressed in human cells. The recombinant RNA was recovered in mitochondria with an even higher efficiency than the wild-type 5S rRNA (Smirnov et al., 2008) (Fig. 4). Whether this further mitochondrial vector system can accommodate large sequences remains to be investigated. Nevertheless, it already seems of special interest in human cells as an alternative for mitochondrial targeting of antigenomic oligoribonucleotides destined to block the replication of mtDNA copies that carry pathogenic mutations. Such a strategy has the potential to shift the level of mutated mtDNA copies below the threshold that generates disease (Smirnov et al., 2008).

8. A shuttle protein for RNA targeting

Other assays aimed at using a customized shuttle protein to deliver cargo RNAs into the organelles. The aminoacyl-tRNA synthetases have been implicated in mitochondrial tRNA import [e.g. (Schneider, 2011;

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Sieber et al., 2011a)]. Although often necessary, they are not sufficient as specific RNA-binding proteins to promote tRNA delivery into the organelles (Dietrich et al., 1996a, 1996b; Mireau et al., 2000). Remarkably, it was established that a general RNA-binding protein, the mammalian dihydrofolate reductase (DHFR), fused to an organelle targeting signal, can fulfill such a role. Known to naturally bind to its own mRNA in vivo (Tai et al., 2002), the mammalian DHFR turned out to dramatically boost tRNA import into isolated plant mitochondria, potentially through co-import (Sieber et al., 2011b). Moreover, the DHFR proved able to shuttle longer RNAs into isolated plant organelles, including an extended tRNA precursor or a full-length mitochondrial mRNA devoid of any tRNA moiety. Upon DHFR-mediated uptake, the tRNA precursor was processed in organello, whereas the mRNA was edited, demonstrating the functional relevance of the approach (Sieber et al., 2011b). Extending the investigations, it was established that the DHFR can also support efficient internalization of a plant tRNA into mitochondria isolated from *S. cerevisiae* or mammalian cells. Finally, two different full-length mRNAs could be translocated into isolated mammalian mitochondria in the presence of the DHFR (Sieber et al., 2011b). Beyond in vitro assays, this strategy is expected to be validated in intact cells following double nuclear transformation with transgenes encoding the cargo RNA and the shuttle protein. If this is the case, it might become an alternative for the translocation of longer RNAs into the organelles in plant or mammalian cells.

9. A multi-subunit complex for RNA targeting

The most documented strategy to target customized RNAs into mitochondria remains also the most controversial. As mentioned, all mitochondrial tRNAs are imported from the cytosol in trypanosomatids (Schneider and Maréchal-Drouard, 2000). Transport of these tRNAs across the inner membrane in *Leishmania tropica* was proposed to be mediated by a large multi-subunit protein complex ("RIC" for "RNA import complex") including several respiratory components (Bhattacharyya et al., 2003; Chatterjee et al., 2006; Mukherjee et al., 2007). Detailed analysis of the process and of the RIC complex was reported (reviewed for instance in Adhya, 2008; Adhya et al., 2011; Lithgow and Schneider, 2010; Rubio and Hopper, 2011; Salinas et al., 2008; Sieber et al., 2011a). However, such a complex could never be characterized in other *Leishmania* species or in *Trypanosoma* and several of its presumed protein components were shown by other groups to be non-essential for mitochondrial tRNA import (Cristodero et al., 2010; Paris et al., 2009). The issue is especially puzzling as an editorial expression of concern (Schekman, 2010) has also been published about one of the manuscripts describing this work (Goswami et al., 2006). Nevertheless, further studies claimed tRNA delivery into isolated human mitochondria mediated by the *L. tropica* multi-subunit complex (Mahata et al., 2005). The complex was subsequently reported to enter living human cells and induce mitochondrial uptake of endogenous tRNAs, restoring organelle function in cybrids carrying a tRNA mutation (Mahata et al., 2006). Moreover, the RIC complex, or a derived sub-complex, was claimed to deliver preloaded exogenous short antisense RNAs (Mukherjee et al., 2008) or long polycistronic coding RNAs (Jash and Adhya, 2011; Mahato et al., 2011) bearing an import signal sequence into mitochondria in human cells, leading accordingly to respiration repression or mitochondrial function rescue (Fig. 4). Ultimately, upon injection, the *L. tropica* complex would distribute preloaded polycistronic coding RNAs encoding parts of the organelle genome to different tissues, into the cells and to mitochondria in a rat model (Jash et al., 2012). As such, this system looks very potent, but in the current context the data need to be confirmed independently by other laboratories.

10. Conclusions

Mitochondrial transformation per se has not been accessed yet in organisms other than yeast and *Chlamydomonas*, but a wealth of strategies has been explored to manipulate the organelle genetic system. As to the transfer of DNA into mitochondria, several approaches currently show promise in mammals, especially those based on carriers like MTD-TFAM (Iyer et al., 2012), MITO-Porters (Yamada and Harashima, 2012) or mitochondriotropic liposomes (Wagle et al., 2011) that can potentially load and distribute regular DNA in whole organisms. But beyond transfection, the challenge of maintaining the exogenous DNA in the organelles is open, in a context where the mechanism of mtDNA replication is still discussed (see references in Ruhanen et al., 2011) and where mtDNA recombination remains controversial (Elson and Lightowlers, 2006; Fan et al., 2012). In this respect, a method was proposed for cloning and replicating the entire mammalian mtDNA in *E. coli* (Yoon et al., 2009). One of the ideas behind that is to subsequently introduce the bacteria into the cytoplasm of mammalian cells as a recombinant enteroinvasive *E. coli* and to look for intracellular conjugative transfer of the cloned mtDNA to the mitochondria (Yoon and Koob, 2012). However, the strategy can also be used to generate customized mtDNA versions to be loaded on one of the above vehicles for in-cell organelle transfection, keeping in mind that mammalian mitochondria might be able to take up DNA of that size (Ibrahim et al., 2011). Whatever the strategy, selectable markers would ultimately be welcome for mammalian mitochondrial transformation, at least for fundamental studies. Recoded drug-resistance genes have recently been put forward for that purpose (Yoon and Koob, 2008, 2011). For obvious reasons, much effort has been concentrated on mammalian mitochondria so that, despite the pioneering work on organelle natural competence for DNA uptake (Koulintchenko et al., 2003), most approaches still need to be assessed in plants. Regarding the above comments, there are some advantages for that, as exogenous DNA introduced into plant mitochondria can be inserted into the mtDNA by homologous recombination (Mileshina et al., 2011). But for plants too, the issue of selection markers for mitochondrial transformation remains open. Conversely, progress in the knowledge on cellular RNA trafficking already allowed the development of a number of unprecedented alternatives to DNA transfection for the investigation, manipulation and rescue of the mitochondrial genetic system. The strategies of customized RNA targeting already diversified and have the potential to fulfill in the near future a number of expectations. These include functional complementation of mtDNA mutations and repression of mutated mtDNA replication, but also ribozyme-mediated elimination of poisonous mutated RNAs, knockdown or increase of organelle RNAs for fundamental studies, and introduction of new coding or noncoding RNAs. Thus, besides continued effort to manipulate the DNA content in the organelles, exploiting RNA technologies might bring a major contribution to mitochondrial genetics.

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RESULTS, CHAPTER I

Based on the novel possibility to target customized RNAs into mitochondria in plant cells (§ Introduction III.I.4), the general outline of the project was to downregulate specific organelle RNAs to reveal genetic coordination and cross-talk pathways. In the absence of information on putative regular antisense mechanisms in plant mitochondria, we chose to trigger downregulation by introducing *trans*-cleaving hammerhead ribozymes into mitochondria. Once annealed to their specific target, such catalytic RNAs develop their own cleaving activity, without need for elusive mitochondrial factors. However, formation of the active ribozyme/target complex has its own structural constraints, especially when the ribozyme is associated with another strongly structured moiety like the TYMV PKTLS that serves as a mitochondrial shuttle. This implied individual design and validation. These steps are the subject of the first part of the present "Results and Discussion" section. Validated chimeric ribozymes enabled the successful development of several *in vivo* RNA knockdown model systems in cell suspensions and whole plants. These are described and discussed in the second part of the "Results and Discussion" section. Finally, the third part details the impact of directed knockdown of mitochondrial RNAs on the organellar and nuclear transcriptomes, highlighting novel genetic regulation mechanisms.

I. Development of chimeric catalytic RNAs

I.1 Design of chimeric *trans*-cleaving ribozymes

A major question raised by the approach was to design *trans*-cleaving ribozymes retaining their catalytic activity when associated with the TYMV PKTLS as a chimeric RNA. On the other hand, a main obstacle in applying hammerhead ribozymes *in vivo* is the requirement of their common forms for high, non-physiological free magnesium concentrations. Although magnesium is considered not to be directly involved in catalysis, it helps the formation of the active complex configuration (Doudna and Lorsch, 2005). Earlier ribozyme cleavage data from *in vitro* model studies (Uhlenbeck, 2003) showed dependence on magnesium concentrations up to 25 mM, whereas the concentrations estimated to be present in plant cells and organelles would be in the range of 1–2 mM (Igamberdiev and Kleczkowski, 2001). We designed the *trans*-cleaving hammerhead ribozymes against the *nad9* mRNA (*Rz1nad9* and *Rz2nad9*, Figure 11), *sdh3* mRNA (*Rz1sdh3* and *Rz2sdh3*, Figure 14), *cob* mRNA (*Rz1cob* and *Rz2cob*, Figure 17), *cox3* mRNA (*Rz1cox3* and *Rz2cox3*, Figure 20) and *atp9* mRNA (*Rzatp9*, Val *et al*., 2011) based on a shortened catalytic core derived from the results of *in vitro* selection for variants active in low magnesium conditions (Persson *et al.*, 2002). Such ribozymes, with 2 instead of 4 bp in helix II and a UUUU tetraloop, showed

high activity at very low $MgCl_2$ concentration both *in vitro* and *in vivo* (Fedoruk-Wyszomirska *et al.*, 2009).

According to the work of Haseloff and Gerlach (1988), the GUC triplet is the best target site for cleavage by hammerhead ribozymes. That is why, when possible, we selected a GUC triplet as a target for our ribozymes in the substrate RNAs. However, there is no such triplet in the *atp9* coding sequence. Thus, we selected an AUC triplet as a target for *Rzatp9*. Previous work has shown that an AUC triplet is also efficiently cleaved if placed in an appropriate sequence context (Zoumadakis and Tabler, 1995). To ensure high specificity of the ribozyme, the antisense flanks responsible for target recognition and binding were designed to form 8 and 7 base-pairs in helixes I and III, respectively (Figures 11,14,17 and 20). Flank sequences that would lead to high bias towards G:C base-airs or A:T base-pairs upon annealing of the ribozyme to the substrate RNA were avoided. To have a better chance to obtain active catalytic RNA systems, two distinct ribozymes, targeting different regions, were designed against each substrate RNA (Figures 11,14,17 and 20).

The hammerhead *trans*-ribozymes were associated with the mitochondrial shuttle PKTLS *via* a linker (L) that provided spacing, to minimize the risk of interactions between the two moieties. The linker sequence was developed by bioinformatics. Initially, a sequence of 40 nucleotides was selected from a computer-generated pool of random sequences. In a second step, the selection was refined, based on predictions of RNA secondary structures using MFOLD (Mathews *et al.*, 1999). The final linker was chosen on the basis of its inability to form stable structures, so as to insert a sequence as neutral as possible between the PKTLS and the hammerhead ribozyme. Linker and ribozyme design benefited from the experience of the laboratory of Jan Barciszewski (Institute of Bio-organic Chemistry, Poznan, Poland).

I.2 *In vitro* activity of chimeric ribozymes

As mentioned above, cleavage of a target RNA by a *trans*-ribozyme is sensitive to a number of constraints that condition the formation of the active complex. These constraints are extended in our case by the combination with the PKTLS moiety. The designed ribozyme/target systems were thus first tested one by one for their functionality *in vitro*. The different ribozymes, combined with the linker and the PKTLS, were synthesized with T7 RNA polymerase and assayed with several target/ribozyme molar ratios and in different reaction conditions, with special attention to the dependence on the concentration of $MgCl_2$.

Name of primer	**Sequence 5'⟶ 3'**	**Used for**
nad9cibdirT7	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGCTGAGTATTCGGTATAACTCAC	*nad9 in vitro* target
nad9cibrev	TCCGTCGCTACGCTGTTCC	
sdh3cibdirT7	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGCCTATGAGAATTTCTACCAATTC	*sdh3 in vitro* target
sdh3cibrev	CCCGAAAAATCCGTCAATAAATG	
cobcib1dirT7	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGAGCAATCTTAGTTATTGGTGG	*cob in vitro* target 1
cobcib1rev	GCTGTTGAAAGCTAGATCCAC	
cobcib2dirT7	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGCATGCTAATGGGGCAAGTATG	*cob in vitro* target 2
cobcib2rev	ATCCTATAAAAGCTGTCACAATC	
coxcib1dirT7	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGCATGGTTTTCATGTGATTATAGG	*cox3 in vitro* target 1
coxcib1rev	GCCAGTACCATGCAGCTGC	
coxcib2dirT7	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGTGATTGAATCTCAGAGGCATTC	*cox3 in vitro* target 2
coxcib2rev	GTGCATGTACATCACACCTCC	

Table 2: List of the primers used to produce the different targets for the *in vitro* experiments.

Name of primer	**Sequence 5'⟶ 3'**	**Used for**
nad9RzLPKTLS	GAGCTTTTGCGAAACCCTCGGACCAGCAGCTGCCAGTAACACACCT ACGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	PCR1 *Rz1nad9*
T7nad9Rz	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGTAATGGATCT GATGAGCTTTTGCGAAACCCTCG	PCR2 *Rz1nad9*
nad9RzLPKTLS2nd	TGAGCTTTTGCGAAACTACCGGACCAGCAGCTGCCAGTAACACACC TACGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	PCR1 *Rz2nad9*
T7nad9Rz2nd	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGAATAGACTCT GATGAGCTTTTGCGAAACTACCG	PCR2 *Rz2nad9*
sdh3Rz1LPKTLS	GAGCTTTTGCGAAACGGAGAGACCAGCAGCTGCCAGTAACACACCT ACGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	PCR1 *Rz1sdh3*
T7sdh3Rz1	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGTAATCTCCTG ATGAGCTTTTGCGAAACGGAGAG	PCR2 *Rz1sdh3*
sdh3Rz2LPKTLS	GAGCTTTTGCGAAACAGGGCGACCAGCAGCTGCCAGTAACACACCT ACGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	PCR1 *Rz2sdh3*
T7sdh3Rz2	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGATGATAGCTG ATGAGCTTTTGCGAAACAGGGC	PCR2 *Rz2sdh3*
cobRz1LPKTLS	GATGAGCTTTTGCGAAACTAAACGACCAGCAGCTGCCAGTAACACA CCTACGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	PCR1 *Rz1cob*
T7cobRz1	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGATCTGAATCT GATGAGCTTTTGCGAAACTAAAC	PCR2 *Rz1cob*
cobRz2LPKTLS	GAGCTTTTGCGAAACTGCTAGACCAGCAGCTGCCAGTAACACACCT ACGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	PCR1 *Rz2cob*
T7cobRz2	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGTTCCCTAGCT GATGAGCTTTTGCGAAACTGCTAG	PCR2 *Rz2cob*
coxRz1LPKTLS	GAGCTTTTGCGAAACCAAGAGACCAGCAGCTGCCAGTAACACACCT ACGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	PCR1 *Rz1cox3*
T7coxRz1	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGTCAGATCTGA TGAGCTTTTGCGAAACCAAGAG	PCR2 *Rz1cox3*
coxRz2LPKTLS	GAGCTTTTGCGAAACTTGGAGACCAGCAGCTGCCAGTAACACACCT ACGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	PCR1 *Rz2cox3*
T7coxRz2	AGCAAGAATTCGAATTGTAATACGACTCACTATAGGAGGCCATGCT GATGAGCTTTTGCGAAACTTGGAG	PCR2 *Rz2cox3*
TLS3p	TGGTTCCGATGACCCTCGGA	Reverse primer

Table 3: List of the primers used to produce the different Rz-L-PKTLS constructs used for the *in vitro* experiments.

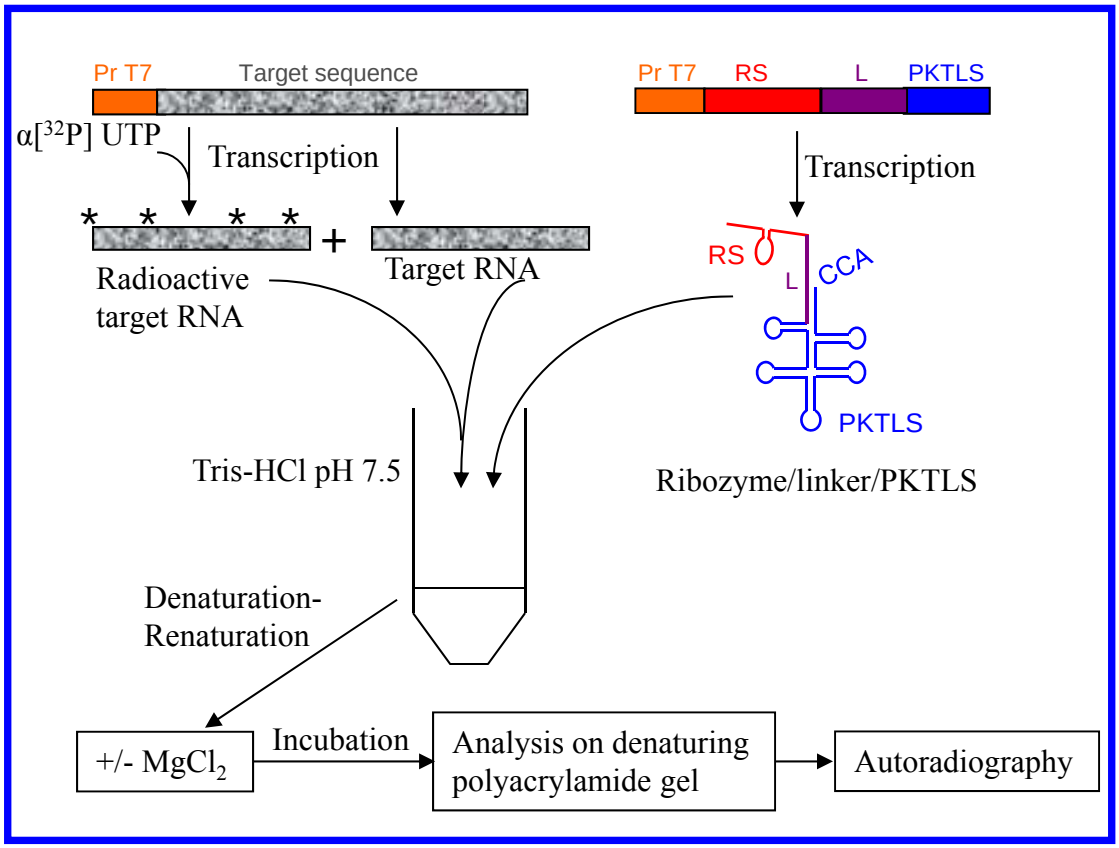


Figure 9: Schematic diagram of the method used to test the *in vitro* activity of ribozymes linked to the PKTLS

Pr T7: bacteriophage T7 promoter, RS: ribozyme sequence, L: linker, PKTLS: Pseudoknot tRNA-like structure.

The chimeric ribozyme against *atp9* (*Rzatp9*) has been validated previously (Val *et al*., 2011).

I.2.1 Synthesis of the target RNAs

DNA templates for transcription of *nad9, sdh3, cob* and *cox3* target RNAs were prepared in a single PCR step (Materials and Methods, §.II.2.5), using *A. thaliana* or *N. tabacum* total DNA as a template. The primers used for these PCR reactions are mentioned in Table 2. The direct primer contained the sequence for the promoter of the T7 RNA polymerase. From these PCR products, three types of transcripts were synthesized *in vitro* with T7 RNA polymerase for each target: unlabeled, labeled with [α^{32}P]UTP during transcription or 5' end-labeled with kinase and [γ^{32}P]ATP after transcription (Figure 9, Materials and Methods, §.II.3.4). A mixture of labeled and unlabeled transcripts at a defined concentration was used for *in vitro* cleavage assays.

I.2.2 Synthesis of the ribozyme/linker/PKTLS chimeric RNAs

The templates for the ribozyme/linker/PKTLS chimeras were synthesized by two successive PCR steps (Materials and Methods, §.II.2.5). The template for the first step (PCR1) was a previous construct in the pER8 vector containing the sequence coding for the PKTLS. The direct primer contained, from 5' to 3', the sequence corresponding to the 3'-end of the ribozyme, the linker and a sequence complementary to the 5'-end of the PKTLS (Table 3). The reverse primer hybridized to the 3'-end of the PKTLS sequence (TLS3P, Table 3). After PCR1, a fragment containing the 3'-end of the ribozyme, the linker and the PKTLS was obtained. This fragment was the template for a second PCR (PCR2) for which the direct primer contained the promoter of the T7 RNA polymerase upstream of the full sequence coding for the ribozyme (Table 3) and the reverse primer was again TLS3P. Thus, the PCR2 product contained the promoter of the T7 RNA polymerase, the sequence coding for the ribozyme, the linker and the PKTLS. PCR2 products were transcribed with T7 RNA polymerase.

I.2.3 *In vitro* cleavage of the target RNAs by the ribozymes linked to the PKTLS

The overall strategy used to test the cleavage of the target RNAs with the different ribozymes linked to the PKTLS is given in Figure 9. As designed, each target was tested with two ribozymes that had different cleavage sites. *In vitro* activity tests were performed as described in Materials and Methods, §.II.3.7, with the target RNAs and

AUGGAUAACCAAUUCAUUUUCAAAUAUAGUUGGGAGACUUUACCCAAGAAAUGGGUCAAAAAAAUGGAAAGAUCGGAAC
AUGGGAAUAGAUCUGAUACCAAUACGGACUACCUAUUUCAAUUGUUGUGCUUUCUGAAAUUGCAUACCUAUACAAGGGU
UCAAGUUUCGAUCGAUAUUUGCGGAGUUGAUUAUCCCUCUCGAAAACAAAGAUUUGAAGUGGUCUAUAAUUUACUGAGU
AUUCGGUAUAACUCACGCAUUCGUGUACAAACCAGUGCAGACGAAGUAACACGAAUAUCUCCGGUAGUCAGUCUAUUUC
CAUCAGCCGGCCGGUGGGAGCGAGAAGUUUGGGAUAUGUUUGGUGUUUCUUCCAUCAAUCAUCCGGAUCUACGCCGUAU
AUCAACAGAUUAUGGUUUCGAGGGUCAUCCAUUACGAAAAGACCUUCCUCUGAGUGGAUAUGUGGAAGUACGCUAUGAU
GAUCCAGAGAAACGUGUGGUUUCUGAACCCAUUGAGAUGACCCAAGAAUUUCGCUAUUUCGAUUUUGCUAGUCCUUGGG
AACAGCGUAGCGACGGAUAA

Figure 10: The full 573 nt sequence of the *nad9* mRNA in *N. tabacum*

The sequences that are underlined correspond to the primers used to amplify the 339 nt-long target.. The sequence in red is the site for attachment of *Rz1nad9* and the sequence in green is the site for attachment of *Rz2nad9*. Inside the green and red sequences, the blue "C" indicates the point of cleavage for the ribozymes.

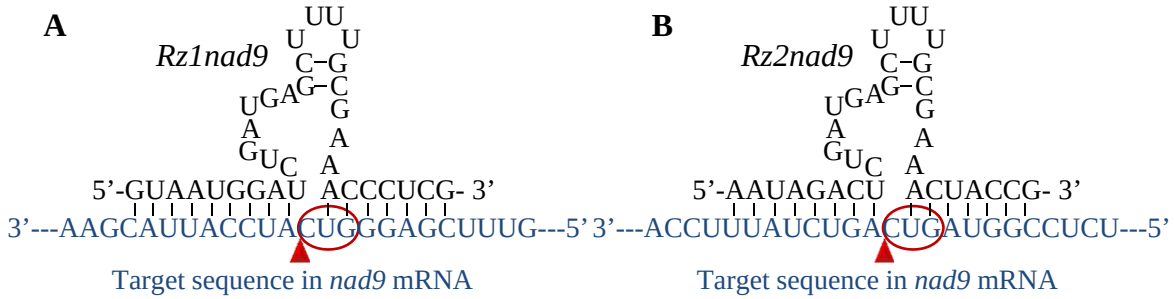


Figure 11: Structure of the *trans*-ribozymes as annealed to the *nad9* target RNA

The ribozyme sequence is in black, while the portion of the target sequence is in blue. The arrow indicates the cleavage site and the circle indicates the triplet that is identified by the *trans*-ribozyme for cleavage. **A)** *Rz1nad9*, **B)** *Rz2nad9*.

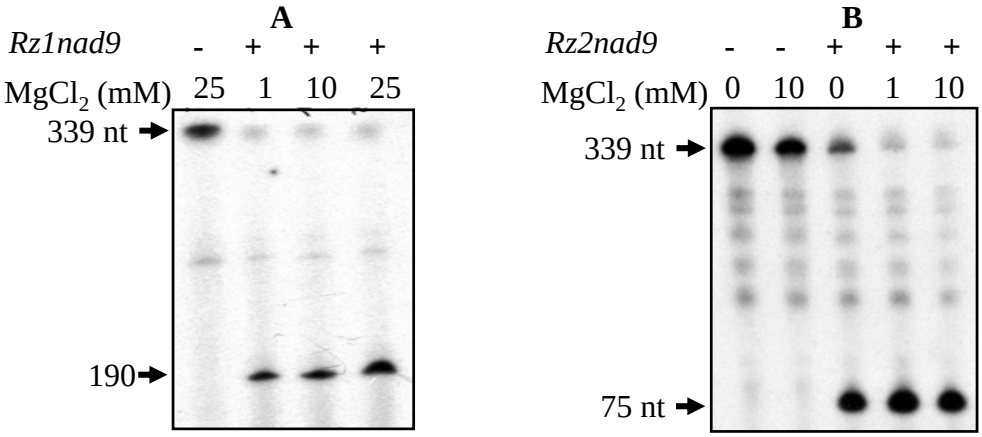


Figure 12: *In vitro* cleavage of the *nad9* target RNA by different ribozymes

The reactions were run with different concentrations of $MgCl_2$. **A)** *In vitro* cleavage mediated by *Rz1nad9* with a target/ribozyme molar ratio of 1/1000. Migration of the full-size target (339 nt) and the 5' end-labeled cleavage product (190 nt) are indicated. **B)** *In vitro* cleavage mediated by *Rz2nad9* with a target/ribozyme molar ratio of 1/10. Migration of the full-size target (339 nt) and the 5' end-labeled cleavage product (75 nt) are indicated. Note: Migration of the fragments is not comparable between A and B, as the electrophoresis conditions were different.

ribozyme/linker/PKTLS chimeric RNAs synthesized from the corresponding PCR products with T7 RNA polymerase.

I.2.3.1 Cleavage of the *nad9* target RNA

The synthesized *nad9* target RNA was 339 nucleotide-long (Figure 10) and was labeled at the 5'-end. It was used to test the cleavage activity of both *Rz1nad9* and *Rz2nad9*. The sequence of the *Rz1nad9* ribozyme, as annealed to its target at the site of cleavage, is shown in Figure 11A. Specific cleavage of the *nad9* target RNA with *Rz1nad9* was expected to produce two fragments of 190 and 149 nucleotides, respectively. The PKTLS-associated *Rz1nad9* did not significantly cleave its target at $MgCl_2$ concentrations up to 5 mM and target/ribozyme molar ratios up to 1/100. The experiments were thus run again with a target/ribozyme molar ratio of 1/1000 and with $MgCl_2$ concentrations up to 25 mM. The results are shown in Figure 12A. As the target was 5'-end-labeled in this case, the only cleavage product observed was the largest fragment of 190 nucleotides. At this higher molar ratio, we detected some cleavage even without added $MgCl_2$, good cleavage at 1 or 10 mM $MgCl_2$ and somehow better activity at 25 mM $MgCl_2$.

The sequence of the *Rz2nad9* ribozyme, as annealed to its target at the site of cleavage, is shown in Figure 11B. Specific cleavage of the *nad9* target RNA with *Rz2nad9* should generate two fragments of 75 and 264 nucleotides, respectively. As shown in Figure 12B, the PKTLS-associated *Rz2nad9* significantly cleaved its target at target/ribozyme molar ratios of 1/10, even without added $MgCl_2$. Only the smaller fragment of 75 nucleotides was visible because of 5'-end labeling. Cleavage efficiency was almost the same for both concentrations of $MgCl_2$ tested: 1 mM and 10 mM.

I.2.3.2 Cleavage of the *sdh3* target RNA

The synthesized *sdh3* target RNA was 132 nucleotide-long (Figure 13) and was tested with *Rz1sdh3* and *Rz2sdh3*. The sequence of the *Rz1sdh3* ribozyme, as annealed to its target at the site of cleavage, is shown in Figure 14A. Specific cleavage of the *sdh3* target RNA with *Rz1sdh3* was expected to produce two fragments of 60 and 72 nucleotides, both detectable thanks to the use of [α^{32}P]UTP during transcription. The PKTLS-associated *Rz1sdh3* was first tested for 1/10, 1/50 and 1/100 target/ribozyme molar ratios. Without added $MgCl_2$, no significant cleavage activity was observed with a 1/10 target/ribozyme molar ratio, but the reaction occurred at a 1/100 ratio. Further assays were run with a target/ribozyme molar ratio of 1/10 and with $MgCl_2$ concentrations up to 10 mM. The results are shown in Figure

ATGAATATCCTTCGCCCCTTATCTCCTCATCTTCCTATTTATAAGCCACAGCTTACTTCGACGTTTTCAATTTCCCATA
GAATCTCCGGAGCTTTCCTAGCCACTATAGTTTTCTTTTTTTATCTTCTTTGTCTGAAAATTGGTTTGATTTGCTTCAC
CTATGAGAATTTCTACCAATTCTTCTTTTATTCATCAAAGCTCATCCTAATCTCCGTCGAGATTACTGCCTTAGCCCTG
TCCTATCATCTGTATAATGGAGTTCGTCATTTATTGACGGATTTTTCGGGATTTTTCTTTCTTAGAATTGGAAGAAAAA
GATTGAAATGA

Figure 13: The full 327 nt sequence of the *sdh3* mRNA in *N. tabacum*.

The sequences that are underlined correspond to the primers used to amplify the 132 nt-long target. The sequence in red is the site for attachment of *Rz1sdh3* and the sequence in green is the site for attachment of *Rz2sdh3*. Inside the green and red sequences, the blue "C" indicates the point of cleavage for the ribozymes.

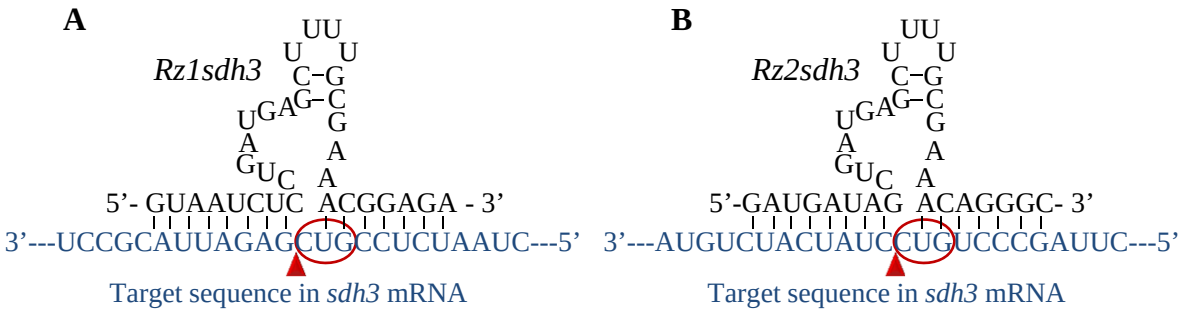


Figure 14: Structure of the *trans*-ribozymes as annealed to the *sdh3* target RNA

The ribozyme sequence is in black, while the portion of the target sequence is in blue. The arrow indicates the cleavage site and the circle indicates the triplet that is identified by the *trans*-ribozyme for cleavage. **A)** *Rz1sdh3*, **B)** *Rz2sdh3*.

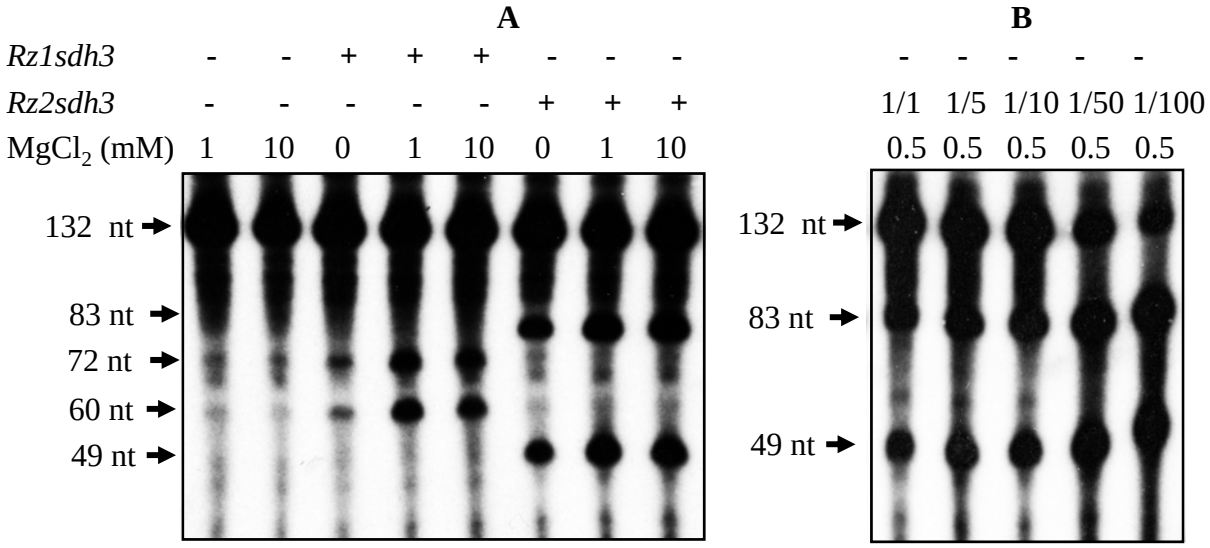


Figure 15: *In vitro* cleavage of the *sdh3* target RNA by different ribozymes

A) *In vitro* cleavage mediated by *Rz1sdh3* and *Rz2sdh3* with a target/ribozyme molar ratio of 1/10. Migration of the full-size target (132 nt) and the cleavage products (72 and 60 nt for *Rz1sdh3*, 83 and 49 nt for *Rz2sdh3*) are indicated. The reaction was run with different concentrations of $MgCl_2$. **B)** *In vitro* cleavage mediated by *Rz2sdh3* with different target/ribozyme molar ratios of 1/1, 1/5, 1/10, 1/50 and 1/100. The reaction was run with 0.5 mM $MgCl_2$.

ATGACTATAAGGAACCAACGACTCTCTCTTCTTAAACAACCTATATCCTCCACACTTAATCAGCATTTGATAGATTATCCAACCC
CGAGCAATCTTAGTTATTGGTGGGGGTTCGGTTCGTTAGCTGGTATTTGTTTAGTCATTCAGATAGTGACTGGCGTTTTTTTAGC
TATGCATTACACACCTCATGTGGATCTAGCTTTCAACAGCGTAGAACACATTATGAGAGATGTTGAAGGGGGCTGGTTGCTCCGT
TATATGCATGCTAATGGGGCAAGTATGTTTTTCATTGTGGTTCACCTTCATATTTTTCGTGGTCTATATCATGCCAGTTATAGCA
GTCCTAGGGAATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACAGCTTTTATAGGATATGTACTACCTTG
GGGTCAGATGAGCTTTTGGGGAGCTACAGTAATTACAAGCTTAGCTAGCGCCATACCTGTAGTAGGAGATACCATAGTGACTTGG
CTTTGGGGTGGGTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAGTCTTCATCATTTACTCCCCTTTATTTTAGTAGGCG
CCAGTCTTCTTCATCTGGCCGCATTGCATCAATATGGATCCAATAATCCATTGGGTGTACATTCAGAGATGGATAAAATTGCTTC
TTACCCTTATTTTTATGTAAAGGATCTAGTAGGTTGGGTAGCTTTTGCTATCTTTTTTTCCATTTGGATTTTTTATGCTCCTAAT
GTTTTGGGGCATCCCGACAATTATATACCTGCTAATCCGATGTCCACCCCGCCTCATATTGTGCCAGAATGGTATTTCCTACCGA
TCCATGCCATTCTTCGTAGTATACCTGACAAAGCGGGAGGTGTAGCCGCAATAGCACCAGTTTTTATATGTCTGTTGGCTTTACC
CTTTTTTAAAAGTATGTATGTACGTAGTTCAAGTTTTCGCCCGATTCACCAAGGAATATTTTGGTTGCTTTTGGCGGATTGCTTA
CTACTAGGTTGGATCGGATGTCAACCTGTGGAGGCACCCTTTGTTACTATTGGACAAATTTCTCCTTTAGTTTTCTTCTTGTTCT
TTGCCATAACGCCCATTCTGGGACGAGTTGGAAGAGGAATTCCTAATTCTTACACGGATGAGACTGATCACACCTGA

Figure 16: The full 1182 nt sequence of the *cob* mRNA in *N. tabacum*.

The sequences that are underlined and double underlined correspond to the primers used to amplify the 124 nt-long target 1 and the 151 nt-long target 2 respectively. The sequence in red is the site for attachment of *Rz1cob* and the sequence in green is the site for attachment of *Rz2cob*. Inside the green and red sequences, the blue "C" indicates the point of cleavage for the ribozymes.

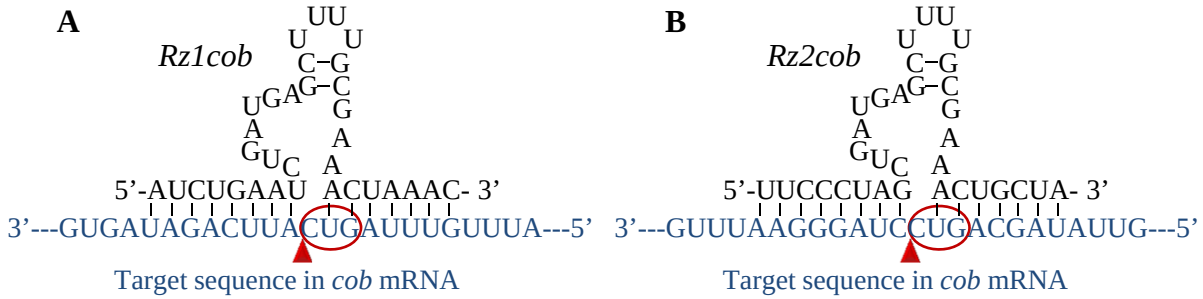


Figure 17: Structure of the *trans*-ribozymes as annealed to the *cob* target RNA

The ribozyme sequence is in black, while the portion of the target sequence is in blue. The arrow indicates the cleavage site and the circle indicates the triplet that is identified by the *trans*-ribozyme for cleavage. **A)** *Rz1cob*, **B)** *Rz2cob*.

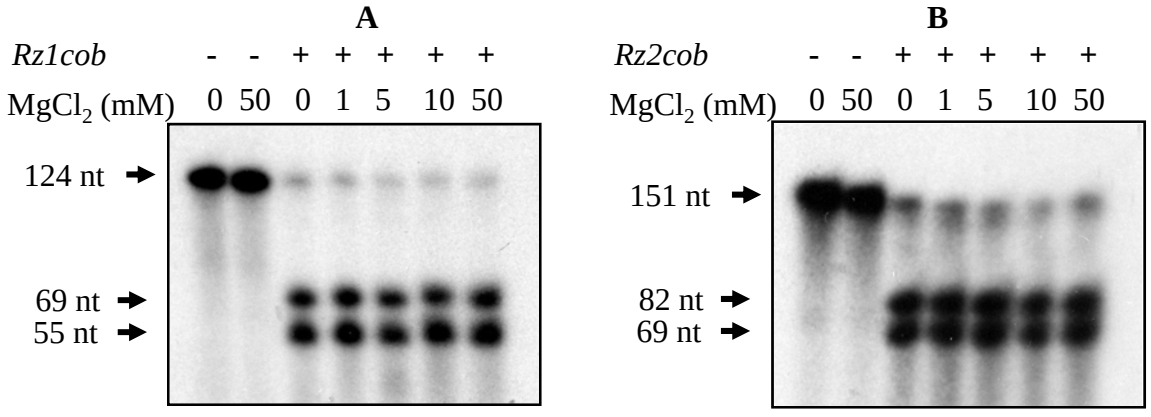


Figure 18: *In vitro* cleavage of the *cob* target RNA by different ribozymes

The reactions were run with different concentrations of $MgCl_2$. **A)** *In vitro* cleavage mediated by *Rz1cob* with a target/ribozyme molar ratio of 1/10. Migration of the full-size target (124 nt) and the cleavage products (69 and 55 nt) are indicated. **B)** *In vitro* cleavage mediated by *Rz2cob* with a target/ribozyme molar ratio of 1/10. Migration of the full-size target (151 nt) and the cleavage products (69 and 82 nt) are indicated.

ATGATTGAATCTCAGAGGCATTCTTATCATTTGGTAGATCCAAGTCCATGGCCTATTTCGGGTTCACTCGGAGCTTTGGCAAC
AACCGTAGGAGGTGTGATGTACATGCACTCATTTCAAGGGGGTGCAACACTTCTCAGTTTGGGCCTCATATTTATCCTATATA
CCATGTTCGTATGGTGGCGCGATGTTCTACGTGAATCCACGTTCGAAGGACATCATACCAAAGTCGTACAATTAGGACCTCGA
TATGGTTTTATTCTGTTTATCGTATCGGAGGTTATGTTCTTTTTTGCTCTTTTTCGGGCTTCTTCTCATTCTTCTTTGGCACC
TACGGTAGAGATCGGAGGTATTTGGCCCCCAAAGGGGATTGCGGTTTTAGATCCTTGGGAAATCCCTTTTCTTAATACCCTTA
TTCCCCTTTCATCCGGAGCTGCCGTAACTTGGGCTCATCATGCTATACTCGCGGGGAAGGAAAAACGAGCAGTTTACGCTTTA
GTAGCTACCGTTTCACTGGCTCTAGTATTCACAGGCTTTCAAGGAATGGAATATTATCAAGCACCCTTCACTATTTCGGATAG
TATTTATGGTTCTACCTTTTTCTTAGCAACTGGCTTTCATGGTTTTCATGTGATTATAGGTACTATTTTCTCGATCATATGTG
GTATTCGCCAATATCTTGGTCATCTGACCAAGGAGCATCACGTTGGCTTTGAAGCAGCTGCATGGTACTGGCATTTTGTAGAC
GTGGTTCGGTTATTCCTATTTGTCTCTATCTATTGGTGGGGAGGTATATGA

Figure 19: The full 798 nt sequence of the *cox3* mRNA in *N. tabacum.*
The sequences that are underlined and double underlined correspond to the primers used to amplify the 120 nt-long target 1 and the 112 nt-long target 2 respectively. The sequence in red is the site for attachment of *Rz1cox3* and the sequence in green is the site for attachment of *Rz2cox3*. Inside the green and red sequences, the blue "C" indicates the point of cleavage for the ribozymes.

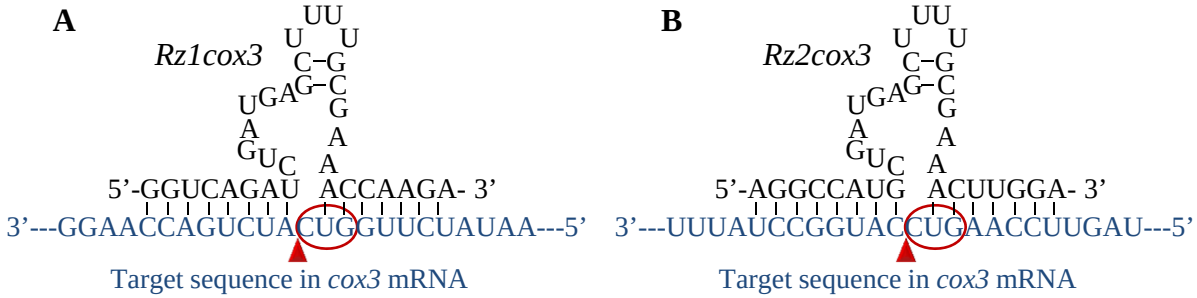


Figure 20: Structure of the *trans*-ribozymes as annealed to the *cox3* target RNA
The ribozyme sequence is in black, while the portion of the target sequence is in blue. The arrow indicates the cleavage site and the circle indicates the triplet that is identified by the *trans*-ribozyme for cleavage. **A)** *Rz1cox3*, **B)** *Rz2cox3*.

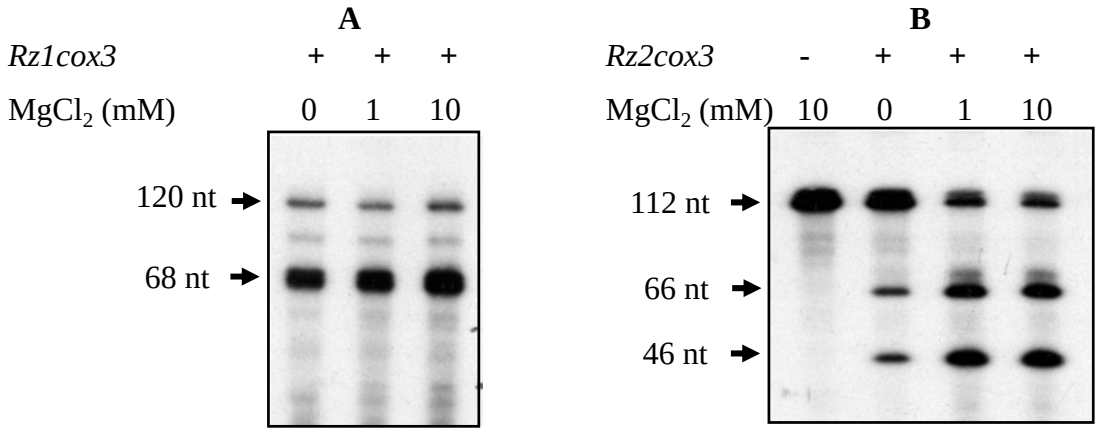


Figure 21: *In vitro* cleavage of the *cox3* target RNA by different ribozymes
The reactions were run with different concentrations of $MgCl_2$. **A)** *In vitro* cleavage mediated by *Rz1cox3* with a target/ribozyme molar ratio of 1/10. Migration of the full-size target (120 nt) and the 5' end-labeled cleavage product (68 nt) are indicated. **B)** The *in vitro* cleavage mediated by *Rz2cox3* with a target/ribozyme molar ratio of 1/10. The reaction was run with different concentrations of $MgCl_2$. Migration of the full-size target (112 nt) and the cleavage products (66 and 46 nt) are indicated.

15A. In these assays, we obtained good cleavage of the target at 1 mM $MgCl_2$ and no significant difference with increased $MgCl_2$ concentrations up to 10 mM.

The sequence of the *Rz2sdh3* ribozyme, as annealed to its target at the site of cleavage, is shown in Figure 14B. Specific cleavage of the *sdh3* target RNA with *Rz2sdh3* should generate two fragments of 83 and 49 nucleotides, respectively. The PKTLS-associated *Rz2sdh3* significantly cleaved its target at target/ribozyme molar ratios of 1/10 already without added $MgCl_2$, as shown in Figure 15B. Cleavage efficiency was almost the same for both concentrations of $MgCl_2$ tested: 1 mM and 10 mM. Then, the cleavage activity of *Rz2sdh3* was tested for lower target/ribozyme molar ratios, *i.e.* 1/1 and 1/5. In both cases, some cleavage was observed without added $MgCl_2$ and the activity was prominent with just 0.5 mM added $MgCl_2$, as shown in Figure 15B.

I.2.3.3 Cleavage of *cob* target RNAs

Two different *cob* target RNAs, 124 (Target 1) and 151 (Target 2) nucleotide-long (Figure 16), corresponding to *Rz1cob* and *Rz2cob*, respectively, were synthesized in the presence of [α^{32}P]UTP. The sequences of the *Rz1cob* and *Rz2cob* ribozymes, as annealed to their targets at the site of cleavage, are given in Figure 17. Specific cleavage of the *cob* Target 1 RNA with *Rz1cob* was expected to produce fragments of 55 and 69 nucleotides, while *cob* Target 2 should generate fragments of 82 and 69 nucleotides upon *Rz2cob* cleavage. Both ribozymes very efficiently cleaved their respective targets even without added $MgCl_2$ and with target/ribozyme molar ratios of 1/10, as shown in Figure 18. Cleavage efficiency was similar for all concentrations of $MgCl_2$ tested: 1 mM, 5 mM, 10 mM and 50 mM. The activity of both ribozymes was also tested for lower target/ribozyme molar ratios, *i.e.* 1/1, 1/2 and 1/5, without added $MgCl_2$. For both ribozymes, some cleavage activity was observed for a 1/5 molar ratio.

I.2.3.4 Cleavage of the *cox3* target RNA

The *cox3* Target 1 RNA was 120 nucleotide-long (Figure 19) and was labeled at the 5'-end. This target was used to test the cleavage with *Rz1cox3*. The sequence of the *Rz1cox3* ribozyme, as annealed to its target at the site of cleavage, is shown in Figure 20A. Specific cleavage of *cox3* Target 1 with *Rz1cox3* was expected to produce fragments of 68 and 52 nucleotides. The PKTLS-associated *Rz1cox3* efficiently cleaved its target at a target/ribozyme molar ratio of 1/10 even without added $MgCl_2$, as shown in Figure 21A

(only the fragment of 68 nucleotides was labeled). Cleavage efficiency was similar for both concentrations of $MgCl_2$ tested: 1 mM and 10 mM.

The *cox3* Target 2 RNA destined to test the cleavage with *Rz2cox3* was 112 nucleotide-long (Figure 19) and synthesized in the presence of [α^{32}P]UTP. The sequence of the *Rz2cox3* ribozyme, as annealed to its target at the site of cleavage, is shown in Figure 20B. Specific cleavage of *cox3* Target 2 with *Rz2cox3* should generate fragments of 46 and 66 nucleotides. The PKTLS-associated *Rz2cox3* significantly cleaved its target at a target/ribozyme molar ratio of 1/10 without added $MgCl_2$, as shown in Figure 21B. Cleavage efficiency was much enhanced at 1 mM $MgCl_2$ and remained similar at 10 mM $MgCl_2$.

I.3 Conclusions from the *in vitro* experiments

The *in vitro* experiments indeed showed diverse cleavage efficiencies of the different ribozyme/target systems, despite a common scheme in the design. The observations thus illustrated the constraints in the fine tuning of the active complex. Nevertheless, almost all of the designed short stem ribozymes in the end showed activity at a realistic target/ribozyme ratio and at low concentrations of magnesium. Many of them were active, or sometimes quite efficient, even in the absence of added $MgCl_2$ in the reaction. Properly devised *trans*-cleaving hammerhead ribozymes can thus develop high catalytic activity at physiological magnesium concentrations. Moreover, the cleavage activity in low magnesium clearly was not impaired by the combination with the linker and TYMV PKTLS. Altogether, these results validated the molecular strategy and opened the way for *in vivo* experiments.

RESULTS, CHAPTER II

Name of primer	Sequence 5'⟶ 3'	Used for
dirRz1nad9pER8Xho	AGCAATCTCGAGGTAATGGATCTGATGAGCTTTTGC GAAACCCTCGGACCAGCAGCTGCCAGTAACACACCT ACGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	*Rz1nad9*
dirRz2nad9pER8Xho	AGCAATCTCGAGAATAGACTCTGATGAGCTTTTGCG AAACTACCGGACCAGCAGCTGCCAGTAACACACCTA CGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	*Rz2nad9*
dirRz1sdh3pER8Xho	AGCAATCTCGAGGTAATCTCCTGATGAGCTTTTGCG AAACGGAGAGACCAGCAGCTGCCAGTAACACACCTA CGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	*Rz1sdh3*
dirRz2sdh3pER8Xho	AGCAATCTCGAGGATGATAGCTGATGAGCTTTTGCG AAACAGGGCGACCAGCAGCTGCCAGTAACACACCTA CGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	*Rz2sdh3*
dirRz1cobpER8Xho	AGCAATCTCGAGATCTGAATCTGATGAGCTTTTGCG AAACTAAACGACCAGCAGCTGCCAGTAACACACCTA CGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	*Rz1cob*
dirRz2cobpER8Xho	AGCAATCTCGAGTTCCCTAGCTGATGAGCTTTTGCG AAACTGCTAGACCAGCAGCTGCCAGTAACACACCTA CGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	*Rz2cob*
dirRz1cox3pER8Xho	AGCAATCTCGAGGGTCAGATCTGATGAGCTTTTGCG AAACCAAGAGACCAGCAGCTGCCAGTAACACACCTA CGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	*Rz1cox3*
dirRz2cox3pER8Xho	AGCAATCTCGAGAGGCCATGCTGATGAGCTTTTGCG AAACTTGGAGACCAGCAGCTGCCAGTAACACACCTA CGTGCGCTGCCAAACACTTCCACCTAAGTTCTCG	*Rz2cox3*
dirRzatp9LHDVpER8Xho	AGCAATCTCGAGCCAATACCCTGATGAGCTTTTGCG AAATAGCAGCTAGCATTGAAATAGCATTCAATGCTC ATACTGTGAACCTACGGGTCGGCATGGCATCTCC	*Rzatp9*
revHDVpER8Spe	GTACAGACTAGTCTCCCTTAGCCATCCGAGTG	Reverse primer

Table 4: List of the primers used to produce the different Rz-L-PKTLS constructs used for the *in vivo* experiments.

Name of primer	Sequence 5'⟶ 3'	Used for
nad9-Fw	CTGAGTATTCGGTATAACTCAC	*nad9* target
nad9-Rv	TCCGTCGCTACGCTGTTCC	
sdh3-Fw	CCTATGAGAATTTCTACCAATTC	*sdh3* target
sdh3-Rv	CCCGAAAAATCCGTCAATAAATG	
cob-Fw1	GAGCAATCTTAGTTATTGGTGG	*cob* target 1
cob-Rv1	GCTGTTGAAAGCTAGATCCAC	
cob-Fw2	CATGCTAATGGGGCAAGTATG	*cob* target 2
cob-Rv2	ATCCTATAAAAGCTGTCACAATC	
cox3-Fw1	CATGGTTTTCATGTGATTATAGG	*cox3* target 1
cox3-Rv1	GCCAGTACCATGCAGCTGC	
cox3-Fw2	TGATTGAATCTCAGAGGCATTC	*cox3* target 2
cox3-Rv2	GTGCATGTACATCACACCTCC	

Table 5: List of the primers used for PCR amplification after reverse transcription of total RNA.

II. Chimeric ribozyme expression and target knockdown *in vivo*

After assessing the cleavage activity of the different ribozyme/linker/PKTLS RNAs *in vitro*, we developed direct manipulation of mitochondrial RNAs in cells and whole plants. For this, we generated expression constructs and stable transformants, aiming at controlled ribozyme-mediated knockdown of individual transcripts in the organelles, along the previously defined RNA trafficking strategy (Val *et al.*, 2011).

II.1 Preparation of gene constructs for *in vivo* expression

PCR products were prepared with long direct primers (Table 4) containing, from 5' to 3', the complete sequence coding for the relevant ribozyme, the linker and the 5' end of the TYMV PKTLS. The reverse primer (Table 4) contained the sequence complementary to the 3'-end of the *cis*-ribozyme of the HDV. The template was a previous construct in which the PKTLS and HDV *cis*-ribozyme were already associated. The obtained PCR products were first cloned into the pUCAP vector using the *Eco*RI and *Hind*III restriction sites. After sequencing**,** the inserts were transferred into the inducible transcription unit (O_{LexA}-46 estradiol-inducible promoter) of the pER8 vector using the *Asc*I and *Pac*I restriction sites. Further details are in Materials and Methods (§ II.7). The resulting plasmids, called pER8-Rz-L-PKTLS-cHDV (Rz standing for the different *trans*-cleaving hammerhead ribozymes as defined in § I.1) were used for transformation of *N. tabacum* BY2 cell suspensions and *A. thaliana* or *N. tabacum* plants.

II.2 Stable nuclear transformants

II.2.2 Nuclear transformants of *N. tabacum* BY2 cells

N. tabacum BY2 cells were transformed by co-culture with *Agrobacterium tumefaciens* carrying the pER8-Rz-L-PKTLS-cHDV plasmids (Materials and Methods, § II.6.1). Transformed calli were selected on hygromycin and the presence of the construct was assessed by PCR with primers P309 and P310 (Table 5) annealing upstream and downstream of the cloning cassette, respectively. Stable transformants were obtained for the chimeric transgenes containing *Rz1nad9, Rz1sdh3, Rz2cob* and *Rz1cox3*. The calli were dispersed in liquid medium to generate transformed cell suspensions. For transgene expression, cells were sub-cultured in liquid medium supplemented with 10 µM of estradiol. In parallel, transformed cells were grown in liquid medium devoid of estradiol, as a control, and non-transformed cells were treated with estradiol. Total RNAs and mitochondrial RNAs were prepared from transformant cells harvested at mid-exponential growth phase.

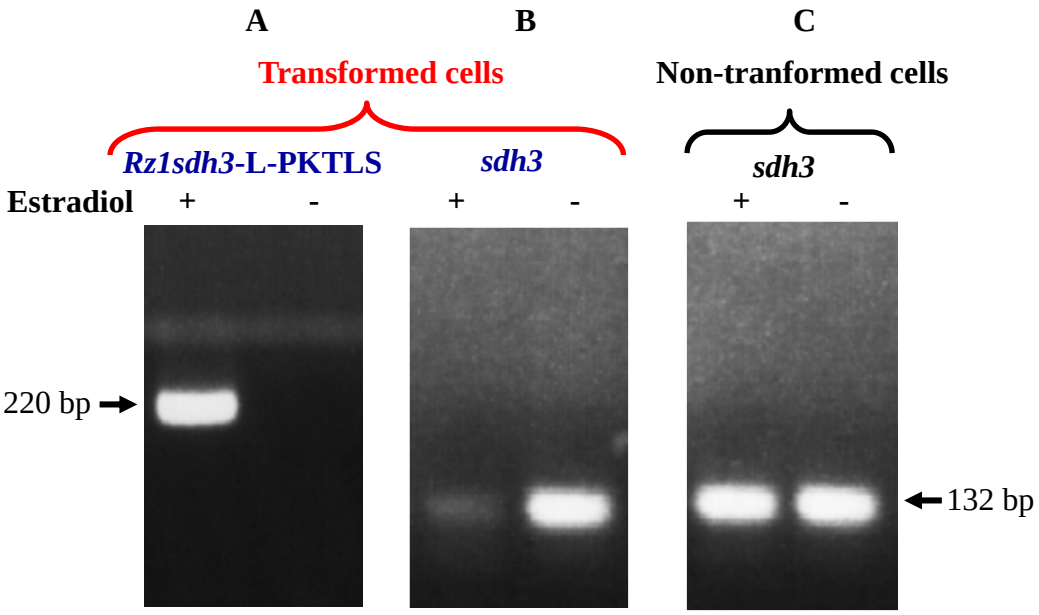


Figure 22: *In vivo* expression of the *Rz1sdh3*-L-PKTLS RNA in *N. tabacum* BY2 cells and knockdown of the *sdh3* target mRNA

Analysis by RT-PCR of total RNA extracted from *Rz1sdh3* transformants (A, B) and wild-type cells (C) cultured in the presence (+) or absence (-) of estradiol and harvested 3 days after onset of the induction. **A)** *In vivo* expression of *Rz1sdh3*-L-PKTLS in transformed *N. tabacum* BY2 cells. **B)** *In vivo* knockdown of the *sdh3* mRNA in transformed *N. tabacum* BY2 cells. **C)** Expression of the *sdh3* mRNA in wild-type *N. tabacum* BY2 cells.

II.2.3 Nuclear transformants of *A. thaliana* plants

A. thaliana plants (ecotype Col-0) were transformed *via A. tumefaciens* through floral dip (Materials and Methods, § II.6.2). Subsequently recovered seeds were germinated on hygromycin for transformant selection. The presence of the construct was assessed by PCR. Stable *A. thaliana* transformants were obtained for the chimeric transgenes comprising *Rz1nad9, Rz2nad9, Rz1sdh3, Rz2sdh3, Rz2cob* and *Rz1cox3*. Homozygous transformants were used for Rz-L-PKTLS RNA expression and target mRNA knockdown analysis. For this, plants were grown on solid agar medium that was overlaid with estradiol-supplemented liquid medium for transgene induction. Alternatively, plants grown on solid agar were transferred to culture plates containing liquid medium supplemented with estradiol and the roots were dipped into the medium. *sdh3* is not a mitochondrial gene in *A. thaliana* and the nuclear SDH3 genes have a distinct sequence. So the ribozyme designed against the *N. tabacum* mitochondrial *sdh3* served as a "no target" control for *A. thaliana* transformants.

II.2.4 Nuclear transformants of *N. tabacum* plants

To target *sdh3* in mitochondria, we generated transformed *N. tabacum* plants by leaf disk transformation (Materials and Methods, § II.6.3). Seeds recovered from regenerated plants were germinated on hygromycin for transformant selection and the presence of the construct was assessed by PCR. Stable *N. tabacum* transformants were obtained for the chimeric transgenes containing *Rz1sdh3* and *Rz2sdh3*. Homozygous transformants were used and induction of transgene expression was as for *A. thaliana* plants.

II.3 Ribozyme expression and target knockdown in *N. tabacum* BY2 cells

Total RNA was prepared from transformant *N. tabacum* cells harvested at mid-exponential growth phase. To analyze the expression of the chimeric ribozymes in induced cells, RT-PCR (Materials and Methods, II.2.5) was performed with a mixture of random hexamers for reverse transcription and the 312direxTraseqpER8 forward primer and TLS3P reverse primer for PCR (Table 5). Uninduced cells were used as a control. The first results were obtained with *Rz1sdh3* and are presented in Figure 22. The *Rz1sdh3*-L-PKTLS RNA was well expressed in induced cells, while it was not detectable in the absence of induction. To confirm that this was the desired sequence, the amplicon was cloned into pGEM®-T Easy Vector (Materials and Methods, II.2.8.4) and sequenced. The results showed that the expected RNA was expressed.

The function of the ribozyme *Rz1sdh3* once imported into the organelles was to cleave the *sdh3* mRNA and trigger knockdown. As described above (§ I.2.3.2) the *Rz1sdh3*-L-PKTLS RNA functioned properly *in vitro* under low $MgCl_2$ concentrations representative for physiological conditions. Subsequent studies showed that it also worked *in vivo* in mitochondria. To demonstrate that the *Rz1sdh3*-L-PKTLS RNA was able to promote a decrease of the *sdh3* mRNA levels in transformed cells, we conducted RT-PCR analysis using total RNA from three different cell cultures. Random hexamer primers were used to generate cDNA from total RNA. PCR in template-limiting conditions was carried out with primers sdh3-Fw and sdh3-Rv (Table 5).

Initially, we determined whether the induction with estradiol could itself cause a decrease in the expression of the *sdh3* gene and thus a decrease in the amount of *sdh3* mRNA. For that purpose, total RNA from non-transformed *N. tabacum* BY2 cells treated with estradiol was used for RT-PCR assays. The results are shown in Figure 22C. The *sdh3* mRNA levels seemed similar in cells cultured with or without estradiol. This information made it possible to assess the *sdh3* mRNA content in control cells cultured in the presence of estradiol.

In a second step, we determined the effects of the expression of the *Rz1sdh3*-L-PKTLS RNA on the amount of *sdh3* mRNA. For that, transformed cells were cultured with estradiol, to induce transcription of the transgene, or without estradiol, as a control. After 3 days of culture, total RNA was extracted and used for RT-PCR experiments (Figure 22B). When the transgene was expressed, a significant decrease in the level of *sdh3* mRNA was observed, compared to uninduced cells. This implied that, after import into mitochondria driven by the PKTLS shuttle, the hammerhead ribozyme *Rz1sdh3* we developed *in vitro* caused a knockdown of the *sdh3* target mRNA.

After obtaining these encouraging results with *Rz1sdh3*, we moved forward with the other ribozyme systems. Apart from *Rz1sdh3,* we had obtained stably transformed lines of *N. tabacum* BY2 cells for *Rz1nad9, Rz2cob* and *Rz1cox3*. Total RNA was prepared from estradiol-induced cells of these lines harvested at mid-exponential growth phase. RT–PCR was performed as above (priming with random hexamers for reverse transcription, 312direxTraseqpER8 forward primer and TLS3P reverse primer for PCR) to establish the expression of the chimeric ribozymes. Uninduced cells were used as control. Similar to *Rz1sdh3*-L-PKTLS, all these ribozymes were well expressed in induced cells and were not

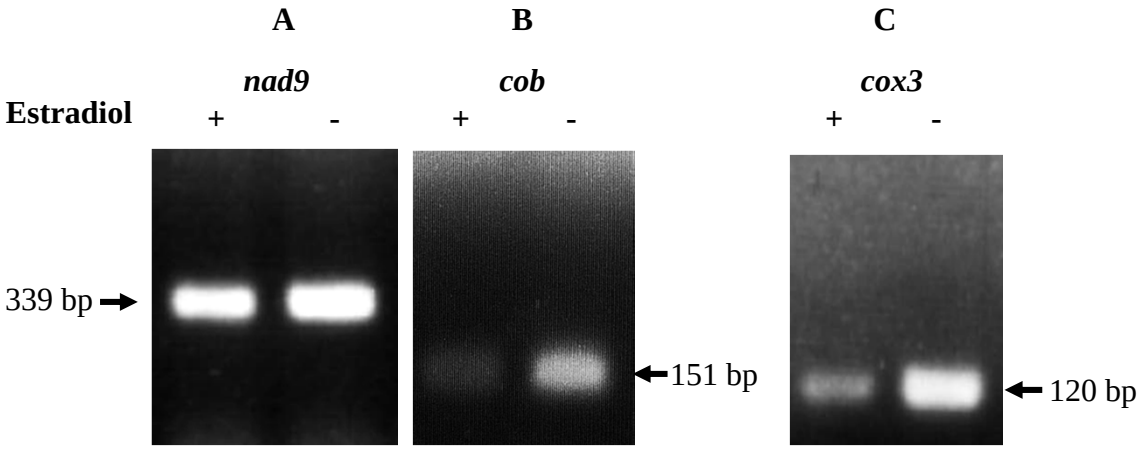


Figure 23: Expression of *nad9, sdh3* and *cob* mRNA in transgenic *N. tabacum* BY2 cells

Analysis by RT-PCR of total RNA extracted from BY2 cells cultured in the presence (+) or absence (-) of estradiol and harvested 3 days after onset of the induction. **(A)** Expression of *nad9* mRNA in transformed BY2 tobacco cells expressing *Rz1nad9*-L-PKTLS RNA. **(B)** Expression of *cob* mRNA in transformed BY2 tobacco cells expressing *Rz2cob*-L-PKTLS RNA. **(C)** Expression of *cox3* mRNA in transformed BY2 tobacco cells expressing *Rz1cox3*-L-PKTLS RNA.

detectable in the absence of induction. The identity of the RT–PCR products was confirmed as before by cloning and sequencing the amplicons.

The *Rz1nad9, Rz2cob* and *Rz1cox3* ribozymes connected to the PKTLS also functioned properly *in vitro* under low $MgCl_2$ concentration (§ I.2.3.1, I.2.3.3 and I.2.3.4) and were expected to cleave the *nad9, cob* and *cox3* mRNAs, respectively, in the mitochondria of induced transformed cells. To demonstrate that these catalytic RNAs were able to decrease the level of their respective target mRNAs *in vivo*, we conducted RT-PCR analysis as for *sdh3* (Figure 23, primers in Table 5). For cells expressing *Rz2cob*-L-PKTLS and *Rz1cox3*-L-PKTLS, the results were comparable to those obtained with *Rz1sdh3*-L-PKTLS, as a significant decrease in the level of the *cob* or *cox3* mRNAs was observed when compared to uninduced cells (Figure 23B and 23C). Thus, after import into mitochondria driven by the PKTLS shuttle, the *Rz2cob* and *Rz1cox3* ribozymes in turn caused a knockdown of their target mRNAs. These observations extended previous data and altogether established that appropriately designed *trans*-cleaving ribozymes are active in mitochondria and promote the knockdown of their specific target.

The results were however different for the transgenic cell lines expressing the ribozyme against *nad9*. Although the *Rz1nad9*-L-PKTLS RNA was well expressed in estradiol-induced transformed cells, the level of *nad9* mRNA remained similar to that of uninduced cells (Figure 23A). So, the *Rz1nad9* ribozyme we developed *in vitro* was not functional *in vivo* in *N. tabacum* transgenic cells. Thus, it is still possible that a hammerhead ribozyme fully functional *in vitro* does not work under *in vivo* conditions.

II.4 Ribozyme expression and target knockdown in whole plants

Having established expression of ribozymes and knockdown of targets in transformed cell lines, we moved forward to whole plant transformants. As mentioned (§ I.3.2.2), homozygous transformed *A. thaliana* plants were obtained for the chimeric transgenes comprising *Rz1nad9, Rz2nad9, Rz1sdh3, Rz2sdh3, Rz2cob* and *Rz1cox3*. There is no sequence in the *A. thaliana* mitochondrial genome to be cleaved by *Rz1sdh3* or *Rz2sdh3*. So, these ribozymes served as controls for *A. thaliana*, whereas their activity and effects were studied in transformed *N. tabacum* plants.

Total RNA was prepared from transformant plants, at a 10-12 leaves stage for *A. thaliana* and a 4-6 leaves stage for *N. tabacum*. Plants were cultivated on agar plates under long day

Name of primer	Sequence 5'⟶ 3'	Used for
14nad1bAtFw	TTCTCGTCTAGCAGAAACTAATCG	*nad1b* Arabidopsis
14nad1bAtRv	TTCTACATTATAGCCTGCAACTGATT	
79nad1cAtFw	TTGGGAGAGTATGCCAATATGA	*nad1c* Arabidopsis
79nad1cAtRv	AAATGGGAAGATCTAGGATAGGC	
133nad2bAtFw	GCTCTAGCCAAAACGAATCCT	*nad2b* Arabidopsis
133nad2bAtRv	GGGGTATTCCTGCGTATGAG	
41nad4AtFw	CCATATGAATCTGGTGACTATTGG	*nad4* Arabidopsis
41nad4AtRv	CGGTCATATAAAACACCAACACA	
128nad5aAtFw	CCACCTACGGCTTTGATTGT	*nad5a* Arabidopsis
128nad5aAtRv	GTTGCCGCAAGGAATGAC	
137nad5bAtFw	CAATTTTTGGGCCAATTCC	*nad5b* Arabidopsis
137nad5bAtRv	TGGTTGGAGCAGCAAACTC	
132nad6AtFw	GATTGGGGATTCAGTGGTGT	*nad6* Arabidopsis
132nad6AtRv	TGGTACGTCAAGATCCGATTG	
132nad7AtFw	GATTGGGGATTCAGTGGTGT	*nad7* Arabidopsis
132nad7AtRv	TGGTACGTCAAGATCCGATTG	
156nad9AtFw	AACTTCTCGCTCCCACCAG	*nad9* Arabidopsis
156nad9AtRv	AGTGCAGACGAAGTAACACGAA	
91sdh3-1AtFw	CAGAGCTTTCGGGTAGGTCA	sdh3-1 Arabidopsis
91sdh3AtRv	CGAGTCAATTGAGCACCAAC	
164cobAtFw	GAACACATTATGAGAGATGTTGAAGG	*cob* Arabidopsis
164cobAtRv	AACATACTTGCCCCATTAGCA	
42cox1AtFw	GTATGCCACGTCGTATTCCA	*cox1*Arabidopsis
42cox1AtRv	CGGATATATAAGAGCCAAAACTGG	
143cox2AtFw	TCCGATGAGCAGTCACTCAC	*cox2* Arabidopsis
143cox2AtRv	AATAAACGTGATTGACCCAATTCT	
136cox3AtFw	CCTTGGGAAATCCCTTTTCT	*cox3* Arabidopsis
136cox3AtRv	TTTCTTTCCCCGCGAGTAT	
78atp1AtFw	GCCTTACCCGTCATTGAAAC	*atp1* Arabidopsis
78atp1AtRv	CACATTGGTGGGAATATAGGC	
60atp4AtFw	CGTTTTCAGGACGATCTAGTCA	*atp4* Arabidopsis
60atp4AtRv	TTTCGAGATACAAGAGTAGGCAAA	
24atp9AtFw	CGGAGCTGCTACAATTGCTT	*atp9* Arabidopsis
24atp9AtRv	CGCCACAGAATGAATCAAAG	
139rps3AtFw	CTCGACCAGCGAGAAAAAGT	*rps3* Arabidopsis
139rps3AtRv	CGAATGAAGTGGGTCAACCT	
143rps4AtFw	TCGGATCCAAACTTCTTTGTTC	*rps4* Arabidopsis
143rps4AtRv	CCCGGTTTTTGCAACAGT	
31rps7AtFw	GCACGTAGGAAAAGGGAGAAT	*rps7* Arabidopsis
31rps7AtRv	ATCTGAAATGCGCGAAACTT	

Table 6: List of the primers used for qPCR analyses (continued).

155rpl2AtFw	TCTGCCTTCTCCTCTCCAAA	*rpl2* Arabidopsis
155rpl2AtRv	GCTATCCTTGGGAAACCAAA	
154rpl5AtFw	CTCGATGGAAACGGAGTTTT	*rpl5* Arabidopsis
154rpl5AtRv	CATTGAACCCCCGAATATGT	
103rpl16AtFw	CGGGAAACCCACAGAAGTAA	*rpl16* Arabidopsis
103rpl16AtRv	ACACACGAGCAATCCAACC	
74rrn18AtFw	CACACTGGGACTGAGACACG	*rrn18* Arabidopsis
74rrn18AtRv	GCCCATTGTCCAAGATTCC	
80rrn26AtFw	GGGAAGGTTTTTGGTGACAA	*rrn26* Arabidopsis
80rrn26AtRv	TCGTTACTCATGTCAGCATTCTC	
72ccb203AtFw	GTGGTTTTTAACCGTAGGCATC	*ccb203* Arabidopsis
72ccb203AtRv	CCACCCCGACCTAATTCAT	
116ccb206AtFw	CGGAATGGATCGGTTAAACA	*ccb206* Arabidopsis
116ccb206AtRv	CCGAACGAGAATGAATACCAC	
31ccb256AtFw	GTGCTGGACCGATCGATATAC	*ccb256* Arabidopsis
31ccb256AtRv	ATGCTCCCAGGTTGATGC	
160ccb382AtFw	GATTGTGGCACTCCACTCG	*ccb382* Arabidopsis
160ccb382AtRv	ATCCAGCAGAGCGAAGCA	
25ccb452AtFw	TGGCTGTTGGTCACGACTAC	*ccb452* Arabidopsis
25ccb452AtRv	GGCAATCGTTGAGTGAACTG	
136aox1aAtFw	TGGTTGTTCGTGCTGACG	aox1a Arabidopsis
136aox1aAtRv	CACGACCTTGGTAGTGAATATCAG	
6aox1dAtFw	CAAACTCTGAAAATACCGGTTCA	aox1d Arabidopsis
6aox1dAtRv	TCTCTAGCAACATCGCATGG	
31actinAtFw	GGATCTGTACGGTAACATTGTGC	actin Arabidopsis
31actinAtRv	TGCTCATACGGTCAGCGATA	
gapdhFwAra	TTGGTGACAACAGGTCAAGCA	gapdh Arabidopsis
gapdhRvAra	AAACTTGTCGCTCAATGCAATC	
expAtFw	GAGCTGAAGTGGCTTCAATGAC	exp Arabidopsis
expAtRv	GGTCCGACATACCCATGATCC	
tipAtFw	GTGAAAACTGTTGGAGAGAAGCAA	tip Arabidopsis
tipAtRv	TCAACTGGATACCCTTTCGCA	
106pp2aa3AtFw	CTGGCTAAGCGACTTTCAGC	pp2aa3 Arabidopsis
106pp2aa3AtRv	AATATGGAAAATCCCACATGCT	
64PKTLSFw	TCGCCAGTTAGCGAGGTCT	PKTLS
64PKTLSRv	GTTCCGATGACCCTCGGAAG	
9sdh3tabFw	GTCGAGATTACTGCCTTAGCC	*sdh3* tobacco
9sdh3tabRv	AATCCGTCAATAAATGACGAACT	
actin-1 Rv	GCCAGTGGCCGTACAACAGGT	*actin* tobacco
actin-1 Fw	CGAAGAATTGCATGAGGAAGGGC	
91rpl2tabFw	CAGTCTCTCCCTTGGCCTTT	*rpl2* tobacco
91rpl2tabRv	AAAAACTTGCGCGAAGGAC	

Table 6: List of the primers used for qPCR analyses.

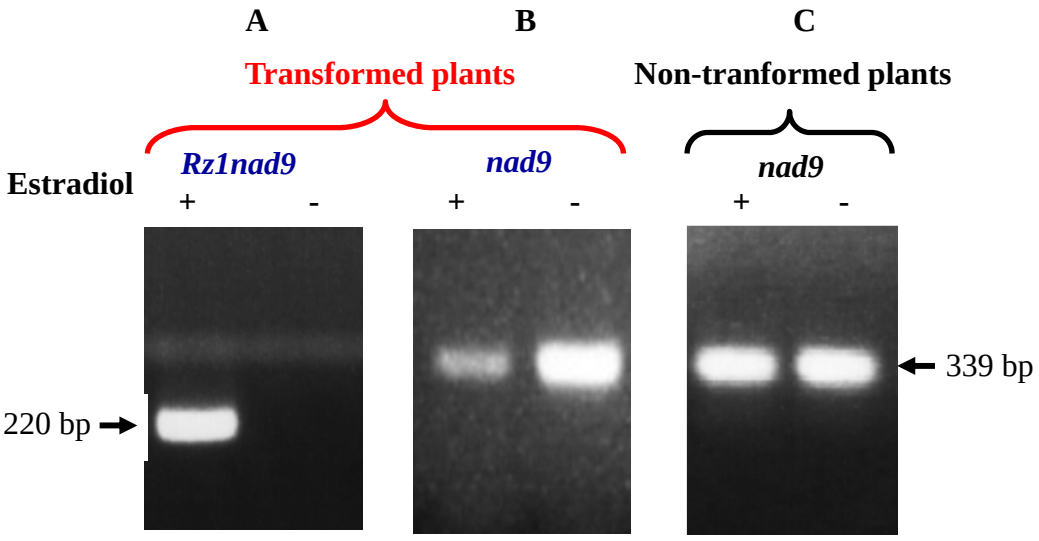


Figure 24: *In vivo* expression of the *Rz1nad9*-L-PKTLS RNA in *A. thaliana* plants and knockdown of the *nad9* target mRNA

Analysis by RT-PCR of total RNA extracted from *Rz1nad9* transformants (A, B) and wild-type plants (C) cultured in the presence (+) or absence (-) of estradiol and harvested 3 days after onset of the induction. **A)** *In vivo* expression of *Rz1nad9*-L-PKTLS in transformed *A. thaliana* plants. **B)** *In vivo* knockdown of the *nad9* mRNA in transformed *A. thaliana* plants. **C)** Expression of the *nad9* mRNA in wild-type *A. thaliana* plants.

conditions (16 h light/8 h dark) and transferred on liquid medium for transgene induction. RT–PCR in template-limiting conditions was performed as before (priming with random hexamers for reverse transcription, 312direxTraseqpER8 forward primer and TLS3P reverse primer for PCR) to establish the expression of the chimeric ribozymes in estradiol-induced plants. Uninduced plants at the same growth stage were used as a control. Similar to what we observed for cell cultures, all chimeric ribozymes were efficiently expressed in plants upon estradiol induction. Cloning and sequencing of the amplicons confirmed that the RNAs were expressed as expected. When RT-PCR was in turn conducted to analyze the levels of the target RNAs, the results implied that, as in cell suspensions, the expressed ribozymes were driven by the PKTLS shuttle into the mitochondria of the transgenic plants and cleaved their respective targets. Decrease of the target RNA level was observed in all cases, to various degrees. Remarkably, whereas no knockdown of the *nad9* mRNA had been detected in transformed *N. tabacum* cell suspensions, expression of the *Rz1nad9*-L-PKTLS RNA led to a large decrease in the level of *nad9* in *A. thaliana* plants, as shown in Figure 24. For the other ribozymes, the results were similar to those described above for cell cultures, with even a better expression of the transgenes and a more pronounced knockdown of the corresponding targets. Following these results, we proceeded with transformed plants for deeper analyses.

II.5 Kinetics of target RNA knockdown in whole plants

In order to study in parallel the expression of the ribozyme and the decrease in target mRNA, kinetic analyses were performed. For this, the plants (10-12 leaves stage for *A. thaliana*, 4-6 leaves stage for *N. tabacum*) were induced with estradiol and harvested each day till four days after induction. As a control, wild-type plants were grown and treated in the same way. Total RNA was extracted from these plants and analyzed by RT-PCR. From that stage and for all further experiments, we switched to RT-quantitative PCR (RT-qPCR, Materials and Methods § II.2.6). The RT reactions were as above primed with a mix of random hexamers, whereas qPCR was run with dedicated couples of primers (Table 6). Three mRNAs were chosen as references for studies in *A. thaliana*, according to the protocol described in Materials and Methods (§ I.7). Two of them, actin and *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase), correspond to nuclear mRNAs. No relationship between these RNAs and mitochondria has been demonstrated to date, these are extra-mitochondrial references. The primers used for qPCR were designed as explained in Materials and Methods (§ I.7). The pair of primers used for the amplification of *actin* and

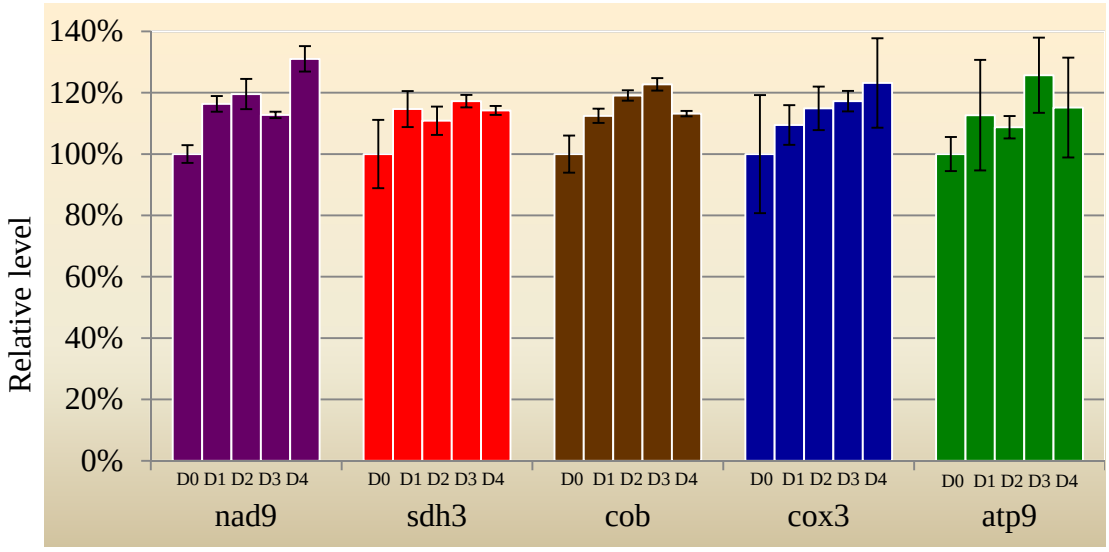


Figure 25: Kinetics of the *nad9, sdh3, cob, cox3* and *atp9* mRNA levels in wild-type *A. thaliana* plants cultured in the presence of estradiol

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. A relative level of 100% was assigned to day 0 in each case.

GAPDH by qPCR were 31actine2araFw/31actine2araRv and GAPDH-Fw/GAPDH-Rv, respectively (Table 6). To minimize any bias due to overall change in mitochondrial RNA within cells, the third reference was the mRNA encoding the mitochondrial ribosomal protein L2 (*rpl2*). It served as an intra-mitochondrial reference. The primers used were 133rpl2araFw and 133rpl2araRv (Table 6). For studies in *N. tabacum*, only *actin* and *rpl2* behaved as valuable references. The primer pairs in that case were actine-1Rv/actine-1Fw and 91rpl2tabFw/91rpl2tabRv (Table 6). The cDNAs corresponding to the *nad9, cob, cox3* and *atp9* mRNAs in *A. thaliana* were amplified with the primers 156nad9araFw/156nad9araRv, 164cobaraFw/164cobaraRv, 136cox3araFw/136cox3araRv and 24atp9araFw/24atp9araRv. Finally, primer pairs 91sdh3araFw/91sdh3araRv and 9sdh3tabFw/9sdh3tabRv were used to probe for *sdh3* in *A. thaliana* and *N. tabacum*, respectively.

RT-qPCR was first performed to investigate a possible negative or positive effect of estradiol treatment itself on the expression of the *nad9, sdh3, cob, cox3* or *atp9* mRNAs. To do this, total RNA from wild-type *A. thaliana* Col-0 plants, exposed or not to estradiol, was used to perform RT-qPCR. The results are shown in Figure 25 as a percentage of the level in wild-type plants grown without estradiol, after normalization according to the reference genes. The presence of estradiol actually triggered a slight increase in the content of the above mRNAs. These values were subsequently considered as the reference mRNA levels in plants cultured in the presence of estradiol but without the action of a ribozyme. Accurate further analyses were thus carried out by comparing the level of a given mRNA in samples from estradiol-treated transformant plants to that in estradiol-treated wild-type plants

The results of RT-qPCR assays performed to check the expression level of the chimeric ribozymes are presented in Figures 26 to 31, Panels A. Maximum expression of the various Rz-L-PKTLS RNAs was on day two after the onset of the induction (D2), and then the level droped to some extent till four days of induction (D4). The rate of transgene transcript in non-induced tranformant plants (D0) was extremely low but not necessarily zero. Control RT-qPCR experiments with reverse transcription reactions performed without reverse transcriptase gave no amplification, demonstrating the absence of residual DNA and effective treatment with RNase-free DNase (Materials and Methods, § II.3.3). Trace expression of ribozymes in non-induced transformed plants was thus likely due to a slight

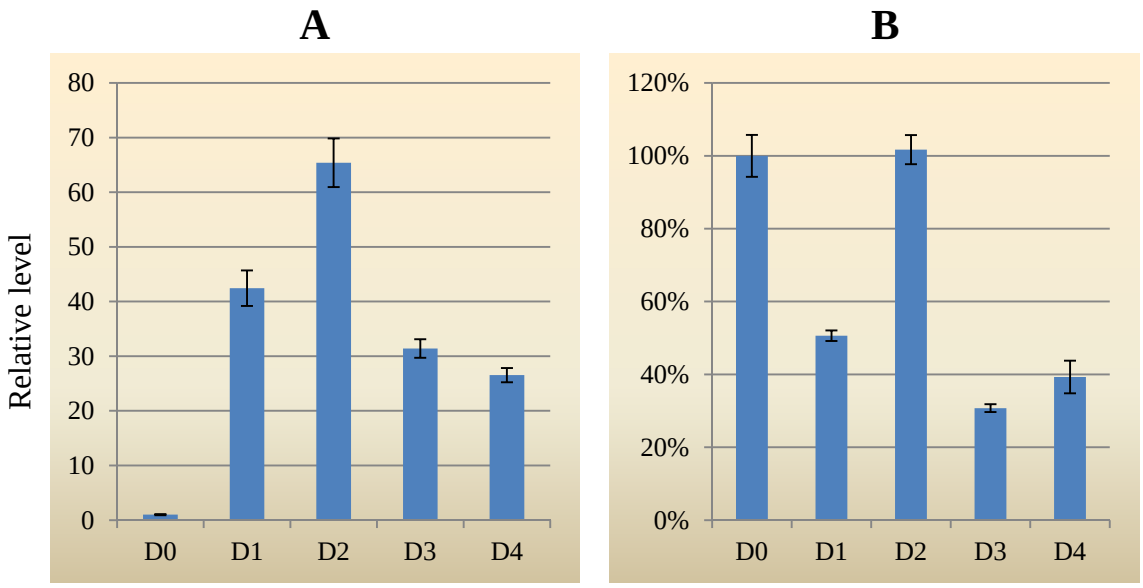


Figure 26: Kinetics of the *Rz1nad9*-L-PKTLS RNA expression and of the *nad9* target RNA level in 10-12 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. **A)** Relative expression of the *Rz1nad9*-L-PKTLS RNA in *Rz1nad9* transformants as compared to the expression at D0. **B)** Percentage of *nad9* mRNA in *Rz1nad9* transformants as compared to wild-type plants.

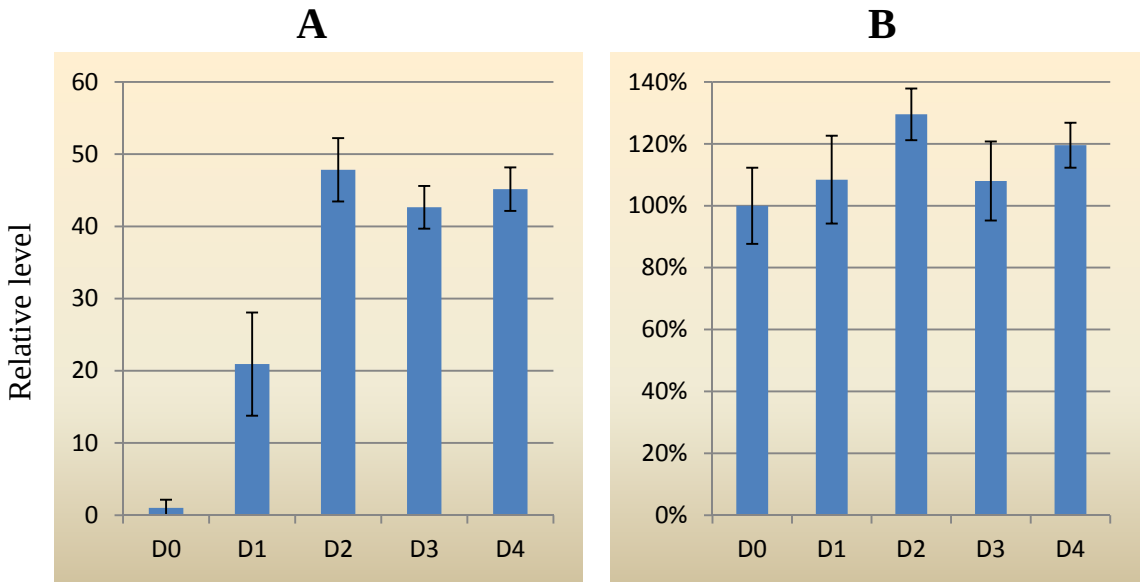


Figure 27: Kinetics of the *Rz1sdh3*-L-PKTLS RNA expression and of the *sdh3* target RNA level in 10-12 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. **A)** Relative expression of the *Rz1sdh3*-L-PKTLS RNA in *Rz1sdh3* transformants as compared to the expression at D0. **B)** Percentage of *sdh3* mRNA in *Rz1sdh3* transformants as compared to wild-type plants.

leakage of the XVE inducible promoter system, possibly mediated by some endogenous plant compounds.

The next step was to determine the effects of expressing the *Rz*-L-PKTLS chimeric ribozymes on the levels of their specific target mRNAs in transformed plants. The experiments were repeated thrice and the results are presented in Figures 26 to 31, Panels B. We will discuss them one by one.

We showed above that the *Rz1nad9* ribozyme connected to the linker and PKTLS did not function properly in *N. tabacum* transformed cell lines, although its expression was quite prominent (§ II.3). On the contrary, this ribozyme was the most efficient in transformed *A. thaliana* plants. One day after onset of transgene induction and expression of the *Rz1nad9*-L-PKTLS RNA (D1), about 50% decrease in the level of *nad9* mRNA was observed, as compared to estradiol-treated wild-type plants (Figure 26B). This implies mitochondrial import of the *Rz1nad9*-L-PKTLS transcript and cleavage of the *nad9* target mRNA. Strikingly, two days after onset of induction (D2), the level of *nad9* mRNA was back to the level of the control, whereas a second decrease reaching 70% occurred on the third day (D3) and remained around 60% on the fourth day (D4) (Figure 26B). The "bounce back" effect on D2 is still unexplained. May be when the demand for mitochondrial activity is high, the drop in the *nad9* level can be sensed and possibly trigger increased synthesis. Such observations are in favor of mRNA control mechanisms in mitochondria. These results were similar with transgenic *A. thaliana* plants expressing the *Rz2nad9*-L-PKTLS ribozyme. That *Rznad9* did not function in *N. tabacum* transformed cell lines but worked in *A. thaliana* plants also showed that the activity of the ribozymes may vary in different organisms and under different physiological conditions.

The results obtained with *Rz1sdh3* in *A. thaliana* transformants are presented in Figure 27. As mentioned, there is no specific target RNA for this ribozyme in *A. thaliana* mitochondria and the nuclear-encoded *sdh3* RNAs have a different sequence. On the other hand, the *Rz1sdh3*-L-PKTLS ribozyme functioned properly in transformed *N. tabacum* cell lines, assessing its activity *in vivo* when its target is present (§ II.3). When the *Rz1sdh3*-L-PKTLS RNA was expressed in *A. thaliana* transformants, there was no decrease of the nuclear *sdh3* mRNAs, as compared to D0. Even there was a slight increase, as for estradiol-treated wild-type plants. Similar observations were made with *Rz2sdh3*. Therefore, *Rz1sdh3* and *Rz2sdh3* also served as controls in further experiments with transformed *A. thaliana* plants.

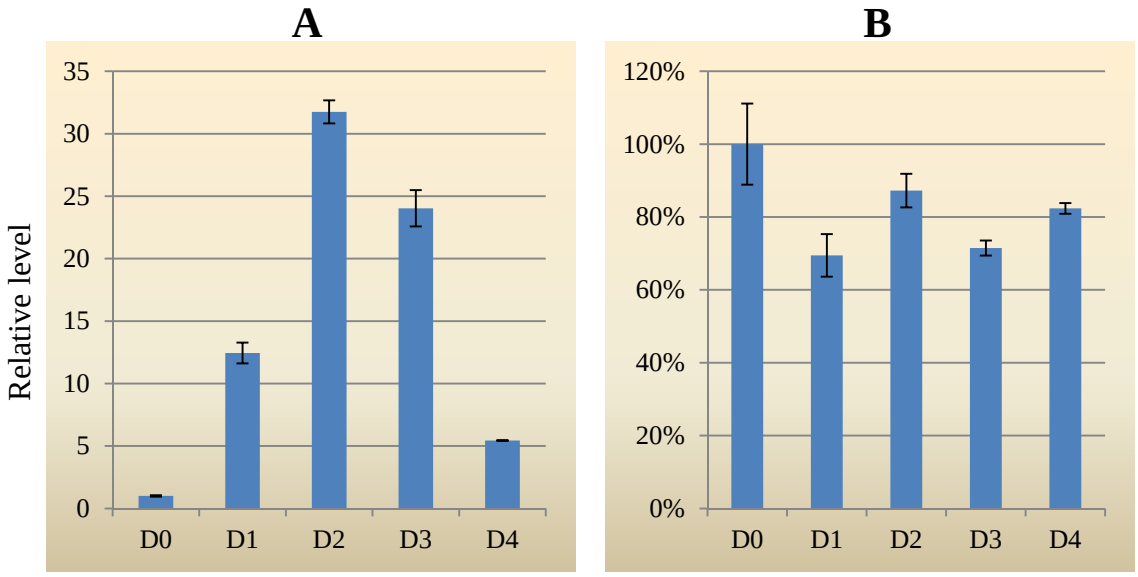


Figure 28: Kinetics of the *Rz1sdh3*-L-PKTLS RNA expression and of the s*dh3* target RNA level in 4-6 leaf stage transformed *Nicotiana tabacum* plants

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. **A)** Relative expression of the *Rz1sdh3*-L-PKTLS RNA in *Rz1sdh3* transformants as compared to the expression at D0. **B)** Percentage of *sdh3* mRNA in *Rz1sdh3* transformants as compared to wild-type plants.

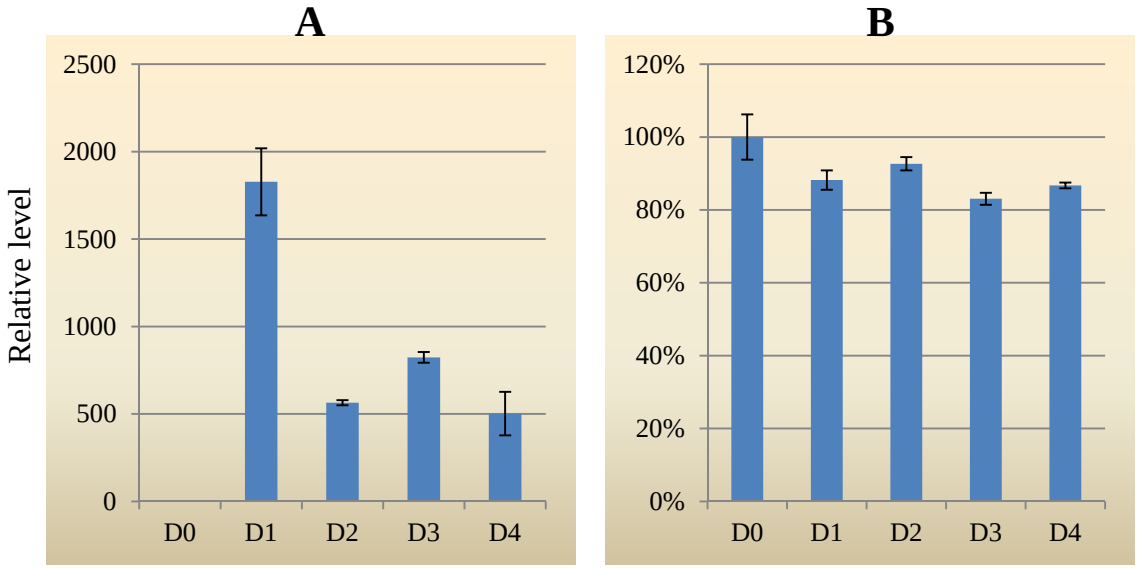


Figure 29: Kinetics of the *Rz2cob*-L-PKTLS RNA expression and of the *cob* target RNA level in 10-12 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. **A)** Relative expression of the *Rz2cob*-L-PKTLS RNA in *Rz2cob* transformants as compared to the expression at D0. **B)** Percentage of *cob* mRNA in *Rz2cob* transformants as compared to wild-type plants.

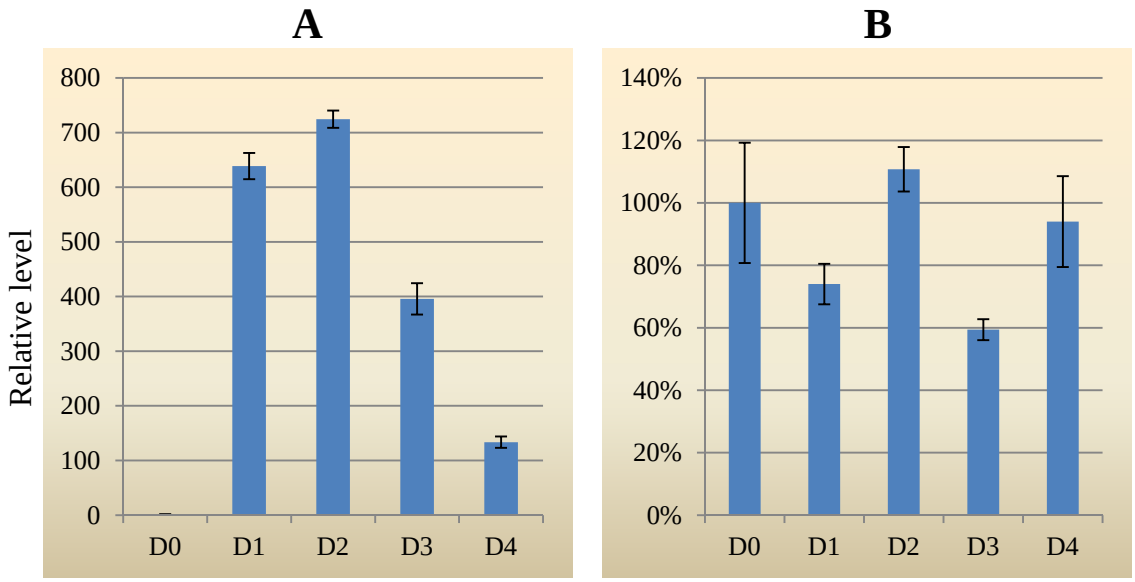


Figure 30: Kinetics of the *Rz1cox3*-L-PKTLS RNA expression and of the *cox3* target RNA level in 10-12 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. **A)** Relative expression of the *Rz1cox3*-L-PKTLS RNA in *Rz1cox3* transformants as compared to the expression at D0. **B)** Percentage of *cox3* mRNA in *Rz1cox3* transformants as compared to wild-type plants.

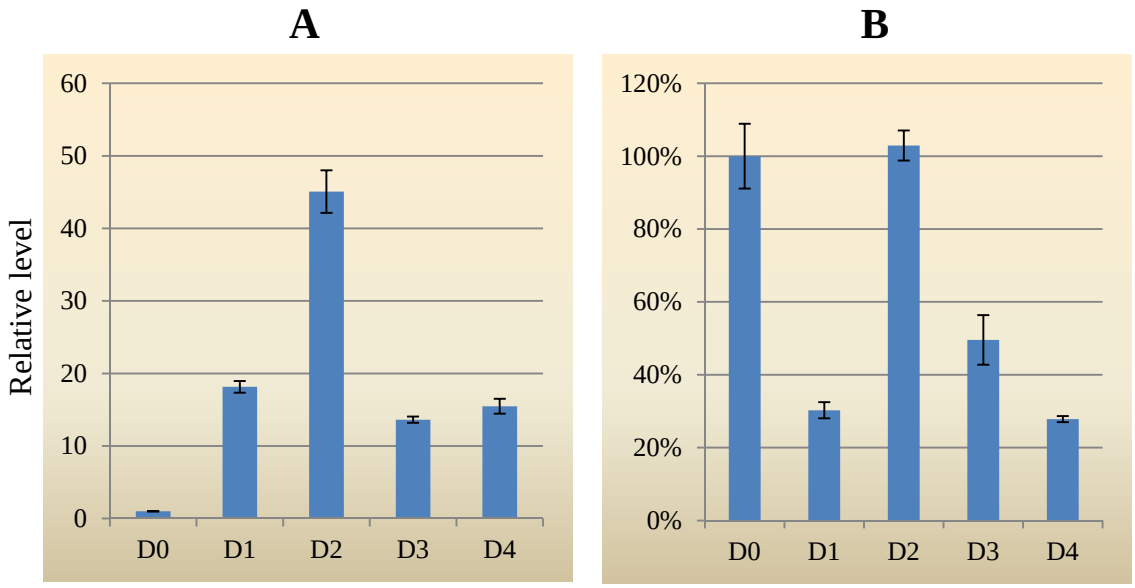


Figure 31: Kinetics of the *Rzatp9*-L-PKTLS RNA expression and of the *atp9* target RNA level in 10-12 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. **A)** Relative expression of the *Rzatp9*-L-PKTLS RNA in *Rzatp9* transformants as compared to the expression at D0. **B)** Percentage of *atp9* mRNA in *Rzatp9* transformants as compared to wild-type plants.

Whereas it functioned properly in transformed *N. tabacum* cell lines (§ II.3), the *Rz1sdh3*-L-PKTLS ribozyme appeared to be less efficient when expressed in transgenic *N. tabacum* whole plants, where it had its specific target. Also, *Rz1sdh3* in *N. tabacum* plants was less efficient than *Rz1nad9* in *A. thaliana*, as only about 30% decrease in the level of the *sdh3* mRNA was observed on D1 compared to estradiol-treated wild-type plants (Figure 28B). Limited decrease by 20-25% was also characterized on D3 and D4 and a bounce back effect was observed on D2, as for *nad9* in *A. thaliana*. Similar results were obtained with *Rz2sdh3*.

The data for *Rz2cob* are shown in Figure 29B. Expression of the *Rz2cob*-L-PKTLS RNA in *A. thaliana* transformants had only a limited effect on the level of the *cob* mitochondrial mRNA, with a maximum decrease of less than 20% on D3 and a subtle bounce back effect on D2. Finally, the *Rz1cox3* ribozyme caused a significant knockdown of the *cox3* target mRNA in *A. thaliana* transformants, although it was not as efficient as *Rz1nad9*. About 25% decrease of *cox3* was observed on D1 and over 40% on D3, with a prominent bounce back effect on D2 (Figure 30B).

An additional system that worked efficiently was with the previously designed *Rzatp9* ribozyme (Val *et al.*, 2011). Further data obtained upon expression of *Rzatp9*-L-PKTLS in transformed *A. thaliana* plants are presented in Figure 31B. As for *Rz1nad9*, about 70% decrease of the *atp9* target mRNA was observed on D1, compared to estradiol-treated wild-type plants. On D2, as for other systems, the level of *atp9* mRNA was back to the level of the control. The bounce back was followed by a second decrease on D3 and a maximum knockdown of 75% was reached on D4. The bounce back effect at D2 was thus very prominent.

So, among the ribozyme/target systems that we designed and characterized functionally, those involving *Rz1nad9*/*Rz2nad9* and *Rzatp9* were the most efficient and were chosen for further studies. To confirm that target knockdown was due to import of the ribozyme into mitochondria, we generated an additional *A. thaliana* transformant carrying an *Rzatp9*-L-cHDV transgene. In that case, the sequence encoding the *Rzatp9* ribozyme was connected, through the linker, directly to the sequence coding for the HDV *cis*-ribozyme in the transgene. An *Rzatp9*-L RNA devoid of the PKTLS moiety was thus expressed. Lacking the PKTLS mitochondrial shuttle, this *Rzatp9*-L RNA was expected not to be imported into the organelles. Such a system also provided a further control for possible side effects of ribozyme expression not related to mitochondrial import and specific target cleavage. The

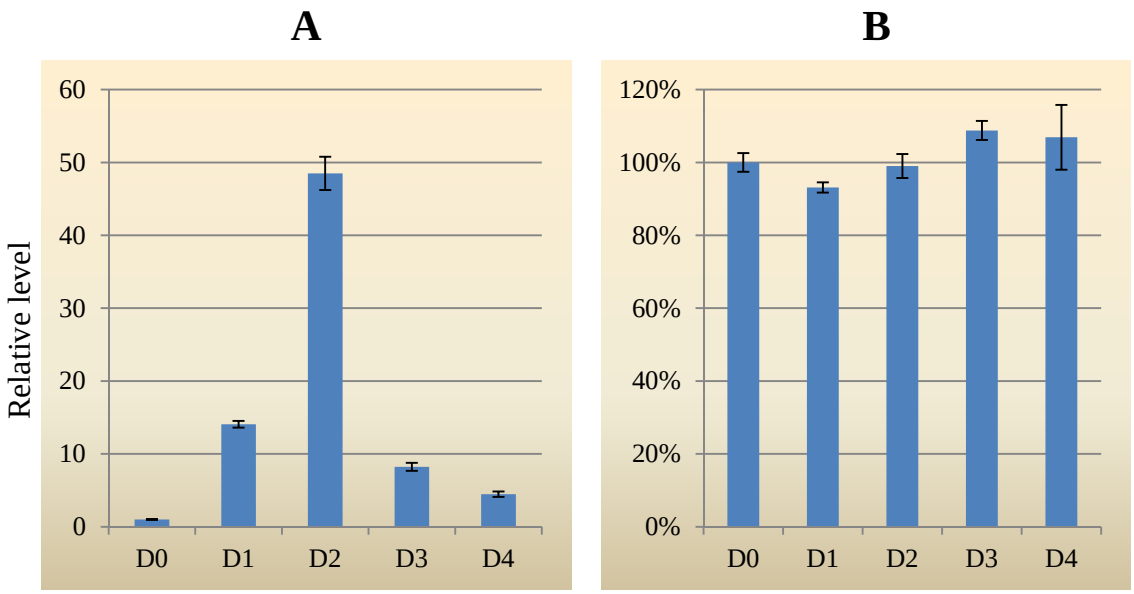


Figure 32: Kinetics of the *Rzatp9*-L RNA expression and of the *atp9* target RNA level in 10-12 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. **A)** Relative expression of the *Rzatp9* RNA in *Rzatp9* transformants as compared to the expression at D0. **B)** Percentage of *atp9* mRNA in *Rzatp9*-L transformants as compared to wild-type plants.

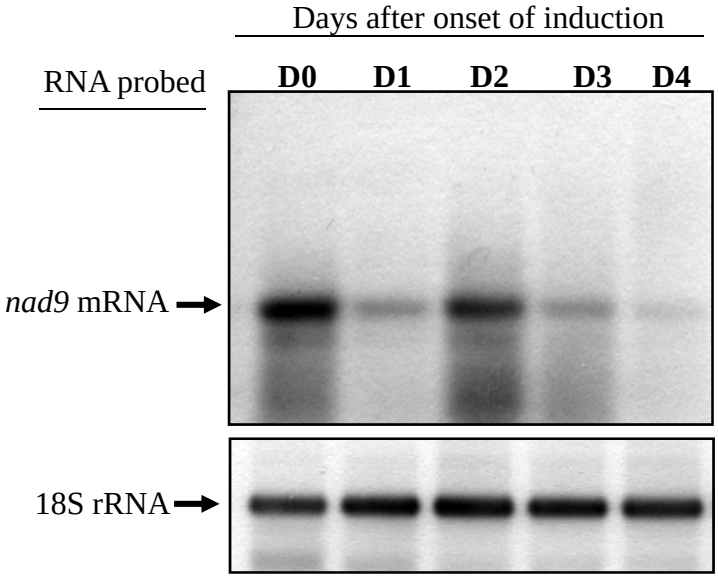


Figure 33: Kinetic analysis of the *nad9* mRNA level in transformed *A. thaliana* plants

Analysis by northern blot of total RNA extracted from *Rz1nad9* transformants. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. Total RNA amounts loaded were standardized using an 18S rRNA probe.

results are presented in Figure 32. The expression of the *Rzatp9*-L RNA was as efficient as that of *Rzatp9*-L-PKTLS. But, contrary to what was observed for *Rzatp9*-L-PKTLS, the presence of the *Rzatp9*-L chimeric ribozyme had no significant effect on the level of *atp9* mRNA. This result clearly demonstrated that the knockdown of *atp9* was related to the import of the ribozyme into the mitochondria and definitely confirmed the functionality and specificity of our approach. It excluded in particular the possibility that the ribozyme would cleave its target during RNA preparation and handling, where both are present in the same fraction. Worthy to notice, RNA samples from induced transformant plants at D2 already contained highest levels of *Rzatp9*-L-PKTLS RNA but retained normal *atp9* mRNA levels (Figure 31). Similar observations were made with *Rz1nad9*-L-PKTLS (Figure 26), also excluding target cleavage during RNA extraction and handling.

Finally, to confirm the results with a second method, we conducted northern blot experiments with total RNA, using digoxigenin-labeled probes (Materials and Methods § II.3.9). The results obtained with RNA from *A. thaliana* transformants expressing *Rz1nad9*-L-PKTLS are presented in Figure 33. The amounts of loaded RNA were balanced using a probe for the 18S rRNA. The pattern obtained with a probe for the *nad9* mRNA fitted exactly the RT-qPCR results (Figure 26B), with a strong knockdown on D1, a bounce back on D2, and further strong knockdown on D3 and D4.

Thus, the data altogether established mitochondrial import of our chimeric ribozymes expressed from nuclear transgenes, followed by specific knockdown of their target RNAs. Our molecular approach appeared to be extremely efficient to knockdown mitochondrial RNAs in whole plants, showing an important potential for the investigation of organelle regulation mechanisms. The bounce back effect observed with all ribozyme/target systems was a first indication in that direction. More hints in favor of mRNA control mechanisms in mitochondria were obtained when analyzing target knockdown in relation to the plant developmental stage or culture conditions.

II.6 Target RNA knockdown and plant developmental stage

The experiments were further extended with the *Rz1nad9*/*Rz2nad9* and *Rzatp9* ribozymes to study the knockdown of the *nad9* and *atp9* target RNAs at different plant growth stages and under different growth conditions. For this, transformed *A. thaliana* plants were grown on solid agar medium (Materials and Methods, § II.6.2) and transferred on liquid medium for transgene induction with estradiol at three different growth stages: young plants (4-6 leaves

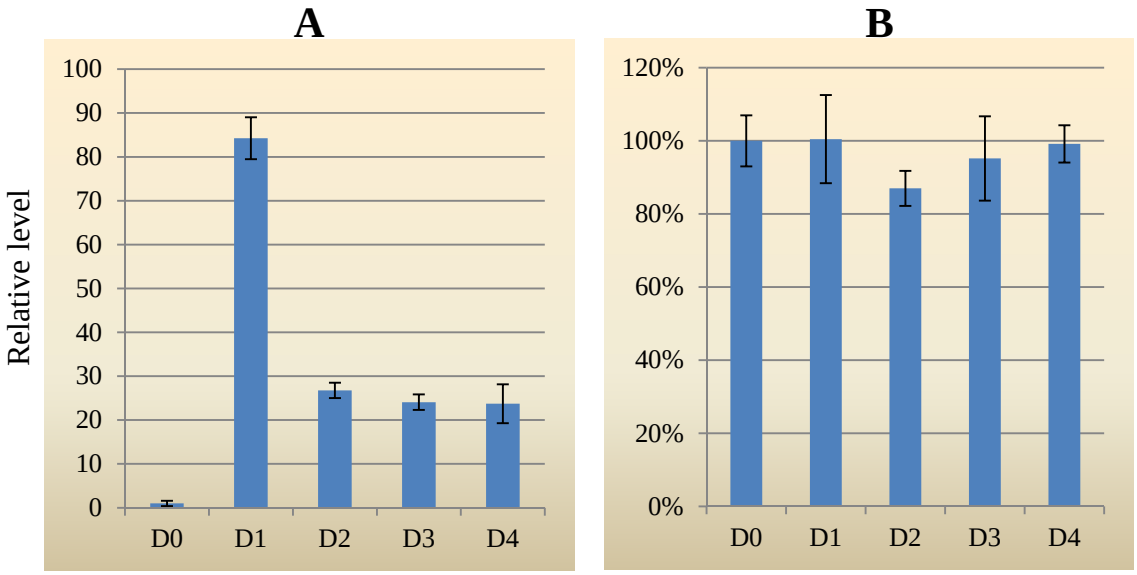


Figure 34: Kinetics of the *Rz1nad9*-L-PKTLS RNA expression and of the *nad9* target RNA level in 4-6 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. **A)** Relative expression of the *Rz1nad9*-L-PKTLS RNA in *Rz1nad9* transformants as compared to the expression at D0. **B)** Percentage of *nad9* mRNA in *Rz1nad9* transformants as compared to wild-type plants.

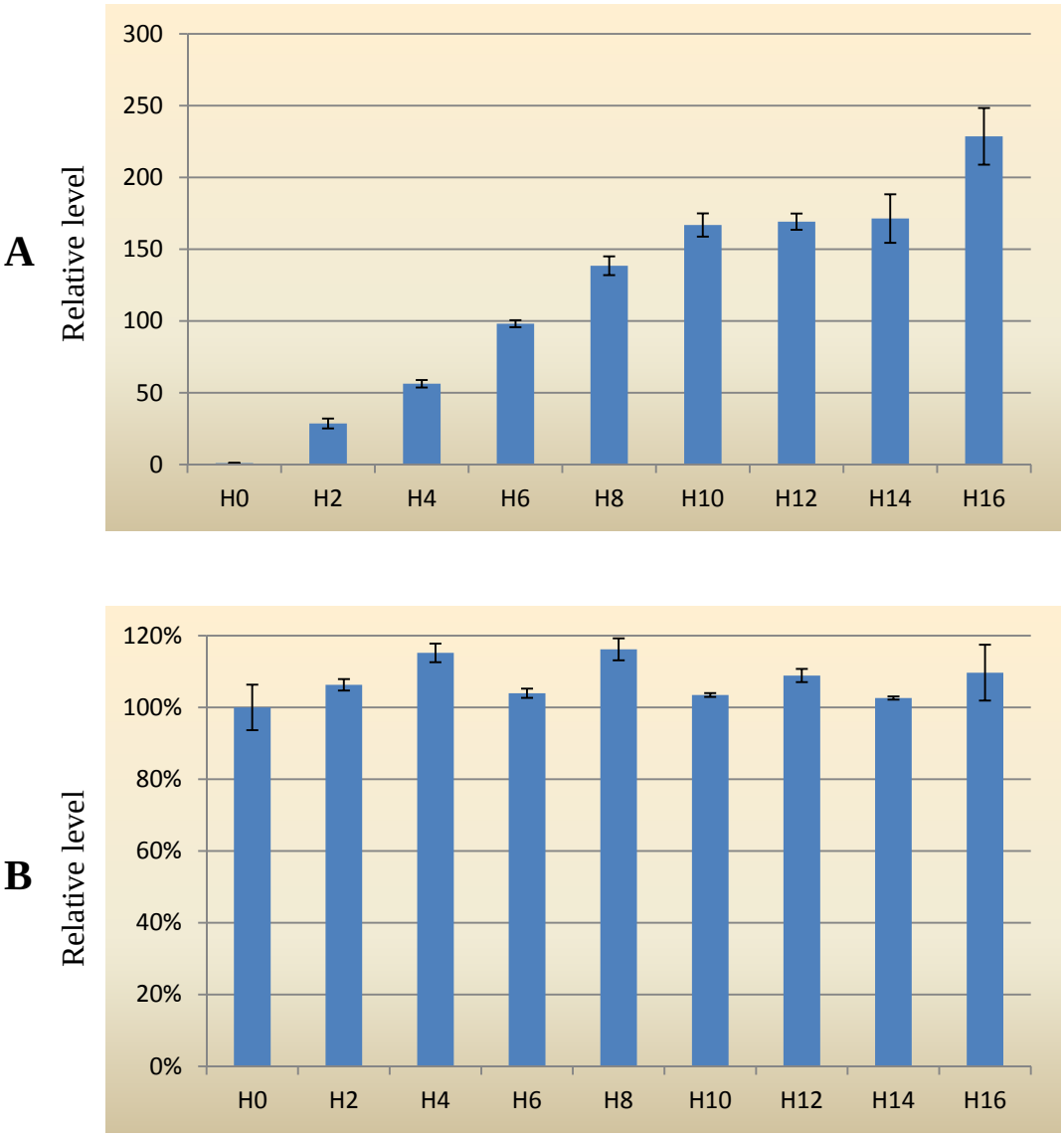


Figure 35: Short time kinetics of the *Rz1nad9*-L-PKTLS RNA expression and of the *nad9* target RNA level in 4-6 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA from *A. thaliana* plants. H0 : before treatment with estradiol; H2 to H16 : hours after induction. **A)** Relative expression of the *Rz1nad9*-L-PKTLS RNA in *Rz1nad9* transformants as compared to the expression at D0. **B)** Percentage of *nad9* mRNA in *Rz1nad9* transformants as compared to wild-type plants.

stage, about 10 days after germination), fully grown plants (10-12 leaves stage, about 20 days after germination) and old plants (16-18 leaves stage, about 30 days after germination). Plants were harvested each day till four days after induction. As a control, wild-type *A. thaliana* plants were grown and treated in the same way. Total RNA was prepared from all samples and analyzed by RT-qPCR as before, with *actin*, *gapdh* and *rpl2* as reference genes.

The results obtained with young *A. thaliana* plants expressing the *Rz1nad9*-L-PKTLS chimeric ribozyme are presented in Figure 34. The *Rz1nad9*-L-PKTLS RNA was properly expressed in induced plants. The maximum was on D1, the level dropped to a large extent on D2 and then remained constant till D4, still in a range expected to be appropriate for target knockdown (Figure 34A). Unexpectedly, despite proper expression of *Rz1nad9*-L-PKTLS, the level of the *nad9* target mRNA did not change significantly, as compared to that in wild-type plants at the same developmental stage treated with estradiol in the same way (Figure 34B). Several interpretations can be put forward for these observations. One cannot exclude that the physiological conditions in young plants were as a whole unfavorable for ribozyme cleavage, which may have affected target *trans*-cleavage by *Rz1nad9* but also self-cleavage of the HDV *cis*-ribozyme. In the latter case, import of *Rz1nad9* into mitochondria could have been impaired. The other possibility is that both ribozymes worked and *Rz1nad9* cleaved its target in mitochondria but the bounce back effect was so quick and efficient that the knockdown was not detectable. Such an explanation would support the hypothesis that the drop in *nad9* level was sensed and triggered increased *nad9* synthesis, as in very young plants the demand for mitochondrial activity is likely to be very high. To test this second possibility, the experiment was run again in the same conditions but, following the onset of the induction, the plants were harvested every two hours till 16 hours. The results are presented in Figure 35. Over that period of time, the level of *Rz1nad9*-L-PKTLS ribozyme was continuously increasing, but still after 16 hours of expression, the *nad9* mRNA was unaffected. After these results, both possibilities remained open.

The results obtained with fully grown *A. thaliana* plants expressing the *Rz1nad9*-L-PKTLS RNA are presented in Figure 26 and were discussed above. These plants showed strong knockdown after expression of the ribozyme, with a prominent bounce back effect on D2 (Figure 26B).

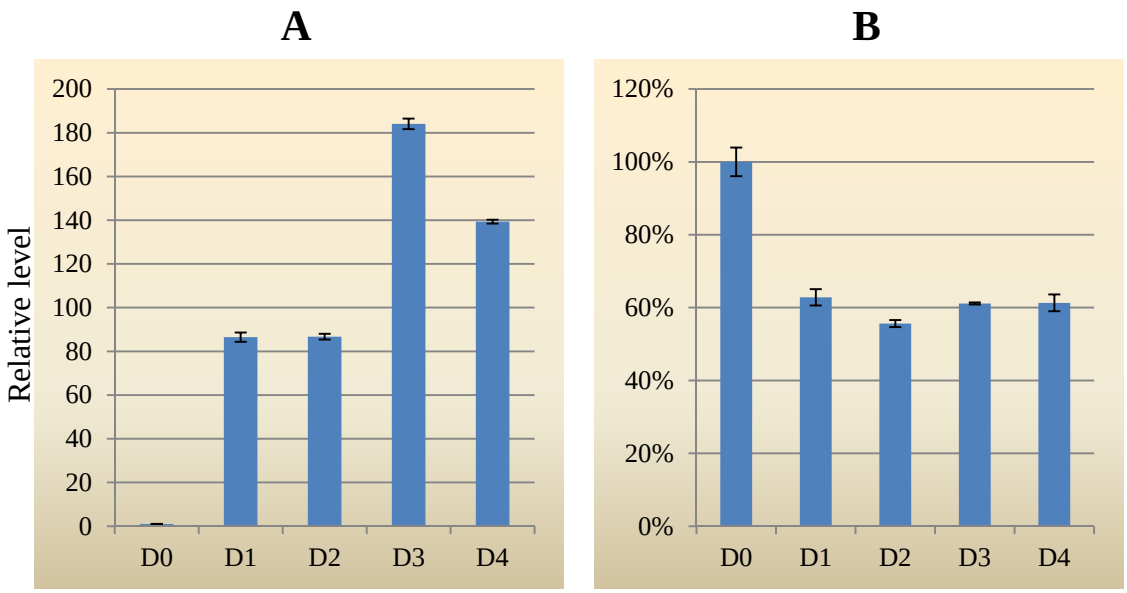


Figure 36: Kinetics of the *Rz1nad9*-L-PKTLS RNA expression and of the *nad9* target RNA level in 16-18 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. **A)** Relative expression of the *Rz1nad9*-L-PKTLS RNA in *Rz1nad9* transformants as compared to the expression at D0. **B)** Percentage of *nad9* mRNA in *Rz1nad9* transformants as compared to wild-type plants.

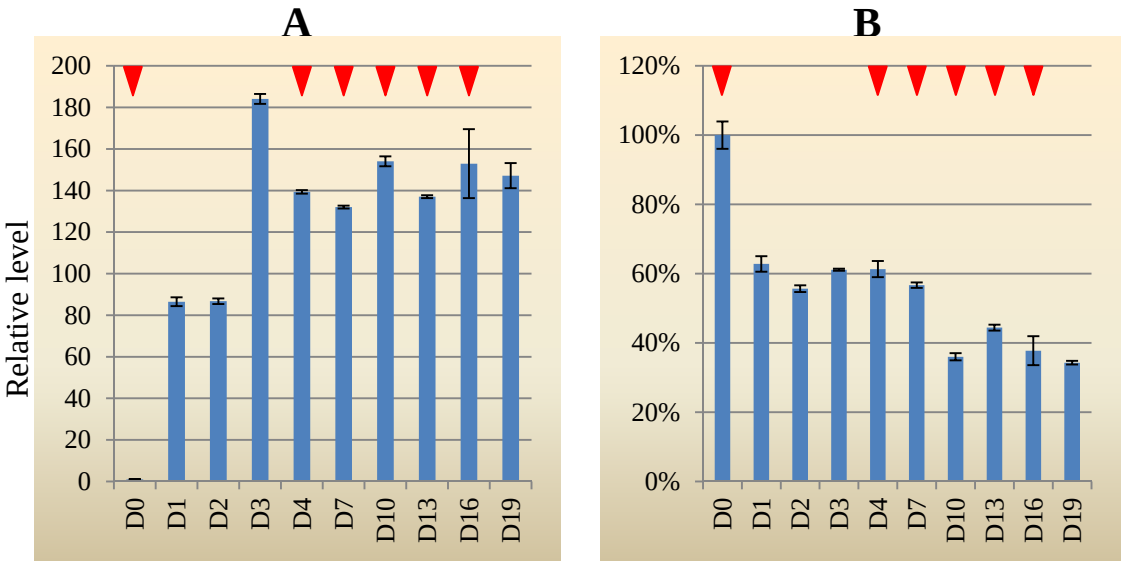


Figure 37: Long time kinetics of the *Rz1nad9*-L-PKTLS RNA expression and of the *nad9* target RNA level in 16-18 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. For induction, estradiol was added on different time points as indicated by red triangles. D0 : before treatment with estradiol; D1 to D19 : days after onset of the induction. **A)** Relative expression of the *Rz1nad9*-L-PKTLS RNA in *Rz1nad9* transformants as compared to the expression at D0. **B)** Percentage of *nad9* mRNA in *Rz1nad9* transformants as compared to wild-type plants.

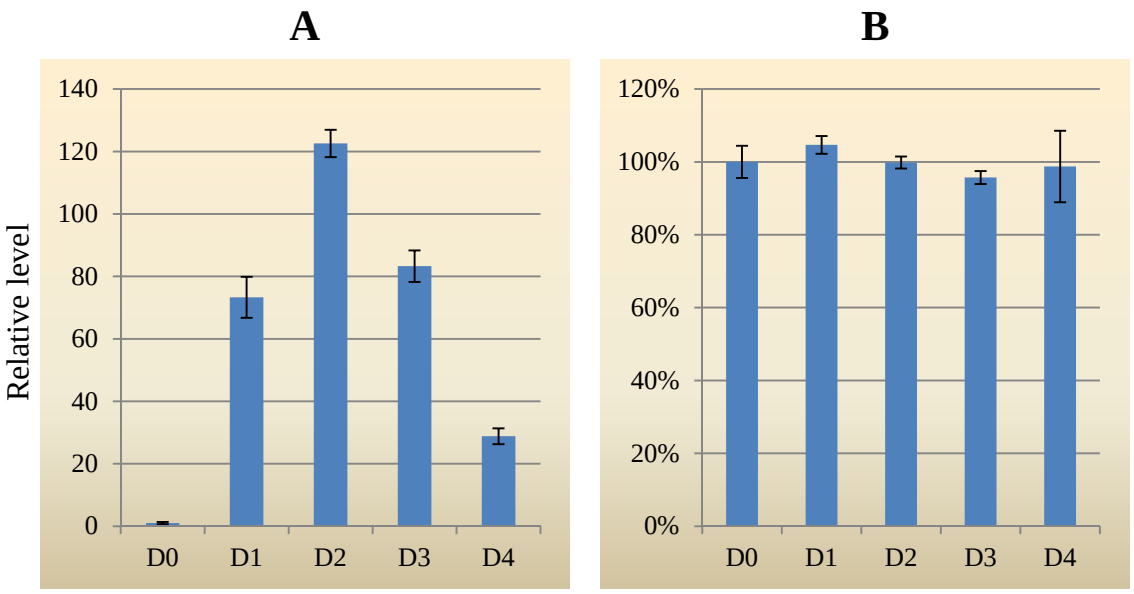


Figure 38: Kinetics of the *Rzatp9*-L-PKTLS RNA expression and of the *atp9* target RNA level in 4-6 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D4 : days after onset of the induction. **A)** Relative expression of the *Rzatp9*-L-PKTLS RNA in *Rzatp9* transformants as compared to the expression at D0. **B)** Percentage of *atp9* mRNA in *Rzatp9* transformants as compared to wild-type plants.

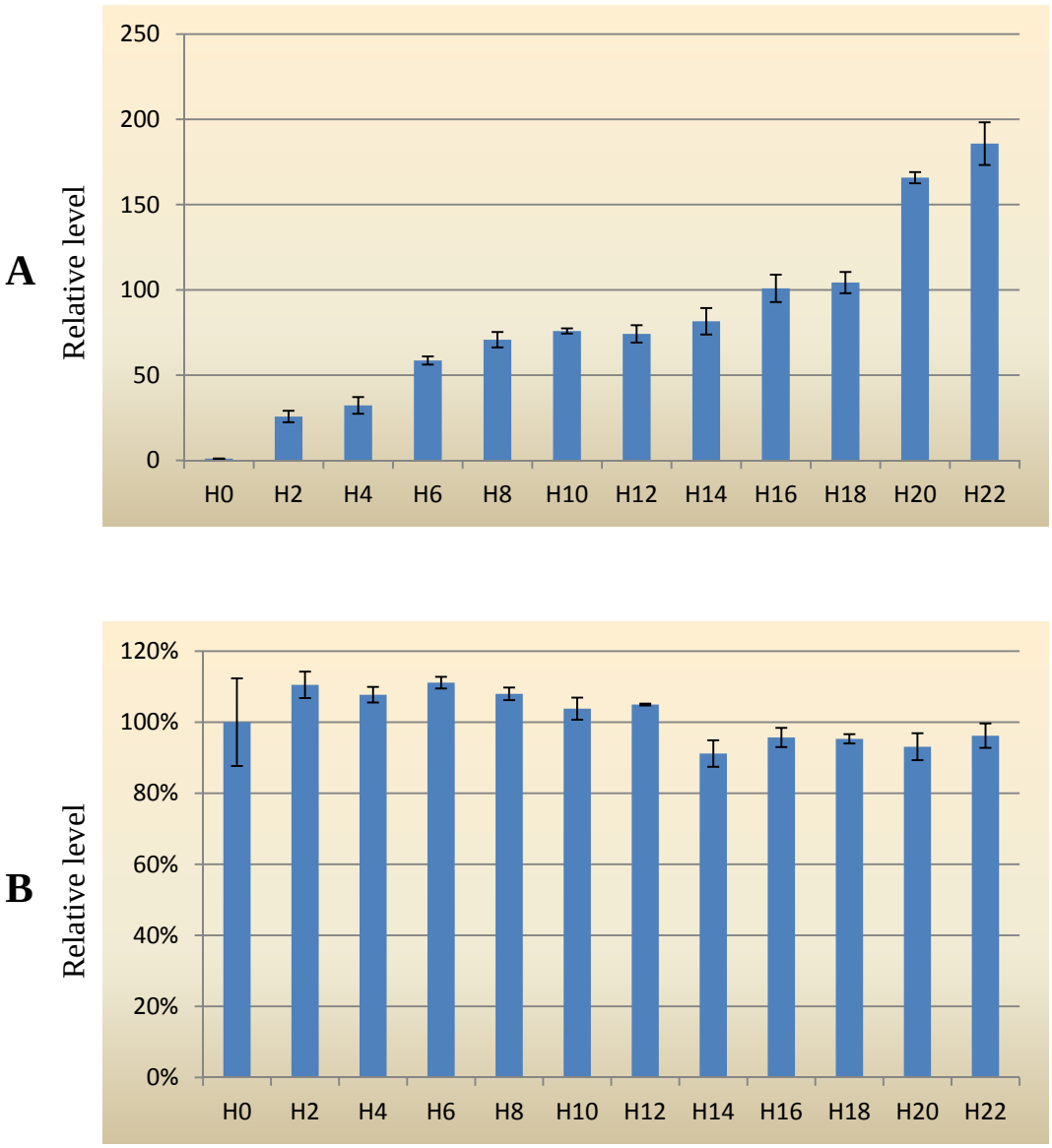


Figure 39: Short time kinetics of the *Rzatp9*-L-PKTLS RNA expression and of the *atp9* target RNA level in 4-6 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. H0 : before treatment with estradiol; H2 to H16 : hours after induction. **A)** Relative expression of the *Rzatp9*-L-PKTLS RNA in *Rzatp9* transformants as compared to the expression at D0. **B)** Percentage of *atp9* mRNA in *Rzatp9* transformants as compared to wild-type plants.

The results obtained with older *A. thaliana* plants are presented in Figure 36. The chimeric *Rz1nad9*-L-PKTLS RNA was properly expressed in induced plants. Its level gradually increased, reaching a peak on D3, and then decreased to some extent on D4 (Figure 36A). Expression of *Rz1nad9*-L-PKTLS RNA correlated with target knockdown, as there was about 40% decrease in the level of the *nad9* mRNA, as compared to that in estradiol-treated wild-type plants at the same developmental stage (Figure 36B). The *nad9* decrease was persistent for all four days. We extended this experiment for longer induction times. Estradiol was again added on D4 and then every three days till 19 days (Figure 37). After D4, plants were harvested every three days till 19 days. RT-qPCR analysis showed that the expression of the *Rz1nad9*-L-PKTLS RNA was maintained till the end at a high level similar to that present at D4 (Figure 37A). So, when estradiol was added regularly, the expression of the chimeric ribozyme could be maintained at highest level. This high expression of *Rz1nad9*-L-PKTLS was correlated with a continuous and gradual decrease in the level of the *nad9* target mRNA, with a final knockdown close to 70% (Figure 37B). No bounce back effect was observed in this case. These were older plants near flowering stage. It is likely that, at that stage, mitochondrial activity was low in somatic tissues. The above results with young plants raised the hypothesis that, when the demand for mitochondrial activity is high, the drop in *nad9* level can be sensed and possibly trigger increased synthesis. Conversely, it seems from the data with older plants that, when the demand is low, a strong knockdown of a mitochondrial mRNA can be maintained continuously over a long period.

The results obtained with young *A. thaliana* plants expressing the *Rzatp9*-L-PKTLS RNA are presented in Figure 38. Expression of the chimeric ribozyme was high and followed the regular pattern, with a maximum on D2 (Figure 38A). However, as for *Rz1nad9*-L-PKTLS, despite the presence of the ribozyme, the level of the mitochondrial target mRNA did not change significantly. Again, the hypothesis of a quick sensing and compensation of target RNA degradation was tested by inducing the young plants and harvesting samples every two hours till 22 hours. The level of *Rzatp9*-L-PKTLS ribozyme was continuously increasing (Figure 39A), but after 22 hours the level of *atp9* mRNA was still not significantly affected (Figure 39B). The question of fast regulation or ribozyme function impairment in young plants thus remained open and we have already started other approaches to understand this phenomenon.

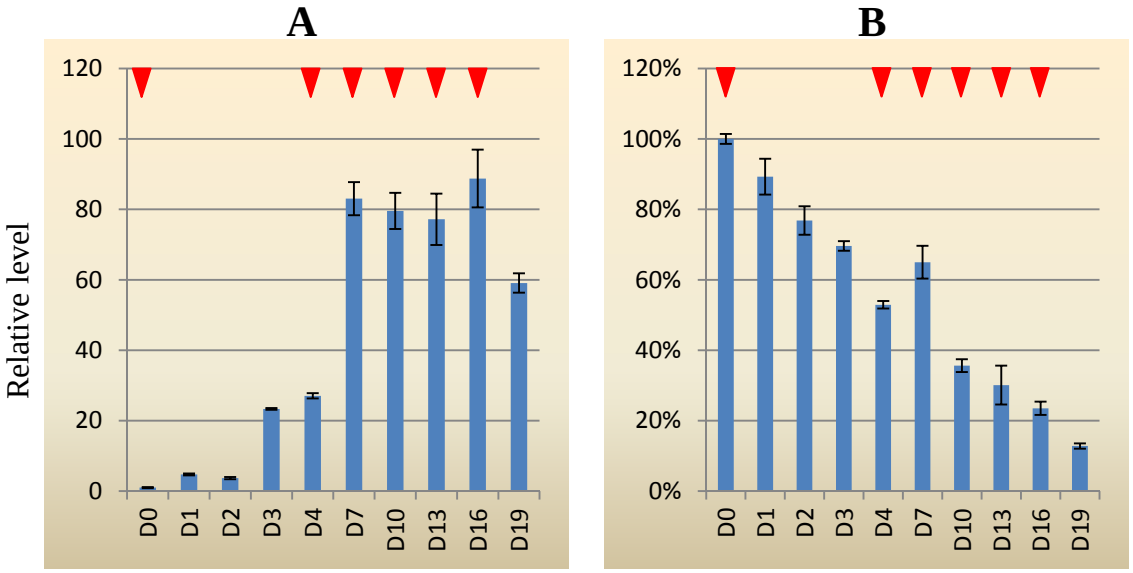


Figure 40: Kinetics of the *Rzatp9*-L-PKTLS RNA expression and of the *atp9* target RNA level in 16-18 leaf stage transformed *A. thaliana* plants

Analysis by RT-qPCR on total RNA. For induction, estradiol was added on different time points as indicated by red triangles. D0 : before treatment with estradiol; D1 to D19 : days after onset of the induction. **A)** Relative expression of the *Rzatp9*-L-PKTLS RNA in *Rzatp9* transformants as compared to the expression at D0. **B)** Percentage of *atp9* mRNA in *Rzatp9* transformants as compared to wild-type plants.

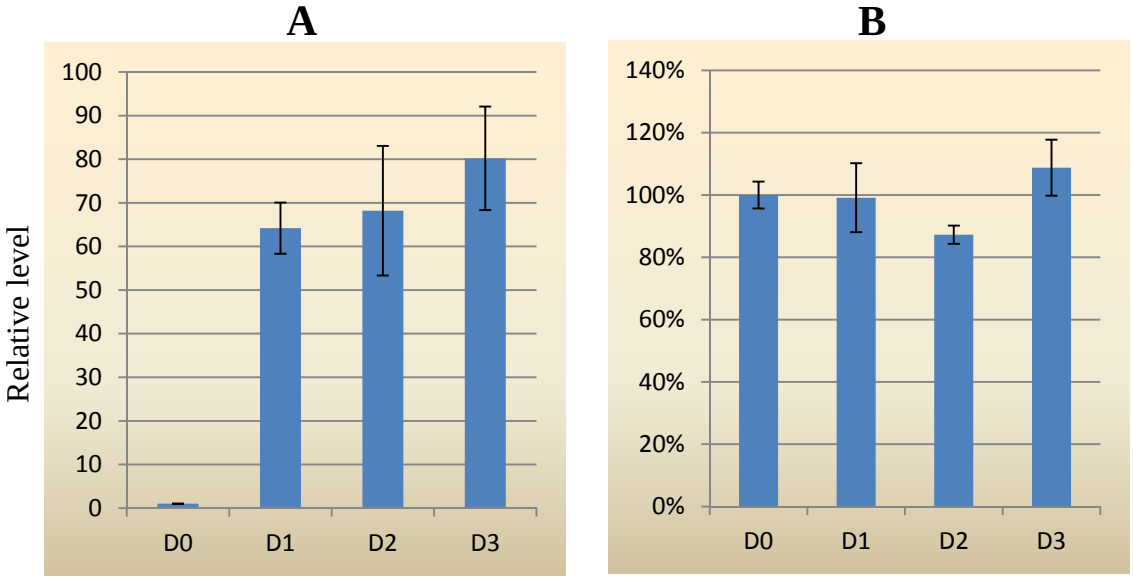


Figure 41: Kinetics of the *Rzatp9*-L-PKTLS RNA expression and of the *atp9* target RNA level in 8 days old hydroponic seedling cultures of transformed *A. thaliana*

Analysis by RT-qPCR on total RNA. D0 : before treatment with estradiol; D1 to D3 : days of induction. **A)** Relative expression of the *Rzatp9*-L-PKTLS RNA in *Rzatp9* transformants as compared to the expression at D0. **B)** Percentage of *atp9* mRNA in *Rzatp9* transformants as compared to wild-type.

The results obtained upon expression of *Rzatp9*-L-PKTLS in fully grown plants are presented in Figure 30 and discussed above. As mentioned, the data showed a strong knockdown and a pronounced bounce back effect on D2. For older *A. thaliana* plants carrying the transgene encoding *Rzatp9*-L-PKTLS, long kinetics were again performed, with an initial estradiol treatment and further induction every three days from D4. As before, plants were harvested every day till D4 and every three days till D19. *Rzatp9*-L-PKTLS RNA expression increased to high levels till D7, remained similar till D16 and decreased somehow on D19 (Figure 40A). Ribozyme expression was correlated with a gradual and very strong knockdown of the *atp9* target mRNA, ending with a decrease close to 90% at D19 (Figure 40B). As in the case of *Rz1nad9*-L-PKTLS, there was no bounce back effect in older plants.

Finally, we tested the behavior of our system in different culture conditions. Special hydroponic seedling cultures of *A. thaliana* have been introduced in plant biological research, especially for extraction of mitochondria. In that case, seeds can be germinated directly as a suspension in liquid culture medium (Sweetlove *et al.*, 2007) (Materials and Methods, § I.3). Growth on large scale is easy as compared to other *in vitro* conditions. This is also helpful to work with mitochondrial nucleic acids. We took this method as an alternative culture condition to check the efficiency of ribozyme-mediated knockdown of mitochondrial RNAs. The experiments were developed with the *Rzatp9*-L-PKTLS *A. thaliana* transformants. Eight-day-old seedlings directly grown in liquid medium were used. The seedling were either harvested without estradiol induction (D0) or estradiol was added to the medium one (D1), two (D2) or three (D3) days before harvesting. As a control, wild-type *A. thaliana* (Col-0) seedlings were grown and treated with estradiol in the same way. The RT-qPCR results obtained with hydroponic seedling cultures (Figure 41) were similar to those for young plants (Figure 38). The *Rzatp9*-L-PKTLS RNA was efficiently expressed, with a slight increase with increasing induction time (Figure 41A). However, the level of the *atp9* target mRNA did not change significantly, as compared to that in control seedlings (Figure 41B).

As a whole, these experiments showed a robust dependence of the efficiency, kinetics and maintenance of ribozyme-mediated target RNA knockdown in mitochondria upon the plant developmental stage and physiological status. Highly similar observations were made with

Rz1nad9-L-PKTLS and *Rzatp9*-L-PKTLS chimeric ribozymes, showing the reproducibility of these data with different ribozyme systems.

RESULTS, CHAPTER III

III. Mitochondrial RNA regulation

It is currently considered that mitochondrial gene expression in plants is mostly constitutive, whereas nuclear-encoded organelle components would be a rate-liming factor. Regulation studies have been restricted so far essentially to the analysis of nuclear mutants. Mutations in the mitochondrial genome are often lethal in plants, which greatly reduces the possibilities for investigation. With our work, for the first time it was possible to downregulate in a directed manner specific mitochondrial RNAs. The bounce back effect and the dependence of RNA knockdown on the plant developmental stage, as described in the previous chapter, point to regulation mechanisms controlling RNA levels in plant mitochondria. On that basis, we deepend our studies and extended the analyses to the whole transcriptome impact resulting from the knockdown of individual RNAs in mitochondria. As these were the most efficient systems, we investigated the response to a strong decline in the major mRNAs *nad9* and *atp9*.

III.1 Collection of RNAs probed by RT-qPCR analysis

We undertook to characterize the behavior of a number of mRNAs encoding subunits of the oxidative phosphorylation (OXPHOS) chain complexes in transformed *A. thaliana* plants expressing *Rz1nad9*-L-PKTLS or *Rzatp9*-L-PKTLS RNA. The vast majority of the mRNAs studied was of mitochondrial origin. For each selected mRNA, we verified by sequence alignment that there was no possibility of undesired cleavage by the *Rz1nad9* or *Rzatp9* *trans*-ribozyme. Among the subunits of the NADH dehydrogenase complex (complex I), we followed the changes in *nad1, nad2, nad4, nad5, nad6, nad7* and *nad9* mRNA. In *A. thaliana*, no subunit of the succinate dehydrogenase complex (complex II) is encoded in the mitochondrial genome. For the cytochrome c reductase complex (complex III), we followed the level of the cytochrome b (*cob*) mRNA and from the cytochrome c oxidase complex (complex IV), we selected *cox1*. For the ATP synthase complex, we chose the *atp1, atp4* and *atp9* mRNAs. The ATP4 and ATP9 proteins are part of the F0 ATP synthase sub-complex and ATP1 is part of the F1 ATPase sub-complex. We also included mRNAs coding for five cytochrome c biogenesis (ccb) proteins: *ccb203, ccb206, ccb256, ccb382* and *ccb452*. In addition to these mRNAs encoding proteins of the oxidative phosphorylation complexes, we also selected RNAs related to mitochondrial protein synthesis, to check how the translational machinery is maintained. Seven RNAs were selected: three mRNAs encoding proteins of the small ribosomal subunit, *rps3, rps4* and *rps7*, two mRNAs

encoding proteins of the large ribosomal subunit, *rpl5* and *rpl16* and two rRNAs, *rrn18* and *rrn26*.

All the previous mRNAs chosen are encoded by the mitochondrial genome. We also selected several nuclear-encoded mRNAs, especially *aox1a* and *aox1b*. These mRNAs code for the alternative oxidase, a protein that is important for the functionality of mitochondria. AOX can deflect the path of the electrons in the respiratory chain to reduce molecules of oxygen. The expression of this protein has often been associated with heat or drought stress, pathogen attack or some malfunction in the respiratory chain. With this mRNA, we aimed to determine whether a cross-talk between the nuclear and mitochondrial genetic systems could be triggered in response to the knockdown of *nad9* or *atp9*. Finally, the nuclear mRNA encoding the EXPRESSED (EXP) protein (AT4G26410), which has no link with the mitochondria, was checked. This is a very stable mRNA and some authors use it as a reference, along with *actin*, for qPCR analysis.

The results presented below correspond to RT-qPCR analyses carried out on total RNA from 16-18-leaf stage transformed *A. thaliana* plants induced for 10 days (D10) with estradiol. Estradiol was added at day 0, day 4 and day 7, so as to keep the level of ribozyme at a maximum level. For *Rz1nad9*-L-PKTLS, the maximum knockdown of the *nad9* target mRNA was observed on day 10, as shown earlier in Figure 37. The same time point was chosen for *Rzatp9*-L-PKTLS-mediated knockdown of the *atp9* mRNA. As a control for non-specific effects, we also included the *Rz1sdh3*-L-PKTLS-expressing *A. thaliana* plants, for which the ribozyme has no target (see above). Reverse transcription reactions were performed using random hexamer primers. Sequences of the primers for qPCR are presented in Table 6. All results were normalized as before with the *actin*, *gapdh* and *rpl2* mRNAs as references.

III.2 Effect of *nad9* knockdown on the steady-state level of other RNAs

The NAD9 protein is part of complex I, which is the major entry point for the mitochondrial electron transport chain in plants. Mitochondrial Complex I is composed of more than 40 subunits in *A. thaliana* (Heazlewood *et al.*, 2003). The majority of these are encoded by nuclear genes, whereas nine genes have been retained in the land plant mitochondrial genome, namely *nad1, nad2, nad3, nad4, nad4L, nad5, nad6, nad7,* and *nad9* (Sugiyama *et al.*, 2005). We aimed to determine whether the knockdown of the *nad9* mRNA had any effect on the level of mitochondrial mRNAs coding for other proteins of the same complex

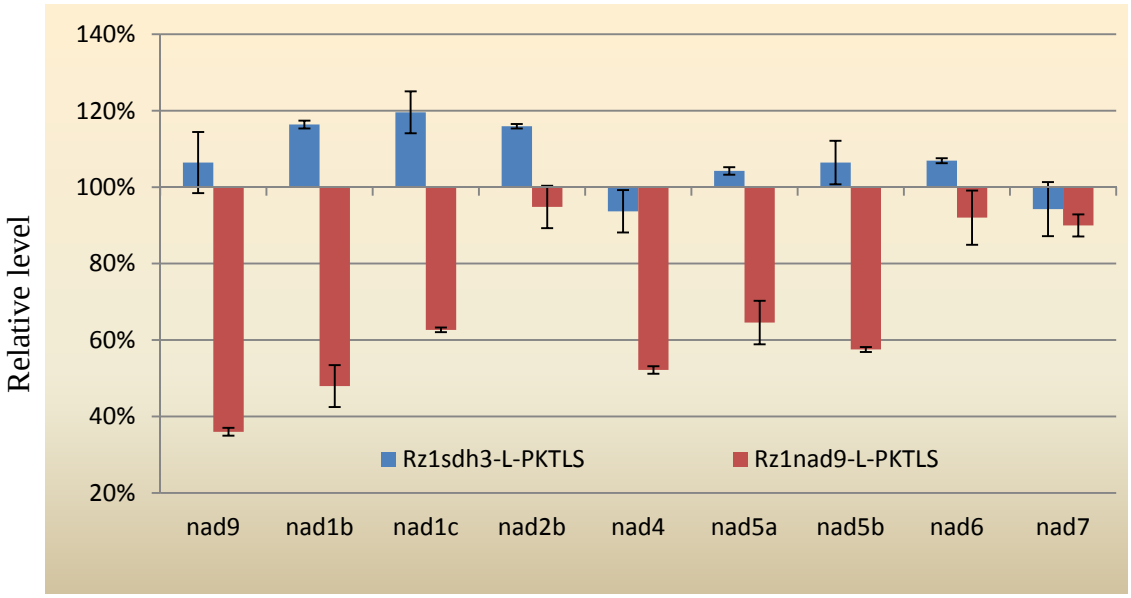


Figure 42: Effect of *nad9* mRNA knockdown on the level of mitochondrial mRNAs encoding complex I subunits in 16-18 leaf stage *A. thaliana* plants

Analysis by RT-qPCR on total RNA. The *nad9*, *nad1b*, *nad1c*, *nad2b*, *nad4*, *nad5a*, *nad5b*, *nad6* and *nad7* mRNA levels were analyzed in *Rz1nad9* transformants (red) and in *Rz1sdh3* transformants (blue). For transgene induction, estradiol was added on day 0, day 4 and day 7. Results are given as the percentage of the different mitochondrial mRNAs at day 10 compared to day 0.

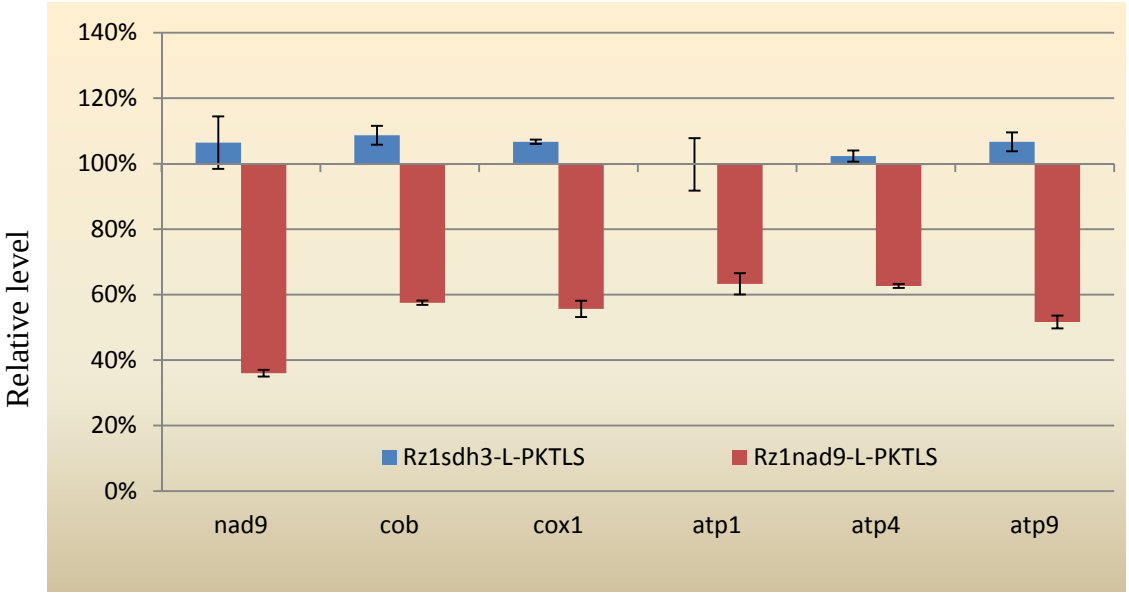


Figure 43: Effect of *nad9* mRNA knockdown on the level of RNAs encoding OXPHOS chain complexes subunits in 16-18 leaf stage *A. thaliana* plants

Analysis by RT-qPCR on total RNA. The *nad9*, *cob*, *cox1*, *atp1* and *atp4* mRNA levels were analyzed in *Rz1nad9* transformants (red) and in *Rz1sdh3* transformants (blue). For transgene induction, estradiol was added on day 0, day 4 and day 7. Results are given as the percentage of the different mitochondrial mRNAs at day 10 compared to day 0.

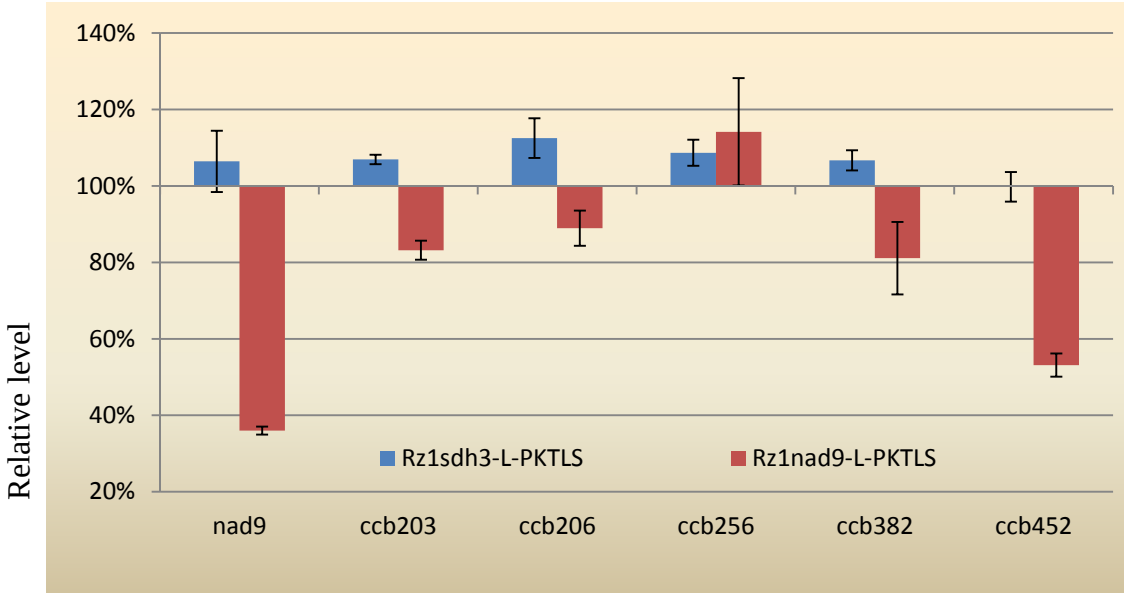


Figure 44: Effect of *nad9* mRNA knockdown on the level of mRNAs encoding cytochrome c biogenesis proteins in 16-18 leaf stage *A. thaliana* plants
Analysis by RT-qPCR on total RNA. The *nad9*, *ccb203*, *ccb206*, *ccb256*, *ccb382* and *ccb452* mRNA levels were analyzed in *Rz1nad9* transformants (red) and in *Rz1sdh3* transformants (blue). For transgene induction, estradiol was added on day 0, day 4 and day 7. Results are given as the percentage of the different mitochondrial mRNAs at day 10 compared to day 0.

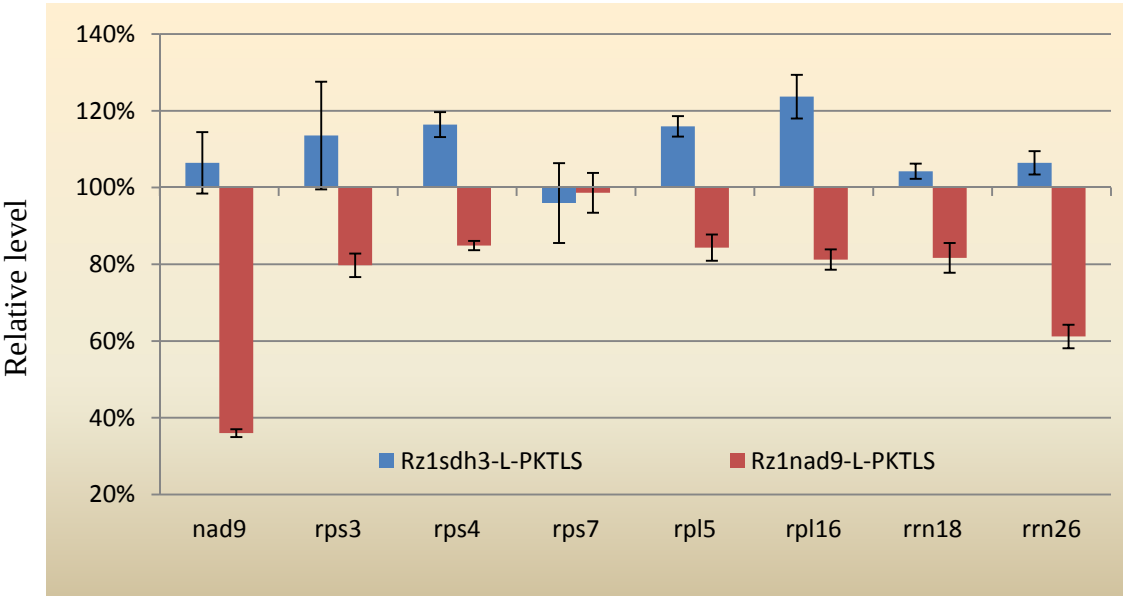


Figure 45: Effect of *nad9* mRNA knockdown on the level of mitochondrial RNAs of the translation system in 16-18 leaf stage *A. thaliana* plants
Analysis by RT-qPCR on total RNA. The *nad9*, *rps3*, *rps4*, *rps7*, *rpl5*, *rpl16*, *rrn18* and *rrn26* RNA levels were analyzed in *Rz1nad9* transformants (red) and in *Rz1sdh3* transformants (blue). For transgene induction, estradiol was added on day 0, day 4 and day 7. Results are given as the percentage of the different mitochondrial RNAs at day 10 compared to day 0.

and on mitochondrial mRNAs coding for proteins of other complexes, so as to reveal possible RNA coordination and regulation mechanisms in mitochondria.

III.2.1.1 Level of mitochondrial mRNAs coding for proteins of the OXPHOS chain

The results obtained with *Rz1nad9*-L-PKTLS and *Rz1sdh3*-L-PKTLS-expressing *A. thaliana* plants are presented in Figures 42 to 44. Knockdown of the *nad9* mRNA in response to the presence of the *Rz1nad9*-L-PKTLS ribozyme caused a significant decrease of five mRNAs coding for subunits of complex I, among the nine tested (Figure 42). As a consequence of a roughly 65% knockdown of *nad9*, the greatest effect was observed for *nad1b*, with a 52% decrease. While *nad1c*, *nad4* and *nad5* mRNAs showed about 40% decrease, the levels of *nad2b, nad6* and *nad7* remained unchanged. Nevertheless, the significant effect observed on the level of a number of these mRNAs strongly suggests that there might exist some regulation between the components of the same complex.

As shown on Figure 43, the levels of all mRNAs tested coding for subunits of the other complexes also decreased substantially. Among them, the most drastic drop was for *atp9* (48%). For *cob*, *cox1*, *atp1* and *atp4,* around 40% decrease was observed. Conversely, there was little effect on the levels of mRNAs encoding CCB proteins, except *ccb452* that showed a 47% drop (Figure 44). It is worthy to note that in control plants expressing the *Rz1sdh3*-L-PKTLS RNA, all the RNAs tested were either unaffected or showed a mild increase, which is possibly the effect of estradiol.

III.2.1.2 Level of mitochondrial RNAs of the translation system

We extended our studies to RNAs involved in mitochondrial protein synthesis, to get an idea on the overall functionality of the organelles facing a drastic knockdown of the *nad9* mRNA. The results are presented in Figure 45. Among the RNAs tested, *rps7* was unaffected, while *rps3, rps4 rpl5*, *rpl16* and *rrn18* showed a limited decrease (15-20%). Only the 26S rRNA (*rrn26)* showed a larger decrease (39%). The effect of *nad9* mRNA knockdown was thus less pronounced on these RNAs than on mitochondrial mRNAs coding for proteins of the OXPHOS complexes. In control plants expressing the *Rz1sdh3*-L-PKTLS RNA, RNAs of the translation system also were either unaffected or showed a mild increase

III.2.1.3 Level of selected nuclear mRNAs

Transformed *A. thaliana* plants expressing the *Rz1nad9*-L-PKTLS ribozyme showed a drastic decrease of the mRNAs encoding the alternative oxidase, *aox1a* (63%) and *aox1d*

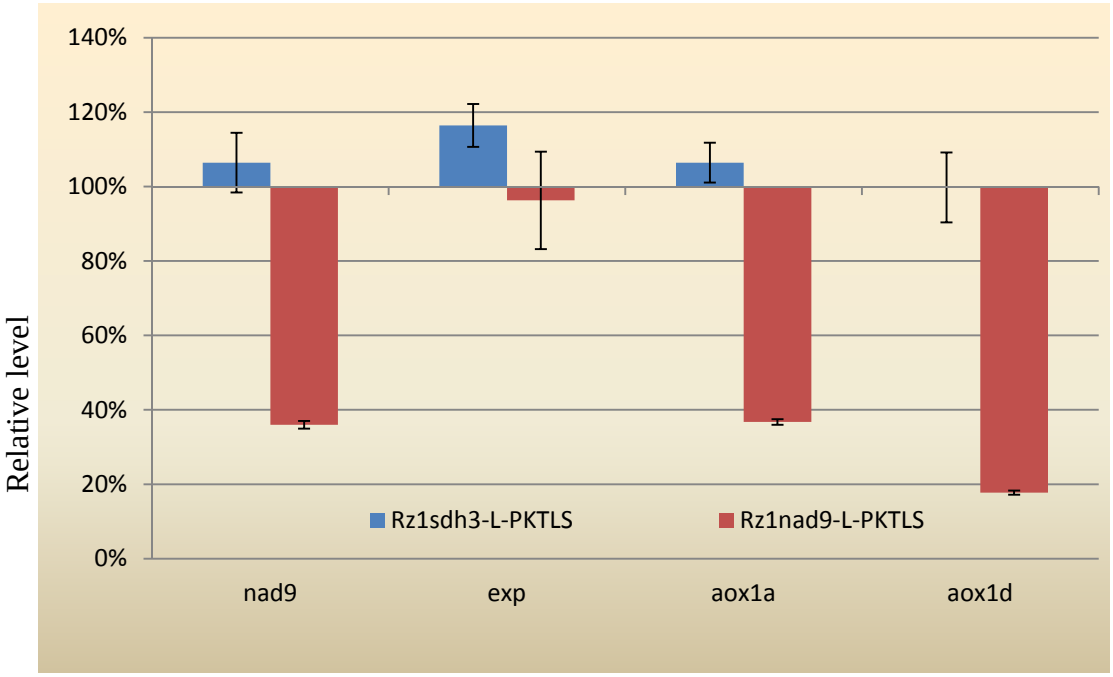


Figure 46: Effect of *nad9* mRNA knockdown on the level of selected nuclear mRNAs in 16-18 leaf stage *A. thaliana* plants

Analysis by RT-qPCR on total RNA. The *nad9*, *exp*, *aox1a* and *aox1d* mRNA levels were analyzed in *Rz1nad9* transformants (red) and in *Rz1sdh3* transformants (blue). For transgene induction, estradiol was added on day 0, day 4 and day 7. Results are given as the percentage of the different mRNAs at day 10 compared to day 0.

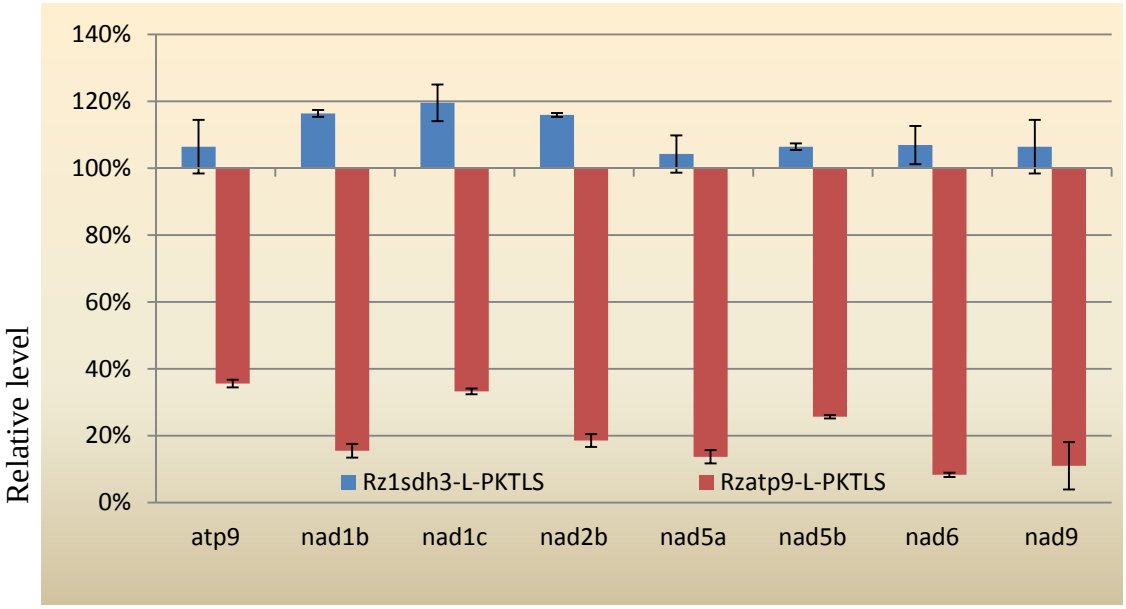


Figure 47: Effect of *atp9* mRNA knockdown on the level of mitochondrial mRNAs encoding complex I subunits in 16-18 leaf stage *A. thaliana* plants

Analysis by RT-qPCR on total RNA. The *atp9*, *nad1b*, *nad1c*, *nad2b*, *nad5a*, *nad5b*, *nad6* and *nad9* mRNA levels were analyzed in *Rzatp9* transformants (red) and in *Rz1sdh3* transformants (blue). For transgene induction, estradiol was added on day 0, day 4 and day 7. Results are given as the percentage of the different mitochondrial mRNAs at day 10 compared to day 0.

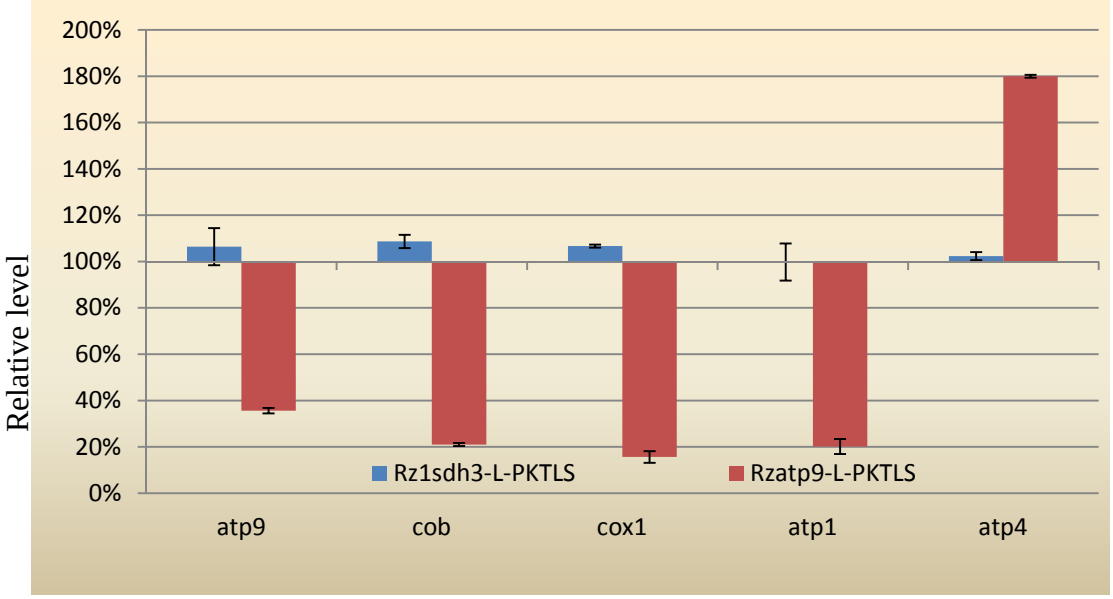


Figure 48: Effect of *atp9* mRNA knockdown on the level of mRNAs encoding OXPHOS chain complexes subunits in 16-18 leaf stage *A. thaliana* plants

Analysis by RT-qPCR on total RNA. The *atp9*, *cob*, *cox1*, *atp1* and *atp4* mRNA levels were analyzed in *Rzatp9* transformants (red) and in *Rz1sdh3* transformants (blue). For transgene induction, estradiol was added on day 0, day 4 and day 7. Results are given as the percentage of the different mitochondrial mRNAs at day 10 compared to day 0.

(82%), whereas these RNAs were unaffected in *Rz1sdh3*-L-PKTLS-expressing control plants (Figure 46). Such results are remarkable, since the AOX protein is encoded by the nuclear genome. Thus, a directed knockdown of a mitochondrial RNA (*nad9* in this case) was associated with a strong decrease of nuclear RNAs encoding a mitochondrial protein, pointing towards a retrograde regulation. On the contrary, the level of the *exp* mRNA, which has no relation with mitochondria, was unaffected (Figure 46).

III.3 Effect of *atp9* knockdown on the steady-state level of other RNAs

The ATP9 protein is part of the ATP synthase complex. This complex has an important function for the cell, the production of ATP using the proton gradient established by the respiratory chain between the intermembrane space and the matrix of the organelles. It can also catalyze the reverse reaction. As complex I, it is composed of subunits encoded by the nucleus and by the mitochondria. Here too, one would assume that some coordination of gene expression is required to ensure proper assembly of the complex (Giegé, 2007). As for *nad9*, we determined whether the knockdown of the *atp9* mRNA had an effect on the level of mitochondrial mRNAs coding for other proteins of the same complex and on mitochondrial mRNAs coding for proteins of other complexes.

III.3.1 Level of mitochondrial mRNAs coding for proteins of the OXPHOS chain

The results obtained with *Rzatp9*-L-PKTLS and *Rz1sdh3*-L-PKTLS-expressing *A. thaliana* plants are presented in Figures 47 and 48. Knockdown of the *atp9* mRNA in response to the presence of the *Rzatp9*-L-PKTLS ribozyme caused a decrease of all tested mRNAs coding for subunits of complex I of the respiratory chain (Figure 47). As a consequence of a 64% knockdown of *atp9*, the greatest effect was observed for *nad6*, with a 92% decrease. Also for all other mRNAs tested, *i.e. nad1b*, *nad1c*, *nad2b, nad5a*, *nad5b* and *nad9*, the decrease was even more important than the specific knockdown of the *atp9* mRNA itself. It is worthy to note that specific knockdown of *nad9* triggered a strong decrease of *atp9*, and to a lower extent of other mRNAs coding for ATP synthase subunits (Figure 43), whereas reciprocally specific knockdown of *atp9* caused a drastic drop of *nad9* and of other mRNAs coding for complex I subunits (Figure 47). This reciprocal effect further suggests that there might be some regulation between the components of different complexes.

As seen on Figure 48, the levels of the *cob, cox1* and *atp1* mRNAs also decreased dramatically (about 80%) upon *atp9* knockdown. Surprisingly, the level of *atp4* showed an increase of 80%. This strong upregulation of the *atp4* mRNA may be to compensate the

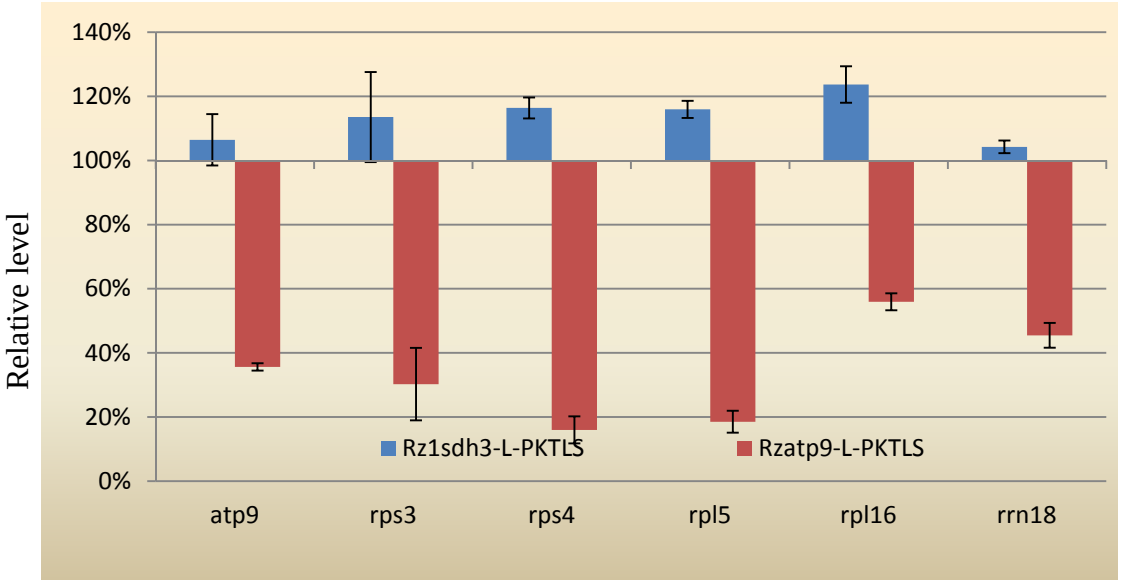


Figure 49: Effect of *atp9* mRNA knockdown on the level of mitochondrial RNAs of the translation system in 16-18 leaf stage *A. thaliana* plants

Analysis by RT-qPCR on total RNA. The *atp9*, *rps3*, *rps4*, *rpl5*, *rpl16*, *rrn18* and *rrn26* RNA levels were analyzed in *Rzatp9* transformants (red) and in *Rz1sdh3* transformants (blue). For transgene induction, estradiol was added on day 0, day 4 and day 7. Results are given as the percentage of the different mitochondrial RNAs at day 10 compared to day 0.

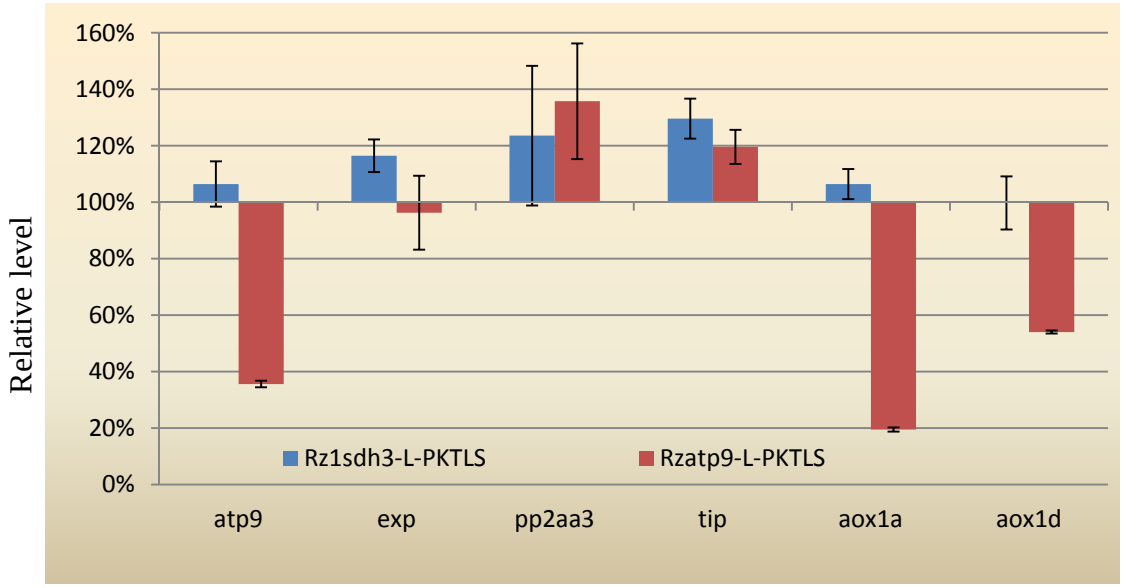


Figure 50: Effect of *atp9* mRNA knockdown on the level of selected nuclear mRNAs in 16-18 leaf stage *A. thaliana* plants

Analysis by RT-qPCR on total RNA. The *atp9*, *exp*, *pp2aa3*, *tip*, *aox1a* and *aox1d* mRNA levels were analyzed in *Rzatp9* transformants (red) and in *Rz1sdh3* transformants (blue). For transgene induction, estradiol was added on day 0, day 4 and day 7. Results are given as the percentage of the different mRNAs at day 10 compared to day 0.

drastic decrease in the level of other mRNAs encoding subunits of the ATP synthase complex. In control plants expressing the *Rz1sdh3*-L-PKTLS ribozyme, all RNAs tested were either unaffected or showed a mild increase, which was possibly the effect of estradiol. Also *atp4* was unaffected, which further supports the hypothesis that the increase of this mRNA upon *atp9* knockdown has a role.

III.3.2 Level of mitochondrial RNAs of the translation system

Contrary to the results obtained with *nad9* mRNA knockdown (§ III.2.2), the effect of *atp9* knockdown on the level of RNAs involved in mitochondrial protein synthesis was very prominent, with in particular a drastic drop (over 80%) of *rps4* and *rpl5* (Figure 49). As before, the level of all these RNAs was unaffected or slighly increased in control plants expressing the *Rz1sdh3*-L-PKTLS RNA.

III.3.3 Level of selected nuclear mRNAs

As shown on Figure 50, also upon *atp9* knockdown we observed a drastic decrease of the *aox1a* (81%) and *aox1d* (46%) nuclear mRNAs coding for the alternative oxidase. These observations further support the hypothesis that a directed knockdown of a mitochondrial RNA can trigger a retrograde response and affect the expression of nuclear RNAs. As before, the level of *exp* mRNA was unaffected (Figure 50). Nevertheless, as there was a more drastic effect on most of the mitochondrial RNAs and nuclear RNAs related to the OXPHOS chain, we tested two more nuclear genes not related to mitochondria. These were mRNAs coding for PP2AA3 (PROTEIN PHOSPHATASE 2A SUBUNIT A3) and TIP (TIP41-like family protein). There was a slight increase in the level of these mRNAs, but it was similar in both *Rzatp9*-L-PKTLS and *Rz1sdh3*-L-PKTLS-expressing plants (Figure 50). So most probably it was just the effect of estradiol.

III.4 Complementary approaches

As the level of many mitochondrial RNAs decreased upon directed knockdown of *nad9* or *atp9*, we verified that this was not due to a general collapse of mitochondria in the cells. For this, we extracted total DNA from 16-18 leaf *A. thaliana* plants expressing the *Rz1nad9*-L-PKTLS ribozyme. Estradiol was added at D0 to start the induction, at D4 and then every three days till 19 days. Plants were harvested each day till D4 and then every three days till 19 days. The extracted DNA served as a template for qPCR analysis with the primers for the *nad9* sequence (Table 6). The data were normalized with *actin* and *gapdh*. The results obtained are presented in Figure 51 and show that there was no decrease in the

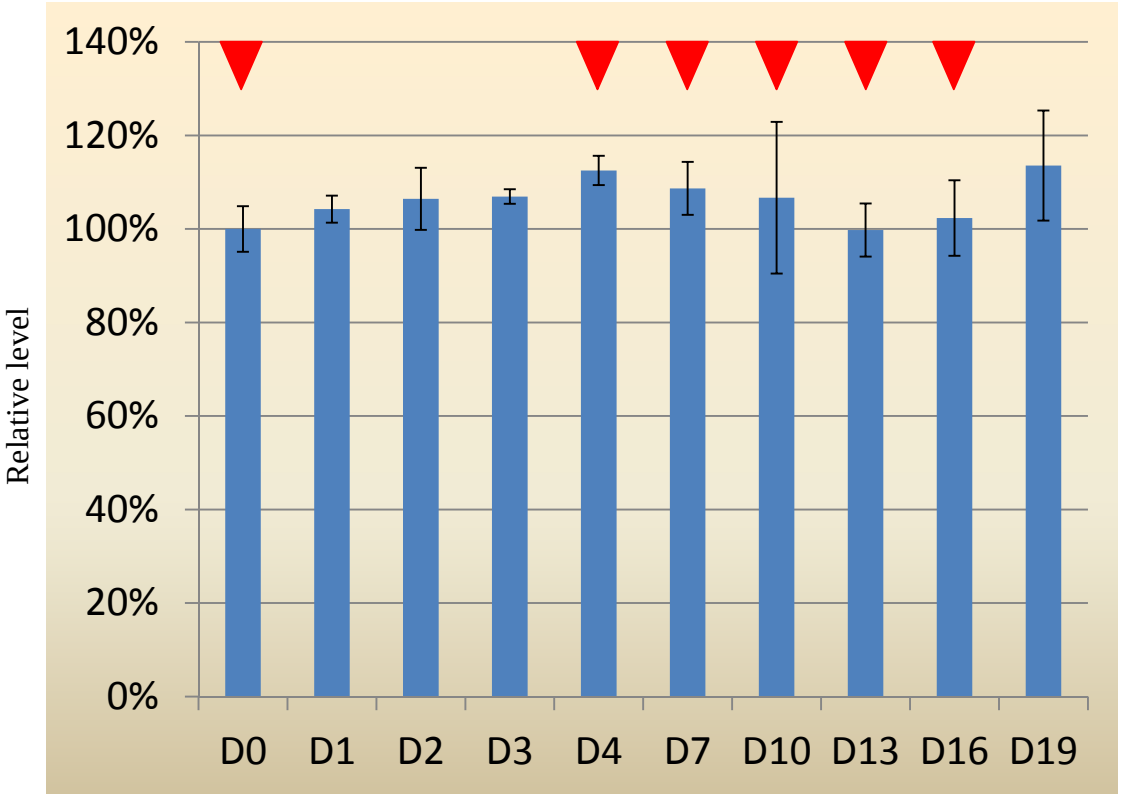


Figure 51: Evaluation of the mitochondrial DNA level in 16-18 leaf stage transformed *A. thaliana* plants

Analysis by qPCR on total DNA. Level of mitochondrial nad9 gene in plants expressing the Rz1nad9-L-PKTLS ribozyme versus control plants. Estradiol was added on day 0, day 4 and day 7 and plants expressing the ribozyme were harvested on day 10. Control plants were harvested on day 0, before addition of estradiol. A relative level of 100% was assigned to day 0.

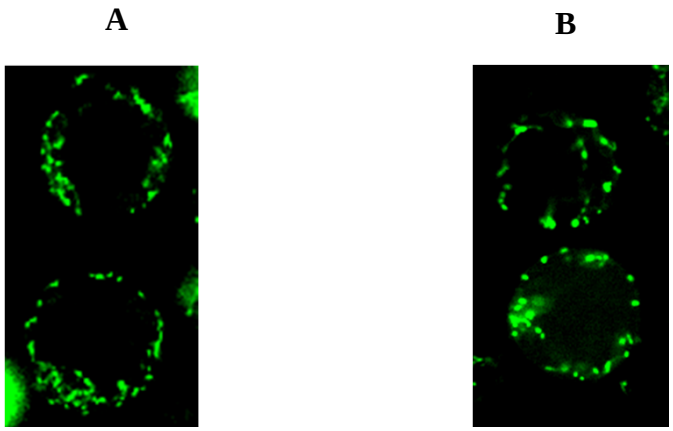


Figure 52: Pattern of mitochondria in 16-18 leaf stage wild-type and *Rz1nad9* transformed *A. thaliana* plants treated with estradiol

Confocal microscope analysis of protoplasts from wild-type (A) and *Rz1nad9* transformants (B) treated with estradiol on day 0, day 4 and day 7 and harvested on day 10.

mitochondrial DNA content associated with *Rz1nad9*-L-PKTLS expression and *nad9* mRNA knockdown.

We also checked by confocal microscopy the pattern of mitochondria in wild-type and *Rz1nad9*-L-PKTLS-expressing 16-18 leaf *A. thaliana* plants harvested 10 days after onset of estradiol treatment (estradiol addition at D0, D4 and D7). For this, we prepared protoplasts from the plant samples (Materials and Methods, § II.5.5). These protoplasts were stained with MitoTracker® Red CMXRRos and microscopic observations were made with a Zeiss LSM 700 laser scanning confocal microscope (Materials and Methods, § II.5.6). We observed similar mitochondrial populations in protoplasts from control and ribozyme-expressing plants (Figure 52).

III.5 Transcriptome analysis with CATMA microarrays

As a whole, the RT-qPCR results described above pointed both to RNA regulation processes within mitochondria and to a retrograde signaling to the nucleus. To strengthen our observations and extend the data to a high throughput level, we moved to transcriptome characterization by microarray technologies, which is a powerful tool for functional analysis of gene expression. The purpose was to explore the regulation mechanisms at a whole transcriptome level, in line with the results obtained with RT-qPCR. On the other hand, whereas exploring the complete transcriptome by RT-qPCR was accessible for mitochondrial genes, extending RT-qPCR analyses on nuclear transcripts needed to find further candidate genes through another approach.

CATMA (Complete Arabidopsis Transcriptome Micro Array) microarray analysis was carried out at the Unité de Recherche en Génomique Végétale (URGV, Evry, France), using the CATMAv6.2 array based on Roche-NimbleGen technology (Materials and Methods, § II. 2.7). Two independent biological replicates were produced. For each biological replicate, a technical replicate with fluorochrome reversal (dye-swap/dye-switch) was performed, leading to a total of 4 hybridizations per comparison. For each biological replicate, total RNA was extracted from 16-18 leaf stage *A. thaliana* plants expressing the *Rz1nad9*-L-PKTLS ribozyme and induced for 10 days. Estradiol was added at day 0, day 4 and day 7 to keep the level of ribozyme at maximum level. For *Rz1nad9*-L-PKTLS, the maximum knockdown of the *nad9* target mRNA in these conditions was observed on day 10, as shown above by RT-qPCR (§ II.6, Figure 37). Control plants were taken before estradiol addition.

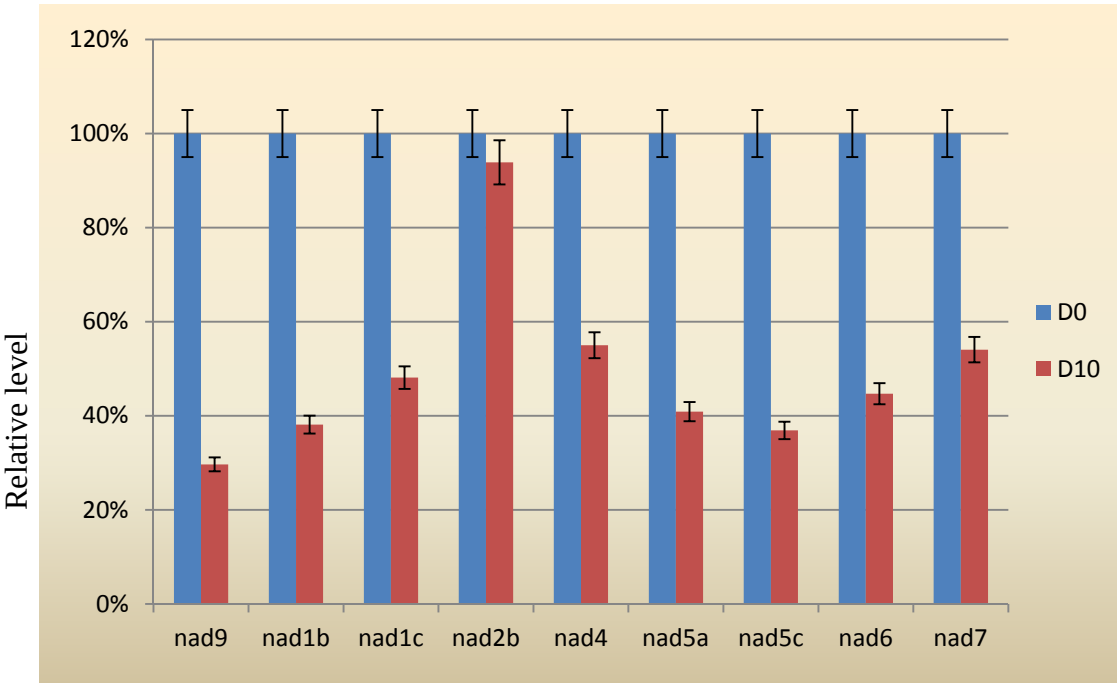


Figure 53: Effect of *nad9* mRNA knockdown on the level of mitochondrial mRNAs encoding complex I subunits in 16-18 leaf stage *A. thaliana* plants

Microarray analysis on total RNA deprived of rRNAs. Levels of *nad9*, *nad1b*, *nad1c*, *nad2b*, *nad4*, *nad5a*, *nad5c*, *nad6* and *nad7* mRNAs in plants expressing the Rz1nad9-L-PKTLS ribozyme versus control plants. Estradiol was added on day 0, day 4 and day 7 and plants expressing the ribozyme were harvested on day 10 (D10, red). Control plants were harvested on day 0 (D0, blue), before addition of estradiol.

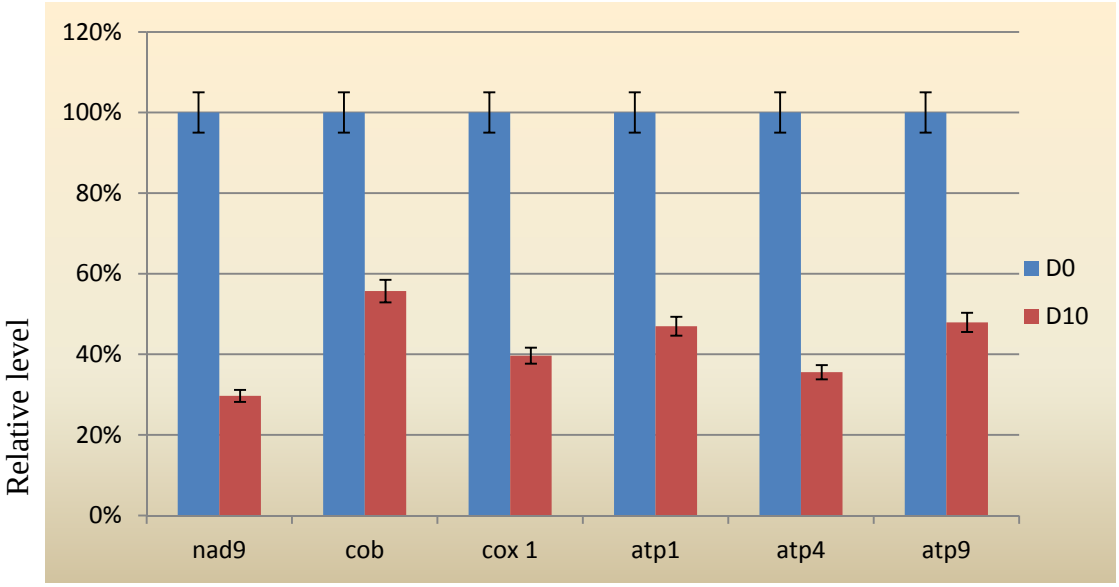


Figure 54: Effect of *nad9* mRNA knockdown on the level of mRNAs encoding OXPHOS chain complexes subunits in 16-18 leaf stage *A. thaliana* plants

Microarray analysis on total RNA deprived of rRNAs. Levels of *nad9*, *cob*, *cox1*, *atp1* and *atp4* mRNAs in plants expressing the Rz1nad9-L-PKTLS ribozyme versus control plants. Estradiol was added on day 0, day 4 and day 7 and plants expressing the ribozyme were harvested on day 10 (D10, red). Control plants were harvested on day 0 (D0, blue), before addition of estradiol.

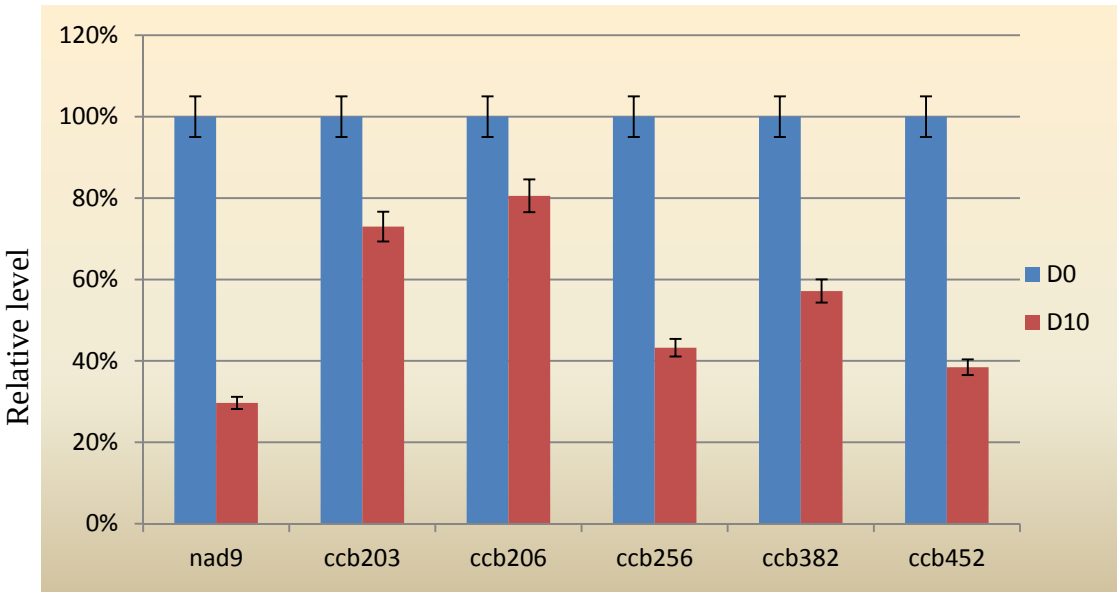


Figure 55: Effect of *nad9* mRNA knockdown on the level of mRNAs encoding cytochrome c biogenesis proteins in 16-18 leaf stage *A. thaliana* plants

Microarray analysis on total RNA deprived of rRNAs. Levels of *nad9*, *ccb203*, *ccb206*, *ccb256*, *ccb382* and *ccb452* mRNAs in plants expressing the Rz1nad9-L-PKTLS ribozyme versus control plants. Estradiol was added on day 0, day 4 and day 7 and plants expressing the ribozyme were harvested on day 10 (D10, red). Control plants were harvested on day 0 (D0, blue), before addition of estradiol.

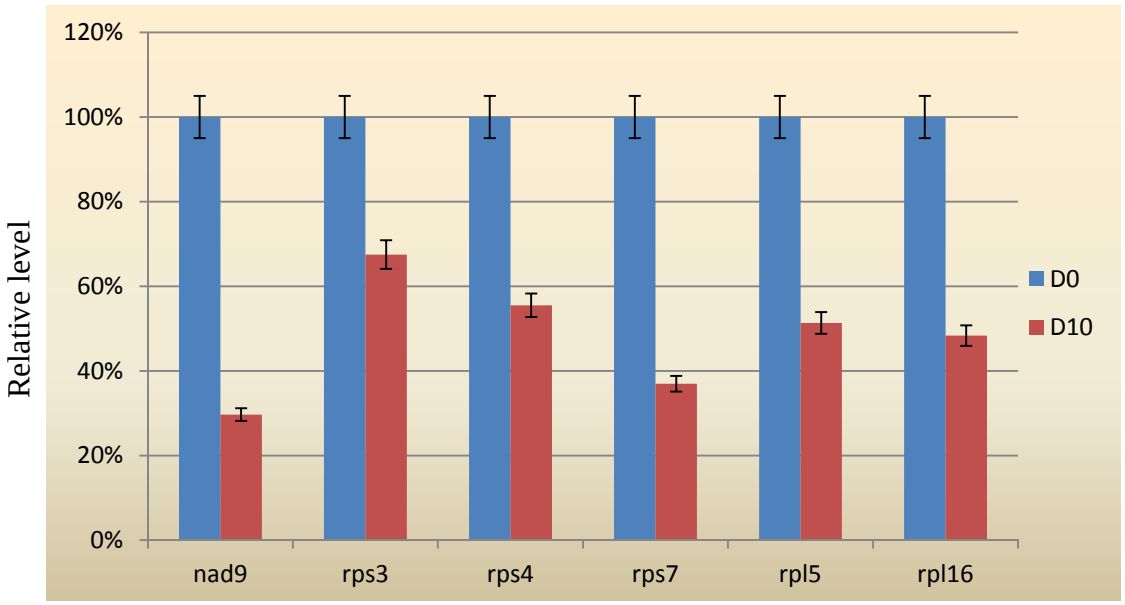


Figure 56: Effect of *nad9* mRNA knockdown on the level of mitochondrial mRNAs of the translation system in 16-18 leaf stage *A. thaliana* plants

Microarray analysis on total RNA deprived of rRNAs. Levels of *nad9*, *rps3*, *rps4*, *rps7*, *rpl5* and *rpl16* mRNAs in plants expressing the Rz1nad9-L-PKTLS ribozyme versus control plants. Estradiol was added on day 0, day 4 and day 7 and plants expressing the ribozyme were harvested on day 10 (D10, red). Control plants were harvested on day 0 (D0, blue), before addition of estradiol.

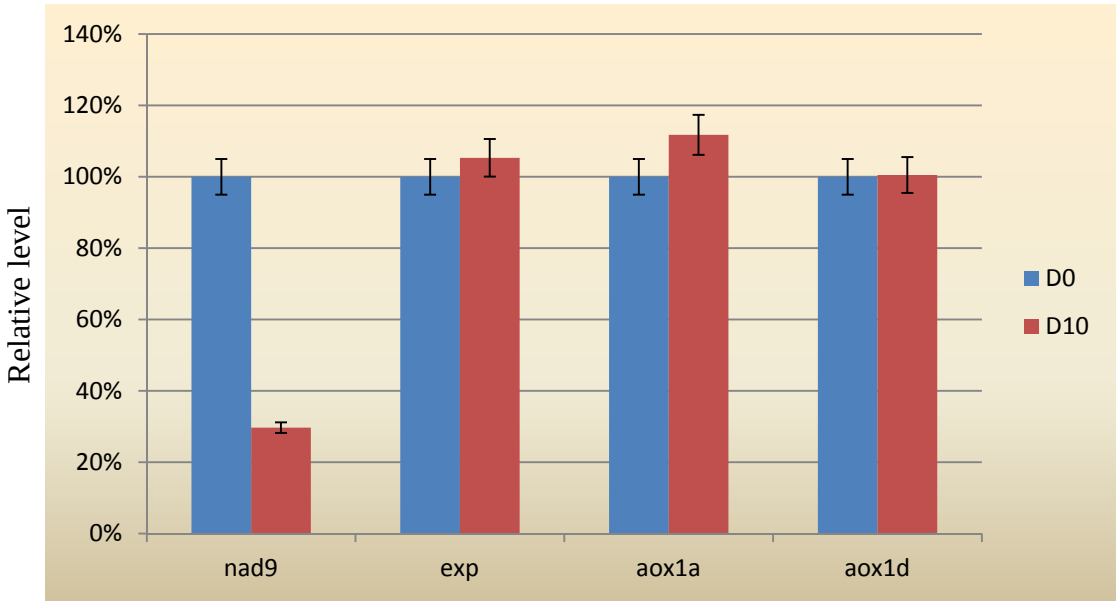


Figure 57: Effect of *nad9* mRNA knockdown on the level of selected nuclear mRNAs in 16-18 leaf stage *A. thaliana* plants

Microarray analysis on total RNA deprived of rRNAs. Levels of *nad9*, *exp*, *aox1a* and *aox1d* mRNAs in plants expressing the Rz1nad9-L-PKTLS ribozyme versus control plants. Estradiol was added on day 0, day 4 and day 7 and plants expressing the ribozyme were harvested on day 10 (D10, red). Control plants were harvested on day 0 (D0, blue), before addition of estradiol.

All the RNA samples were rRNA depleted at the URGV before further steps. cDNA was synthesized with random hexamer primers.

The data were generated in the form of a logarithmic scale (base 2) and adjusted by the Bonferroni method (Material and Methods, § II. 2.7.1). When the Bonferroni *P*-value was lower than 0.05, the spot was declared differentially expressed. Then logarithmic data were converted to percentage for making graphs. We are still analyzing the data obtained with these experiments. About 2500 RNAs showed significant changes; about half of them were upregulated, while others were downregulated. We have primarily undertaken to extract those genes that have some relation with the mitochondrial OXPHOS chain and genes encoding other proteins that are imported into mitochondria. But before all this, we looked at the levels of mRNAs that we had already studied with RT-qPCR.

These results are presented in Figures 53 to 57. First of all, microarray analysis revealed a roughly 70% knockdown of the *nad9* mRNA in plants expressing the *Rznad9*-L-PKTLS ribozyme versus control plants (Figure 53), which confirmed precisely the RT-qPCR results (§ II.6, Figure 37). As the latter approach, microarrays also showed that knockdown of *nad9* had an impact on the level of other mRNAs in mitochondria and in the nucleus. Knockdown of this single mRNA caused a drastic decrease in the level of most of the mRNAs encoding subunits of complex I. As with RT-qPCR, *nad2b* remained unaffected. However, *nad6* and *nad7* showed some decrease, which was not the case before (Figure 53). In agreement with the above assays, a significant decrease was observed for *cob, cox1, atp1* and *atp4* mRNA levels (Figure 54). However, for the mRNAs coding for CCB proteins, a significant decrease was previously observed only for ccb452, but with microarrays ccb256 (57%) and ccb382 (43%) also showed a pronounced decrease (Figure 55). Finally, substantial decrease was evident for mRNAs encoding proteins of the small and large ribosomal subunits (Figure 56). Concerning the nuclear genes, as before, the *exp* RNA remained unaffected. Strikingly, the drastic decrease in the level of *aox1a* and *aox1d* mRNAs repeatedly associated previously with both *nad9* and *atp9* knockdown could not be confirmed with microarrays (Figure 57). Nevertheless, with only a few exceptions, microarray analysis confirmed RT-qPCR data, including the knockdown of *nad9* and the behavior of *nad1b*, *nad1c*, *nad2b, nad4*, *nad5, nad9, cob, cox1, atp1, atp4, ccb203, ccb206, ccb452, rps3, rrn18* and *exp* (Figure 53-57). The results thus reinforce the idea of a mitochondrial regulation at the transcriptome level.

Annotation	Gene	Level
Mitochondrial Genes		
NADH Dehydrogenase Subunit 9	*nad9*	0.30
TatC-like protein	*mttb*	0.63
Cytochrome C Oxidase Subunit 2	*cox2*	0.64
Cytochrome C Oxidase Subunit 3	*cox3*	1.11
ATP Synthase Subunit 6	*nad6*	0.56
Nuclear Genes		
Indole-3-Acetic Acid Inducible 29	*iaa29*	0.04
Phytochrome Interacting Factor 3-Like 2	*pil2*	0.04
Drought-Repressed 4	*atdr4*	0.07
Heat Shock Protein 70	*hsp70*	6.36
Pistillata	*pi*	11.96
Chloroplastic Genes		
Large Subunit Of RUBISCO	*rbcl*	0.20
Ribosomal Protein S12	*rps12*	0.32
Photosystem II Reaction Center Protein A	*psba*	0.17
Psac Subunit Of Photosystem I	*psac*	0.21
Ribosomal Protein S16	*rps16*	1.77

Table 7: Effect of *nad9* mRNA knockdown on the level of other RNAs in 16-18 leaf stage *A. thaliana* plants

Microarray analysis on total RNA deprived of rRNAs. Levels of various mRNAs from mitochondria, nucleus and chloroplasts in plants expressing the Rz1nad9-L-PKTLS ribozyme versus control plants. Estradiol was added on day 0, day 4 and day 7 and plants expressing the ribozyme were harvested on day 10. Control plants were harvested on day 0, before addition of estradiol.

Annotation	Gene	Level
Pentatricopeptide (PPR) repeat-containing proteins		
PPR protein	AT5G13770	4.82
PPR protein	AT5G02830	3.10
PPR protein	AT2G18940	2.73
PPR protein	AT2G01860	2.72
PPR protein	AT3G04760	2.39
PPR protein	AT5G42310	2.36
PPR protein	AT5G25630	2.34
PPR protein	AT3G26630	2.33
PPR protein	AT3G46870	2.26
PPR protein	AT2G31400	2.13
PPR protein	AT5G04810	1.97
PPR protein	AT1G01970	1.96
PPR protein	AT5G46580	1.92
PPR protein	AT3G46610	1.90
PPR protein	AT1G07590	1.88
PPR protein	AT1G69290	0.32

Table 8: Effect of *nad9* mRNA knockdown on the level of some mRNAs coding for PPR proteins in 16-18 leaf stage *A. thaliana* plants

Microarray analysis on total RNA deprived of rRNAs. Levels of some mRNAs coding for PPR proteins in plants expressing the Rz1nad9-L-PKTLS ribozyme versus control plants. Estradiol was added on day 0, day 4 and day 7 and plants expressing the ribozyme were harvested on day 10. Control plants were harvested on day 0, before addition of estradiol.

A second purpose of microarray analysis was to find further interesting candidates that can help to understand the regulation pathways between organelles and the nucleus or within the mitochondria. That the *aox1a* and *aox1d* mRNAs were unaffected in the microarray assay remained surprising, as the drastic decrease of these RNAs in response to *nad9* or *atp9* knockdown was observed many times by RT-qPCR. But microarrays brought further arguments for retrograde regulation, as numerous other nuclear mRNAs were affected, positively or negatively. As mentioned, some 2500 RNAs showed a significant difference as a result of the knockdown of one single major mRNA (*nad9* in this case) in mitochondria. This effect was very diverse. Some RNAs showed a drastic increase, others showed a drastic decrease and others only mild changes. A few examples of mRNAs from all three cell compartments affected by the knockdown of *nad9* are shown in Table 7.

We paid special attention to the genes encoding PPR proteins. As mentioned (Introduction § I.3.2), bioinformatics analyses have identified a family of at least 550 PPR protein genes in *A. thaliana* (tinyurl.com/d4m8bxt). Much of these proteins are predicted to be targeted to mitochondria or chloroplasts (Lurin *et al.*, 2004). PPR proteins bind to RNAs in organelles and recruit different factors for editing, maturation or translation (Sosso *et al.*, 2012; Zehrmann *et al.*, 2012; Zhu *et al.*, 2012). There are a number of publications about the role of PPR proteins in gene regulation (Hammani *et al.*, 2011a, 2011b; Saha *et al.*, 2007; Schmitz-Linneweber and Small, 2008). Due to this importance of PPR proteins in regulation mechanisms, these can be an interesting candidate for further studies. We made some interesting observations for a set of 16 mRNAs encoding such proteins, as presented in Table 8. A significant increase was observed for most of these mRNAs, whereas one mRNA, coding for PPR protein AT1G69290, showed a drastic decrease (68%) (Table 8). The latter protein is targeted to mitochondria. Another important PPR protein is GUN I (AT2G31400), which is involved in many retrograde regulation pathways (Introduction § IV.2). It is considered as a master switch because it appears to be a convergence point for ROS and Redox pathways, and also for PGE and tetrapyrrole pathways from chloroplasts to the nucleus. GUN I may have some role in MRR, which makes it of special relevance for our studies. In our microarray results, the GUN I mRNA showed a substantial increase (113%, Table 8). PPR protein-encoding mRNAs will be further analyzed by RT-qPCR.

A recent study showed that PNM1 (for "PPR protein localized to the nucleus and mitochondria 1") plays a role in gene expression adjustments between mitochondria and the nucleus, with the help of TCP8 that binds the promoter of PNM1 (Hammani *et al.*, 2011a).

Annotation	Gene		Level
TCP family transcription factors			
TCP family transcription factor 23	*tcp23*	AT1G35560	2.06
TCP family transcription factor 17	*tcp17*	AT5G08070	1.89
TCP family transcription factor 14	*tcp14*	AT3G47620	0.48
TCP family transcription factor 11	*tcp11*	AT5G08330	0.41
TCP family transcription factor 15	*tcp15*	AT1G69690	0.39
Alternative NADH dehydrogenases			
Alternative NADH dehydrogenase A1	*nda1*	AT1G07180	1.09
Alternative NADH dehydrogenase A2	*nda2*	AT2G29990	1.09
Alternative NADH dehydrogenase B1	*ndb1*	AT4G28220	1.11
Alternative NADH dehydrogenase B2	*ndb2*	AT4G05020	0.61
Alternative NADH dehydrogenase B3	*ndb3*	AT4G21490	0.91
Alternative NADH dehydrogenase B4	*ndb4*	AT2G20800	1.04
Alternative NADH dehydrogenase C1	*ndc1*	AT5G08740	1.05

Table 9: Effect of *nad9* mRNA knockdown on the level of some mRNAs coding for TCP family transcription factors and alternative NADH dehydrogenases proteins in 16-18 leaf stage *A. thaliana* plants

Microarray analysis on total RNA deprived of rRNAs. Levels of some mRNAs coding for TCP family transcription factors and alternative NADH dehydrogenases proteins in plants expressing the Rz1nad9-L-PKTLS ribozyme versus control plants. Estradiol was added on day 0, day 4 and day 7 and plants expressing the ribozyme were harvested on day 10. Control plants were harvested on day 0, before addition of estradiol.

There are also other studies that showed the importance of the genes coding for TCP family transcription factors in regulation mechanisms between mitochondria and the nucleus (Hammani *et al.*, 2011b). So, this family can also be an interesting candidate. Our microarray results showed a drastic decrease for the *tcp11* (59%), *tcp14* (52%) and *tcp15* (61%) mRNAs. Conversely, a significant increase was observed for *tcp17* (89%) and *tcp23* (106%) (Table 9). TCP protein-encoding mRNAs will be further analyzed as well by RT-qPCR. Finally, in the context of *nad9* knockdown, another important family would be the alternative NAD(P)H dehydrogenases, as these have a role comparable to that of complex I. However, in our microarray assays, only the *ndb2* mRNA showed substantial change, which was actually about 40% decrease, while other alternative NAD(P)H dehydrogenase mRNAs remained unaffected (Table 9).

We have already started to look into different pathways using MapMan (Usadel *et al.*, 2005, mapman.mpimp-golm. mpg.de) and we will explore more with the help of these and other available tools.

III.6 First analyses addressing RNA regulation mechanisms

Our results raised the question of the mechanisms that underlie the regulation processes that we highlighted. The most straightforward hypothesis would be a rapid change in transcription rate but a change in the rate of RNA degradation should be considered as well. We initiated experimental approaches to investigate both possibilities. We began to set up run-on transcription assays with mitochondria isolated from control plants or plants expressing the chimeric ribozymes directed against the *nad9* or *atp9* mRNAs. Notably, the above microarray analyses did not highlight important changes in the expression of the mitochondrial RNA polymerases.

As to RNA degradation analysis, we based our assays on the fact that RNA polyadenylation in bacteria and organelles serves a purpose opposite to the role it plays in nuclear systems. The majority of nucleus-encoded transcripts are characterized by stable poly(A) tails at their mature 3'-ends, which are essential for stabilization and translation initiation. In contrast, in bacteria, chloroplasts, and plant mitochondria, polyadenylation is a transient feature that promotes RNA degradation (Holec *et al.*, 2008). We aim to use this property to test whether the decrease in the level of many mitochondrial RNAs in relation with *nad9* or *atp9* knockdown was due to an increased degradation rate. In initial experiments, total RNA extracted from 16-18 leaf stage plants expressing the *Rz1nad9*-L-PKTLS ribozyme was

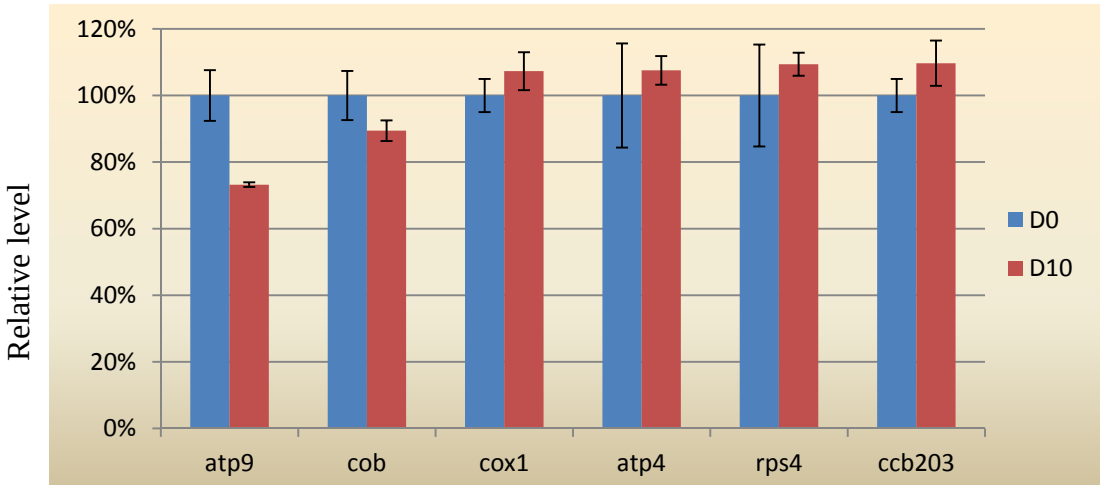


Figure 58: Effect of *nad9* mRNA knockdown on the level of mitochondrial RNA polyadenylated forms in 16-18 leaf stage *A. thaliana* plants

Analysis by RT-qPCR on total RNA. The *atp9*, *cob*, *cox1*, *atp4*, *rps4* and *ccb203* mRNA levels were analyzed in *Rz1nad9* transformants using oligo(dT) as a primer for reverse transcription. For transgene induction, estradiol was added on day 0, day 4 and day 7. Results are given as the percentage of the different mitochondrial mRNAs at day 10 (red) compared to day 0 (blue).

used for RT-qPCR assays but the RT reactions were primed with oligo(dT) instead of random hexamers, so as to amplify degradation intermediates. qPCR was run as before with dedicated couples of primers (Table 6). Ribozyme expression was maintained for 19 days and plant samples were harvested every day from D0 to D4 and then every three days. The results were also compared with reactions in which the RT was primed with a mix of random hexamers. For the qPCR reactions, *actin* and *gapdh* were used as reference genes. We probed for polyadenylated forms of a series of mitochondrial RNAs, especially those showing a strong decrease in relation to *nad9* knockdown. The results obtained at D10 are presented in Figure 58. The samples primed with oligo(dT) did not reveal an increase in the level of detectable polyadenylated forms along with the knockdown kinetics (Figure 58). Rather a limited decrease of polyadenylated forms was observed in some cases. So, at that stage, we have no evidence for increased degradation. Comparing the levels of mRNAs reverse-transcribed with oligo(dT) versus random hexamer primers indicated 2-10% polyadenylation, depending on the RNA considered.

DISCUSSION
AND
CONCLUSION

All the complexes of the mitochondrial oxidative phosphorylation chain consist of subunits encoded by the nuclear genome and subunits expressed from mitochondrial genes. Thus, a functional and responsive OXPHOS chain requires the coordinated expression of the two genomes. This is achieved by a combination of anterograde and retrograde regulatory pathways. It is generally believed that in plants transcription *per se* is not a regulatory point for mitochondrial genes and genetic control would mainly occur at the post-transcriptional level (Giegé *et al.*, 2005; Millar *et al.*, 2011). However, over the past two decades, there have been a number of reports describing changes in the abundance of specific mitochondrial transcripts during growth, such as during the development of wheat leaves (Topping and Leaver, 1990) or seed germination in maize and rice (Howell *et al.*, 2006, 2007; Ishizaki *et al.*, 2005). In this respect, variations in the steady state levels of a number of mitochondrial mRNAs were also observed with *A. thaliana* nuclear mutants deficient for the editing or splicing of a given organelle RNA. For instance, the level of many mitochondrial transcripts was increased in *slo1* mutants lacking a PPR protein required for the editing of specific sites in the *nad4* and *nad9* transcripts (Sung *et al.*, 2010). Similarly, the loss of At-nMat1a, involved in splicing of *nad4*, or of BIR6, needed for *nad7* splicing, was associated with a decrease or increase in the level of a series of other mitochondrial RNAs (Koprivova *et al.*, 2010; Nakagawa and Sakurai, 2006). Also, ATP1 and ATP2 are both subunits of the F0-F1 ATP synthase complex and interact together in the F1 subunit. The former is encoded by the mitochondrial genome, while the latter is encoded by the nuclear genome. It has been shown in *Nicotiana sylvestris* that the levels of the mRNAs encoding these two proteins are highly correlated (Lalanne *et al.*, 1998). However, Giegé *et al.*, (2005) showed in *A. thaliana* that, following sugar starvation, the level of the mRNA encoding the ATP2 protein decreases, while that of the mRNA for ATP1 remains unchanged. The molecular basis and significance of such differential accumulations is unknown. So the field remains open and the idea of regulation mechanisms at the RNA level in mitochondria, connected with mitochondrial retrograde pathways, has to be considered. In the present work, we established specific *in vivo* knockdown of different target mRNAs in mitochondria, using import of *trans*-cleaving ribozymes. Although the development of some of our ribozyme/target systems can still be optimized, nuclear expression and mitochondrial targeting of catalytic RNAs proved effective to knockdown major RNAs in the organelles of plants and to specifically manipulate the transcriptome. The question of the existence of RNA control and coordination processes could then be

Gene	Location	*Rz1sdh3*-L-PKTLS		*Rz1nad9*-L-PKTLS	
nad9	Complex I	=	+106%	↘	36%
nad1b	Complex I	↗	+116%	↘	48%
nad1c	complex I	↗	+119%	↘	63%
nad2b	Complex I	↗	+116%	=	95%
nad4	Complex I	=	-93%	↘	52%
nad5a	Complex I	=	+104%	↘	65%
nad5b	Complex I	=	+106%	↘	58%
nad6	Complex I	=	+107%	=	92%
nad7	Comlpex I	=	94%	=	90%
cob	Complex II	=	109%	↘	58%
cox1	Complex IV	=	107%	↘	56%
atp1	ATP syntase	=	100%	↘	63%
atp4	ATP syntase	=	102%	↘	63%
atp9	ATP syntase	=	107%	↘	52%
ccb203	Heme synthesis	=	107%	↘	83%
ccb206	Heme synthesis	↗	112%	=	89%
ccb256	Heme synthesis	=	109%	↗	114%
ccb382	Heme synthesis	=	107%	↘	81%
ccb452	Heme synthesis	=	100%	↘	53%
rps3	Ribosome/protein synthesis	↗	113%	↘	80%
rps4	Ribosome/protein synthesis	↗	116%	↘	85%
rps7	Ribosome/protein synthesis	=	96%	=	99%
rpl5	Ribosome/protein synthesis	↗	+116%	↘	84%
rpl16	Ribosome/protein synthesis	↗	+124%	↘	81%
rrn18	Ribosome/protein synthesis	=	+104%	↘	82%
rrn26	Ribosome/protein synthesis	=	+106%	↘	61%
exp	Unknown	↗	+116%	=	96%
aox1a	Alternative oxidase	=	+106%	↘	37%
aox1d	Alternative oxidase	=	100%	↘	18%

Table 10: Effect of *nad9* mRNA knockdown on the level of other RNAs in 16-18 leaf stage *A. thaliana* plants – synthetic view

RT-qPCR analysis on total RNA. The data were compiled from Figures 42-46. ↘ indicates decrease, ↗ indicates increase, = indicates no change. The percentage value indicates the residual level as compared to control.

Gene	Location	*Rz1sdh3*-L-PKTLS	*Rz1atp9*-L-PKTLS
atp9	ATP synthase	= 107%	↘ 36%
nad1b	Complex I	↗ 116%	↘ 16%
nad1c	Complex I	↗ 119%	↘ 34%
nad2b	Complex I	↗ 116%	↘ 19%
nad5a	Complex I	= 104%	↘ 14%
nad5b	Complex I	= 106%	↘ 26%
nad6	Complex I	= 107%	↘ 8%
nad9	Complex I	= 106%	↘ 11%
cob	Complex II	= 109%	↘ 21%
cox1	Complex IV	= 107%	↘ 16%
atp1	ATP synthase	= 100%	↘ 20%
atp4	ATP synthase	= 102%	↗ 20%
aox1a	Alternative oxidase	= 106%	↘ 19%
aox1d	Alternative oxidase	= 100%	↘ 54%
rps3	Ribosome/protein synthesis	↗ 114%	↘ 30%
rps4	Ribosome/protein synthesis	↗ 116%	↘ 16%
rpl5	Ribosome/protein synthesis	↗ 116%	↘ 18%
rpl16	Ribosome/protein synthesis	↗ 124%	↘ 56%
rrn18	Ribosome/protein synthesis	= 104%	↘ 45%
exp	nuclear	↗ 116%	= 96%
pp2aa3	nuclear	↗ 124%	↗ 136%
tip	nuclear	↗ 130%	↗ 120%

Table 11: Effect of *atp9* mRNA knockdown on the level of other RNAs in 16-18 leaf stage *A. thaliana* plants – synthetic view

RT-qPCR analysis on total RNA. The data were compiled from Figures 47-50. ↘ indicates decrease, ↗ indicates increase, = indicates no change. The percentage value indicates the residual level as compared to control.

addressed by analyzing the response of the mitochondria, and the rest of the cell, to the knockdown of a single organelle transcript.

The impact of *nad9* and *atp9* mRNA knockdown was analyzed in detail by RT-qPCR. For the plants expressing the *Rz1nad9*-L-PKTLS ribozyme, we tested 32 RNAs other than *nad9*, 29 of mitochondrial origin and 3 of nuclear origin (summarized in Table 10). Of the 29 mitochondrial RNAs analyzed, 12 showed a substantial drop of more than 30%. The decrease of a whole series of mRNAs encoding proteins of the OXPHOS complexes, such as *nad1b, nad1c, nad4, nad5a, nad5b, cob, cox1, atp1, atp4* and *atp9*, suggested that a regulation system exists that would coordinate the expression of the mitochondrial genes encoding the subunits of the same complex or of different complexes. However, we noted that the mRNAs corresponding to the same complex did not necessarily all decrease. This was the case for example for complex I, for which *nad1b, nad1c, nad4, nad5a* and *nad5b* mRNAs decreased, but *nad2b, nad6* and *nad7* were not affected significantly. As to the plants expressing the *Rzatp9*-L-PKTLS ribozyme, we tested 23 RNAs other than *atp9*, 18 of mitochondrial origin and 5 of nuclear origin (summarized in Table 11). The results confirmed much of the data obtained with *nad9* knockdown. Of the 18 mitochondrial RNAs analyzed, all showed a substantial drop, with the exception of *atp4* that increased by 80%. Remarkably, the observations also highlighted RNA control mechanisms in mitochondria with relation to the plant developmental stage and culture conditions. Furthermore, in fully grown plants, a drop of the *nad9* or *atp9* target mRNA was observed one day after the beginning of the expression of the ribozyme, followed by a rise to near normal level the next day, and then a final generally higher knockdown from the third day. Such bounce back profiles suggested a transient defense reaction of the mitochondria against the knockdown of one of their essential mRNAs.

Going further in the analysis of RT-qPCR, we also observed very large variations in the level of some nuclear mRNAs after *nad9* or *atp9* knockdown in the mitochondria. This was particularly the case for the *aox* mRNAs encoding the alternative oxidase (AOX), a mitochondrial enzyme that provides an alternative pathway for the electrons in the respiratory chain when ATP synthesis fails. These observations in turn implied that the knockdown of a mitochondrial RNA triggered retrograde regulatory mechanisms.

The reasons for a decrease of the *aox* mRNAs are difficult to interprete. Indeed, one would have to assume that the knockdown of *nad9* or *atp9* in the mitochondria causes stress. The

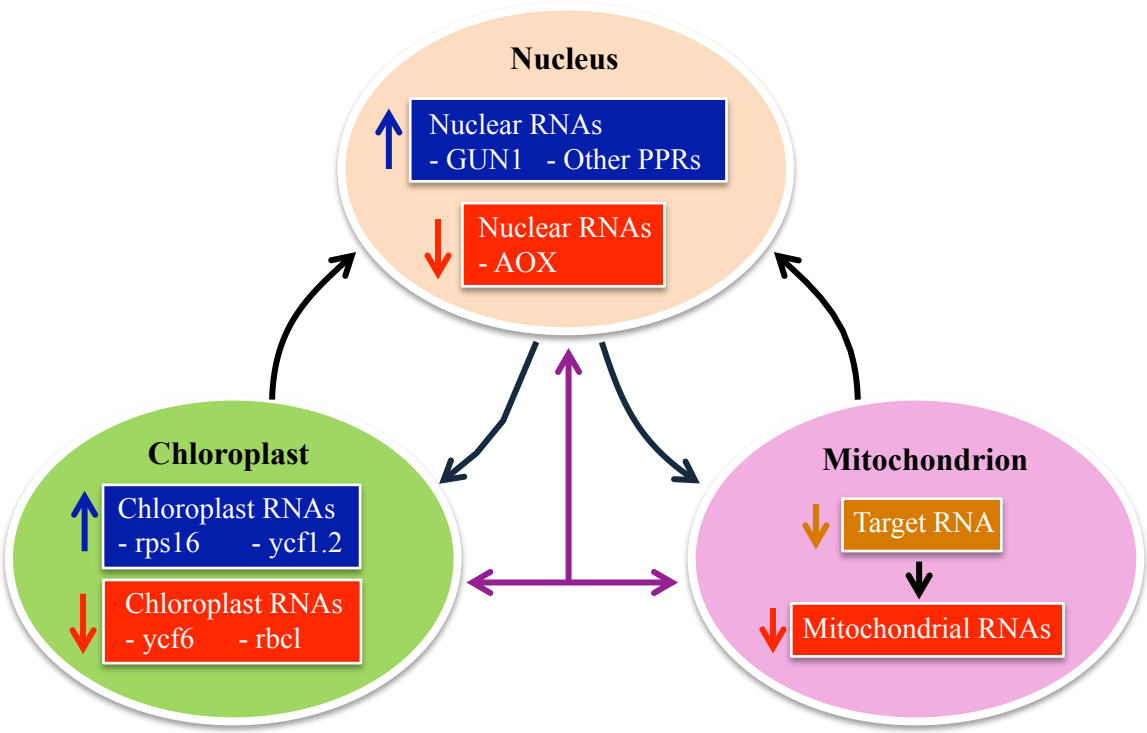


Figure 59: Genome coordination between cell compartments
Upon specific knockdown of a mitochondrial RNA, the level of other mitochondrial RNAs decreases, this affects positively or negatively nuclear gene expression, either directly or through the chloroplast, as we observed an increase in the level of GUN1. Altogether, these results imply that MRR exists in plants at the RNA level.

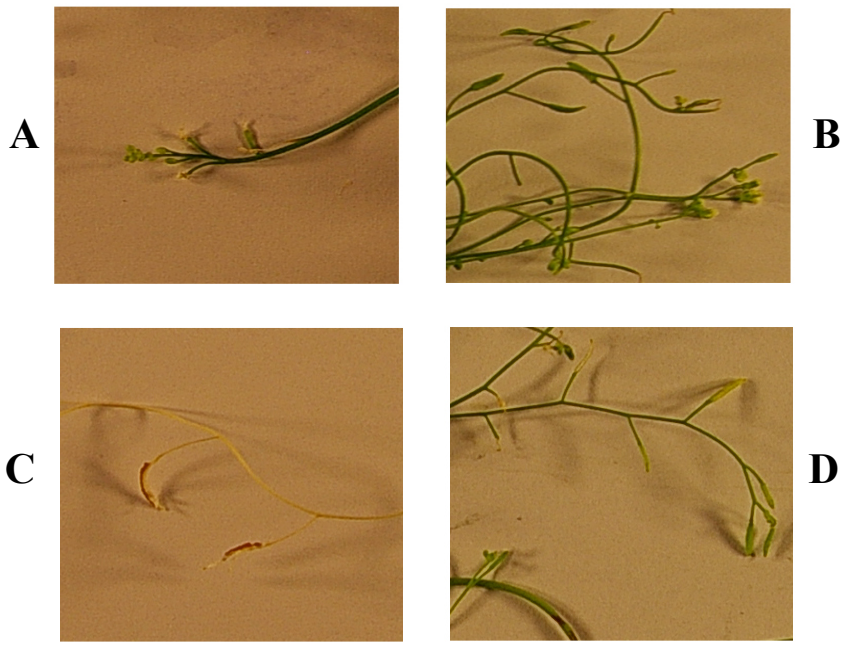


Figure 60: Phenotype analyses of the transformed *A. thaliana* plants
For ribozyme transgene induction, estradiol was added on after every three days. **A)** Plants expressing the *Rz1nad9*-L-PKTLS ribozyme **B)** Uninduced *Rz1nad9* transformants **C)** Plants expressing the *Rz1cox3*-L-PKTLS ribozyme **D)** Plants expressing the *Rzatp9*-L-PKTLS ribozyme.

expected effect would be an increase in the amount of *aox,* to compensate for a possible malfunction of the ATP synthase complex that would lead to an overload of the respiratory chain and ROS production. However, the level of *aox* mRNAs on the contrary decreases. Clifton *et al.*, (2006) proposed that *aox* serve to reprogram the cell metabolism based on potential stress encountered. In view of the changes in the level of *aox* in our experiments, we can hypothesize a more global effect on cell metabolism in transformed *A. thaliana* plants upon knockdown of *nad9* or *atp9* in the mitochondria.

To consolidate the data and generate a comprehensive view of the regulatory process, we began microarray analyses. The results obtained with RT-qPCR were mostly confirmed by our first microarray analyses, which showed a substantial response to the scale of the entire transcriptome. A notable exception was the *aox* mRNAs that were not affected. This discrepancy will have to be sorted out through further microarray assays, as the RT-qPCR results are very strong on that point and were confirmed in a number of independent samples or replicates. In addition to the negative effect of *nad9* knockdown on the level of many other mitochondrial RNAs, the rate of nearly 30 chloroplast transcripts was affected, negatively or positively, in the microarray pattern. Finally, the expression of more than 2500 nuclear genes was significantly altered, half down and the other half up. These results further reinforce and extend the idea of RNA level control mechanisms within mitochondria and between cell compartments (Figure 59). In particular, a conversation between the nuclear and mitochondrial genomes seems to exist. The data presented in this work do not yet allow to determine whether this regulation is the result of genetic or metabolic signaling but they point to expression synchronization.

Finally, in a more applied perspective, some of these manipulations of mitochondrial gene expression may cause a male-sterile phenotype, a relevant agronomical trait. As a preliminary test, our transgenic plants were grown on solid agar plates and estradiol with liquid medium was directly applied every three days to keep permanent high expression of the ribozymes. In our first tests of long-term transgene induction, the expression of the *Rz1nad9*-L-PKTLS ribozyme directed against the *nad9* mRNA seemed to stop the plants at the flowering stage (Figure 60A). On the contrary, uninduced transformed plants (Figure 60B) and plants expressing the ribozymes against *cox3* or *atp9* (Figure 60C and 60D) showed normal silique formation, excluding a negative effect of either the estradiol or the *in vivo* conditions. We are now generating *A. thaliana* transformants expressing the ribozymes

under a constitutive promoter instead of the estradiol-inducible promoter. With this system, it will be possible to study plant growth both under *in vitro* conditions and in greenhouse conditions directly on soil. A more detailed analysis of inflorescences and pollen will better characterize the phenotype.

MATERIALS AND METHODS

I. Materials

I.1 Plant materials

- Potato tubers *(Solanum tuberosum var. Bintje),* untreated against germination and coming from local production are used to prepare mitochondria (§ II.5.3). Tubers are stored at 25°C in the dark.

- Tobacco plants *(Nicotiana tabacum var. Samsun nn),* cultivated *in vitro* are used for leaf disk transformation with *Agrobacterium tumefaciens* (§ II.6.3). The transformed tobacco plants obtained are grown first *in vitro* and then in greenhouses.

- Arabidopsis plants *(Arabidopsis thaliana* ecotype *Colombia)* are used for agro-transformation by floral dip (§ II.6.2). The selected transformants are grown in greenhouses. *A. thaliana* plants are either grown on soil (16 h light and 8 h dark) in greenhouse or *in vitro* (16 h light and 8 h dark) on **germination medium** with appropriate antibiotics. Seeds grown on plates are stratified in the dark at 4°C for 2-4 days to synchronize the germination time.
Germination medium: 4.3 g/L micro and macroelements M0255 (DUCHEFA), 10 g/L agar, 1% (w/v) sucrose, pH 5.7

I.2 Cell cultures

- *N. tabacum* BY2 (from "Bright Yellow 2") suspension cell cultures are used for agrobacterium-mediated transformation (§ II.6.1). Cell culture is cultivated with agitation in the darkness at 25°C in **BY2 medium**. Once a week, 4 mL of suspension is transferred to 80 mL of fresh medium into 250 mL of conical flask.
BY2 medium: 4.3 g/L micro and macroelements M0221 (DUCHEFA), 1 mg/L thiamine, 200 mg/L KH_2PO_4, 0.2 mg/L 2,4D, 100 mg/L myoinositol, 3% (w/v) sucrose, pH 5.8

- *A. thaliana* suspension cell cultures are used for extraction of mitochondria (§ II.5.2). Cell culture is cultivated with agitation (120 rpm) under illumination at 25°C in **culture medium**. Once a week, 10 mL of suspension is transferred to 90 mL of fresh medium into 250 mL of conical flask.
Culture medium: 4.3 g/L micro and macroelements M0256 (DUCHEFA), 1 mg naphthalene acetic acid (NAA) (from 2 mg/mL stock in water), 50 µg kinetin (from 2 mg/mL stock in water), 3% (w/v) sucrose, pH 5.6

I.3 Hydroponic seedling cultures of *A. thaliana*

Hydroponic seedling cultures of *A. thaliana* are used for extraction of mitochondria (§ II.5.1). *A. thaliana* seeds are first sterilized. For this, about 200 seeds are placed in a 2 mL Eppendorf tube and 1 mL of 70% (v/v) ethanol is added. After incubation for 2 min with continuous stirring, the ethanol is removed and 1 mL of **sterilization solution** is added. The seeds are again put with continuous stirring for 15 min and then washed 5 times in sterile water. The seeds are then put in 100 mL of ½ M0231 (DUCHEFA) sterilized liquid medium. Seed germination takes place during culture under continuous stirring (120 rpm) and under continuous light at 24°C. Eight days old seedlings are used for the extraction of mitochondria.

Sterilization solution: 4% (w/v) Sodium Hypochlorate and 4% (v/v) Tween 20

I.4 Bacterial strains

- Escherichia coli

DH5α: *F'/endA1 hsdR17(rk$^-$mk$^+$) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(lacZYA-argF)$_{u169}$ (m80lacZΔM15)*

This *E. coli* strain is cultivated on **LB medium**.

- A. tumefaciens

The *A. tumefaciens* strains used are:

LBA4404 Rif^R cultivated on **LB medium.**

This strain is resistant to rifampicin (a chromosomal marker) as well as spectinomycin and streptomycin (markers on the disarmed Ti plasmid pAL4404) (Hoekema *et al.*, 1983).

GV3101 Rif^R cultivated on **LB medium.**

This strain is resistant to rifampicin, gentamycin and kanamycin. GV3101 carries a disarmed Ti plasmid that possesses the vir genes needed for T-DNA transfer, but has no functional T-DNA region of its own.

LB medium: 10 g/L bacto-trypone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5

I.5 Plasmid vectors

- pUCAP

This 2.7 kb plasmid derives from plasmid pUC18 following the addition of the rare restriction sites *Asc*I and *Pac*I into the cloning cassette. It contains the ampicillin resistance gene and, because of the position of the cloning cassette in the gene coding for the

N-terminal domain of the β-galactosidase, it permits the blue-white selection of transformed bacteria.

- pGEM®-T Easy Vector System I (Promega)

The pGEM®-T Easy vector of 3 kb is a high copy number plasmid used for cloning and sequencing. This vector system is designed for the cloning of PCR products. The vector is linearized and has 3´ terminal thymidine to both ends, this greatly improve the ligation efficiency of a PCR product into the plasmid by preventing recircularization of the vector.
This vector system contains both T7 and SP6 RNA polymerase promoters flanking the multiple cloning regions within the α-peptide coding region of the ß-galactosidase. Bacteria transformed with this vector are resistant to ampicillin. It allows the direct insertion of a DNA PCR fragment in the α-peptide coding region of the lacZ gene and therefore the blue/white selection on plates containing X-Gal and IPTG.

- pER8

The binary plasmid pER8 of 11.5 kb is used for transformation of *N. tabacum* cells, *A. thaliana* and *N. tabacum* plants *via A. tumefaciens*. This vector contains the cassette for inducible expression of transgene in higher plants. It expresses the XVE (for Lex-VP16-ER) chimeric transcription factor. The *trans*-activating activity of this factor is induced with estradiol (Zuo *et al.*, 2000). The presence of the spectinomycin resistance gene allows the selection of the transformed *E. coli* and agrobacteria. The hygromycin phosphotransferase gene (*hpt*) under the control of the promoter of the nopaline synthase gene (NOS), allows the selection of the transformed plant cells.

- pDONR™207

The plasmid pDONR™207 of 5.6 kb is the gateway donor vector from Invitrogen in the Gateway® cloning system. It confers resistance for gentamycin to transformed bacteria. It allows the recombination of DNA PCR fragments. This vector contains two sites of recombination, namely *attP1* and *attP2*, surrounding the gene encoding the ccdB protein. This protein triggers cell death of bacteria that integrate non-recombined vectors.

- pH2GW7

The plasmid pH2GW7 is a binary Gateway overexpression vector. It is the destination vector in the Gateway cloning system. This vector has a 35S Cauliflower Mosaic Virus (CaMV) promoter/terminator and a hygromycin-B resistance cassette (Karimi *et al.*, 2002).

This vector also contains the XVE inducible promoter for 17-β-estradiol inducible expression in plants.

I.6 Primers

Oligonucleotides are synthesized by Sigma-Aldrich and Eurofins. Primer sequences are presented in Tables 2-6. Their fusion temperatures are estimated by the following formula: Tm (°C) = 2 x (A+T) + 4 x (C+G).

I.7 Informatics tools

Sequence alignments

- Sequence alignments are realized with MacVector® (MacVector Inc.) by ClustalW Algorithm, suitable for calculation of homology between nucleotide sequences.

The major sites used for database and sequence alignments are:

- NCBI: http://www.ncbi.nlm.nih.gov/
- *A. thaliana* database TAIR: http://www.arabidopsis.org/

Data on mitochondrial genomes:

- http://megasun.bch.umontreal.ca/gobase/gobase.html
- http://www.mitomap.org/

The site used to draw graphical maps is:

- http://ogdraw.mpimp-golm.mpg.de/

OrganellarGenomeDRAW (OGDRAW) is convenient and user-friendly software tool available online to directly visualize circular genomes as clearly laid out, high-quality graphical maps. It produces clearly laid out, high resolution custom graphical maps of DNA sequences as stored in standard GenBank format entries. The program is specially optimized for the display of mitochondrial genomes (Lohse *et al.*, 2007). We provided the GenBank data as accession numbers.

Designing primers for quantitative PCR

The oligonucleotides used in quantitative PCR are designed from “Universal ProbeLibrary” (tinyurl.com/cowakpk) by using ProbeFinder software (Version: 2.49). For short sequences it does not design primers, so for them Primer 3 software (Untergasser *et al.*, 2012) is used. These softwares determine the sequences of primers used for amplification for a given sequence minimizing the structural effects and nonspecific amplifications.

Evaluating reference genes expression for RT-qPCR

- http://www.leonxie.com/referencegene.php?type=reference#

RefFinder is a user-friendly web-based comprehensive tool that is used to evaluate and screen reference genes. It displays the results from the currently available major computational programs (geNorm, Normfinder, BestKeeper, and the comparative ΔCt method) to compare and rank the tested candidate reference genes just in one step. It not only displays the individual ranking of each program but also assigns an appropriate weight to an individual gene based on the rankings from each program and calculates the geometric mean of their weights for the overall final ranking.

II. Methods

II.1 Techniques related to nucleic acids

II.1.1 Extraction of mitochondrial nucleic acids

To extract mitochondrial nucleic acids, 150 µL of **extraction buffer** are added to a quantity of mitochondria equivalent to 200 µg of proteins and then supplemented with the same volume of phenol. After vortexing during 5 minutes, the mix is spun 10 min at 15,000 g and the supernatant is precipitated 30 min at -20°C with 2.5 volumes of ethanol in the presence of 200 mM of NaCl. The pellet is then resuspended with an appropriate volume of water.

Extraction buffer: 1% (w/v) SDS, 1 mM EDTA, 10 mM Tris-HCl pH 7.5

II.1.2 Nucleic acid quantification

Nucleic acid solutions are quantified by using a NanoDrop device. To directly evaluate their concentrations, the NanoDrop uses the following correspondences:

An A_{260} of 1 corresponds to 50 µg/mL of double-stranded DNA

An A_{260} of 1 corresponds to 37 µg/mL of single-stranded DNA

An A_{260} of 1 corresponds to 40 µg/mL of RNA

II.1.3 Separation of nucleic acids on agarose gel

Nucleic acid molecules are separated as a function of their size by electrophoresis. The solution, supplemented with 1/5 of **loading buffer**, is loaded on a 1% (w/v) agarose gel in **0.5 x TAE buffer**. Electrophoresis is run at 75-150 Volts, depending on the gel size. Fragments are then visualized under UV by the ethidium bromide dissolved in the gel (0.5 µg/µL). DNA size is estimated using a scale DNA marker (Fermentas®) from 100 bp to 10 kb.

0.5 x TAE buffer: 0.5 mM EDTA, 20 mM Tris-Acetate pH 8.0

Loading buffer: 50% (w/v) glycerol, 1% (w/v) SDS, 1 mM EDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue

II.1.4 Purification of a nucleic acids by gel filtration

After an enzymatic reaction or elution, nucleic acids may be purified by gel filtration on a Sephadex G-50. For this, a column of Sephadex G-50 beads is prepared in a 1 mL syringe. The column is dried by centrifugation at 200 g for 2 min. The solution containing nucleic acids (maximum volume 100 uL) is then loaded onto the column which is centrifuged again under the same conditions. Nucleic acids separated from small molecules is recovered in the excluded volume of the column. If necessary, it can be concentrated by vacuum evaporation.

II.2 Specific techniques for DNA

II.2.1 Extraction of total DNA

Approximately 3 g of plant tissues, cell cultures or callus are ground in liquid nitrogen and transferred into a 1.5 mL microcentrifuge tube containing 1 mL of **extraction buffer**. The tubes are placed for 45 min at 65°C with occasional swirling. After adding equal volumes of chloroform, they are mix gently by inverting and centrifuged at 3000 g for 7 min. Supernatant is mixed gently with equal volumes of isopropanol. After incubating 10 min at room temperature, the tube is centrifuged at 16,000 g for 5 min at 4°C. The pellet is then washed with 70% (v/v) ethanol, centrifuged for 25 min at 16,000 *g* at 4°C and dissolved in 30-100 µL of autoclaved and distilled water.

Extraction buffer: 100 mM Tris HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% CTAB

II.2.2 Purification of DNA by phenol-chloroform extraction

To eliminate contaminant proteins from DNA solutions, one volume of phenol/chloroform (1/ 1) is added. The mix is vortexed during 3-4 min and centrifuged 5-10 min at 16,000 g. The aqueous phase is supplemented with one volume of chloroform, vortexed during 3-4 min and centrifuged 5-10 min at 16,000 g. The aqueous phase is then precipitated at -20°C for at least 2 hours with 2.5 volumes of ethanol in the presence of 0.2 M NaCl. After centrifugation during 20 min at 16,000 g, the DNA pellet is resuspended in water or an appropriate buffer.

II.2.3 DNA purification after gel electrophoresis

After migration (§ II.1.3), slices containing the DNA fragments of interest are excised from the agarose gels. DNA is then eluted with a kit (Nucleospin System®, Macherey-Nagel) based on the use of a silica membrane spin column.

II.2.4 DNA separation under denaturing conditions

When it is necessary to use DNA separated under denatured conditions as template for enzymatic reaction, denaturing gels containing NaOH should be avoided. For such experiments, denatured DNAs are separated on a native agarose gel. DNA samples, boiled for 5 min in **denaturing buffer** and immediately transferred on ice for 2-5 min, are loaded on a 1% (w/v) agarose gel in **0.5 x TAE buffer**. Electrophoresis is run at 100-120 Volts depending on the gel size. Fragments are then visualized under UV by the ethidium bromide dissolved in the gel (0.5 µg/µL).

Denaturing buffer (6x): 96% formamide, 20 mM EDTA, 0.05% (w/v) bromocresol green, 0.05% (w/v) xylene cyanol

0.5 x TAE buffer: 0.5 mM EDTA, 20 mM Tris-Acetate pH 8.0

II.2.5 Amplification by Polymerase Chain Reaction (PCR)

PCR sets in motion a chain reaction in which the DNA template is exponentially amplified by a Taq polymerase, using two primers specific to the ends of the interest fragment.

For basic amplification, the Dynazyme Taq polymerase (FINNZYMES) is used. However, to amplify DNA fragments that have to be sequenced, the "Expand High Fidelity" polymerase (ROCHE) is used because of its better pairing fidelity during elongation.

A 50 µL basic PCR set up requires:

DNA template (100 pg of plasmid DNA or 100 ng of mtDNA)

1 µM of each primer

200 µM of each dNTP

5 µL of 10X PCR buffer

2.5 units of *Taq* DNA polymerase

A Master Mix system (Roche) is also used.

For 50 µL PCR set up:

DNA template (100 pg of plasmid DNA or 100 ng of mtDNA)

1 µM of each primer

25 µL of Master Mix (containing *Taq* polymerase, dNTPs and PCR buffer)

The reaction is carried out for 30 cycles in a Gene Cycler automator (BioRad):

Step 1 (1 cycle): 95°C, 2 min (template denaturation)

55°C, 45 sec (primer hybridization)

72°C, 45 sec (DNA elongation)

Step 2 (29 cycles): 95°C, 45 sec (template denaturation)

55°C, 45 sec (primer hybridization)

72°C, 45 sec (DNA elongation)

Step 3 (1 cycle): 72°C, 10 min (DNA elongation)

II.2.6 Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) is performed in 384-well optical plates on a BioRad i-cycler apparatus using 5 µL of PCR master mix containing 480 SYBER® Green I fluorescent reporter (Roche) with 2.5 µM forward and reverse specific primers. DNA or cDNA template and distilled H_20 are added to a total volume of 10 µL. For each sample, PCR is performed in triplicate using fixed amounts of DNA or cDNA template (§ II.3.6). PCR is carried out using the following conditions: pre-heating at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C and 15 sec at 72°C. Melting curves of PCR reactions are checked to insure the quality of PCR reaction and also to avoid any DNA contamination if cDNA is being used as template. The threshold cycle value (CT) is set so that the fluorescent signal is above the baseline noise but as low as possible in the exponential amplification phase.

II.2.7 Microarray analysis

Microarray analysis is carried out at the Unité de Recherche en Génomique Végétale (URGV, Evry, France), using the CATMAv6.2 array based on Roche-NimbleGen technology. A single high density CATMAv6.2 microarray slide contains twelve chambers, each containing 270 000 primers representing all the *A. thaliana* genes: 30834 probes corresponding to TAIRv8 annotation (including 476 probes of mitochondrial and chloroplast genes) + 1289 probes corresponding to EUGENE software predictions. Moreover, it included 5352 probes corresponding to repeat elements, 658 probes for miRNA/MIR, 342 probes for other RNAs (rRNA,tRNA, snRNA, soRNA) and finally 36 controls. Each long primer is triplicate in each chamber for robust analysis and in both forward and reverse orientation. Two independent biological replicates are produced. For each biological repetition, total RNA is extracted using TRI-reagent (§ II.3.1) *A. thaliana* plants, treated twice with RNase-free DNase (§ II.3.3) and purified using NucleoSpin® RNA

Plant kit (Macherey-Nagel). All the RNA samples are sent to the URGV (Evry, France) for the further steps. There they checked for quality control, depleted the ribosomal RNAs and did reverse transcription with random hexamer primer containing T7 promoter followed by *in vitro* transcription with T7 promoter. The labeling of cRNAs with Cy3-dUTP or Cy5-dUTP (Perkin-Elmer-NEN Life Science Products) and the hybridization to the slides are performed as described in Lurin *et al.*, (2004). Two micron scanning is performed with InnoScan900 scanner (Innopsys^{R}, Carbonne, FRANCE) and raw data are extracted using Mapix^{R} software (Innopsys^{R}, Carbonne, FRANCE). For each comparison, one technical replicate with fluorochrome reversal (dye-swap/dye-switch) is performed for each biological replicate (*i.e.* 4 hybridizations per comparison).

II.2.7.1 Statistical Analysis of Microarray Data

Experiments are designed with the statistics group of the Unité de Recherche en Génomique Végétale. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). For each array, a global intensity-dependent normalization using the loess procedure (Yang *et al.*, 2002) is performed to correct the dye bias. The differential analysis is based on the log-ratios averaging over the duplicate probes and over the technical replicates. Hence the numbers of available data for each gene equals the number of biological replicates and are used to calculate the moderated t-test (Smyth, 2004). Under, the null hypothesis, no evidence that the specific variances vary between probes is highlighted by Limma and consequently the moderated t-statistic is assumed to follow a standard normal distribution. To control the false discovery rate, adjusted p-values found using the optimized FDR approach (Storey and Tibshirani, 2003) are calculated. We considered as being differentially expressed the probes with an adjusted p-value ≤ 0.05. Analysis is done with the R software. The function SqueezeVar of the library limma has been used to smooth the specific variances by computing empirical Bayes posterior means. The library kerfdr has been used to calculate the adjusted p-values.

II.2.7.2 Data Deposition

Microarray data from this manuscript are deposited at CATdb (http://urgv.evry.inra.fr/CATdb/; Project: RS12-04_RNAnonPolyA, Gagnot *et al.*, 2008) according to the "Minimum Information About a Microarray Experiment" standards (Brazma *et al.*, 2001).

II.2.8 Cloning techniques

II.2.8.1 Preparation of plasmid DNA

After overnight culture of bacteria at 37°C in **LB medium**, plasmidic DNA is extracted from 2 mL of culture using the NucleoSpin system® (Macherey-Nagel) kit. By this method, 40 µg of plasmid can be obtained. The technique is based on the optimization of purification by alkaline lysis (Birnboim and Doly, 1979), DNA is eluted through micro-spin columns containing silica gel.

LB medium: 10 g/L bacto-trypone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5

II.2.8.2 Digestion of DNA by restriction enzymes

To linearize or fragmentize DNA, restriction enzymes such as *Xho*I and *Spe*I, are used. Digestions are carried out during 1-2 h at 37°C in the appropriate buffer given by the supplier. One unit of enzyme is added to 1 µg of DNA.

II.2.8.3 Ligation of two DNA fragments

Two fragments with sticky or cohesive ends are ligated using the T4 DNA ligase (Fermentas). For this, 15 µL of reaction contains: 3 µL of **5x ligation buffer**, 2 units of ligase, 10-20 ng of linear vector and the fragment to insert with a stoichiometric molar ratio insert/vector comprised between 3 and 5. The reaction is developed at room temperature during 2 hours and 1 to 5 µL are used for transformation of competent bacteria(§ II.2.8.7, § II.2.8.8 and § II.2.8.9).

5 x ligation buffer: 250 mM Tris-HCl pH 7.5, 25% PEG 8,000, 50 mM $MgCl_2$, 5 mM ATP and 5 mM DTT

II.2.8.4 Cloning by pGEM®-T Easy Vector System I

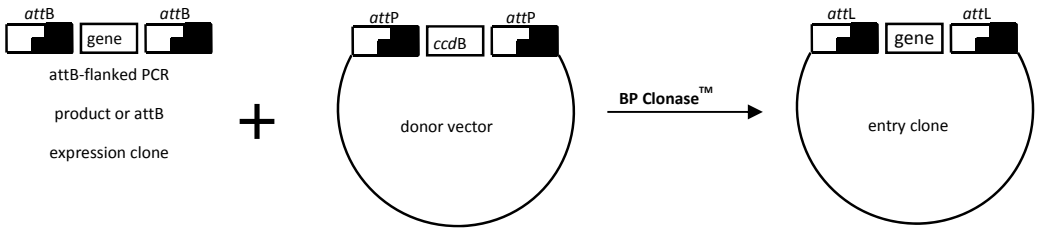
To directly clone a PCR fragment, the pGEM®-T Easy Vector System I (Promega) kit is used. For this, 3 µL (15 ng) of PCR fragment (§ II.2.5) purified from gel (§ II.2.2) is incubated overnight at 15°C or 3 hours at room temperature in the presence of 50 ng of pGEM-T Easy Vector, 3 units of T4 DNA ligase and **rapid ligation buffer** in a final volume of 10 µL. Transformation of competent bacteria is done as described below (§ II.2.8.7 and § II.2.8.8).

2 x rapid ligation buffer: 60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% PEG

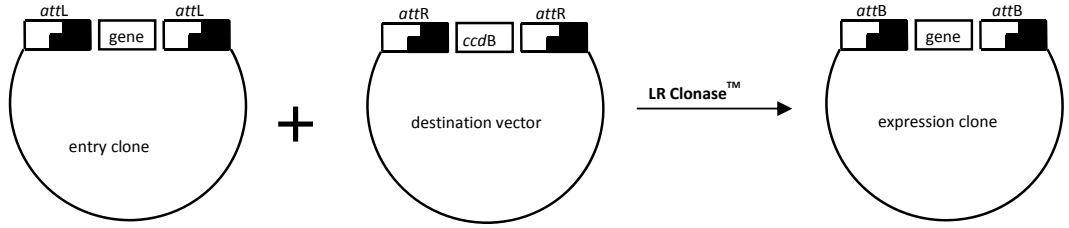
II.2.8.5 Recombinational cloning with Gateway® vectors

Gateway® (Invitrogen) is a cloning technology that takes advantage of the site-specific recombination properties of bacteriophage lambda to move a gene of interest into multiple vector systems. This cloning technique is divided in two main steps:

- Genes of interest are cloned using the BP reaction into Gateway® entry vectors (e.g. pDONR207) to create entry clones.



- Expression clones are generated by performing LR recombination reactions between entry clones and Gateway® destination vectors (e.g. pB7FWG2.0)



The BP reaction is a recombination between *attB* and *attP* sequences mediated by the BP clonase™ enzyme. The reaction is performed between an *attB*-flanked DNA fragment and an *attP*-containing donor vector (pDONR207) to generate an entry clone. It results in an entry clone that contains the gene of interest flanked by *attL* sequences. In practice, 1 µL of *attB*-flanked PCR product is incubated 1 h at RT in presence of 1 µL of pDONR vector (150 ng), 1 µL of BP clonase™ (Invitrogen), 1 µL of 5X BP clonase™ buffer (Invitrogen) and **TE buffer** to a final volume of 5 µL. After incubation, 1 µL of proteinase K (Invitrogen) (2 µg/µL) is added and incubated 10 min at 37°C. The reaction mixture can directly be used for transformation of competent cells (§ II.2.8.7 and § II.2.8.8).

The LR reaction is a recombination between *attL* and *attR* sequences mediated by the LR clonase™ enzyme. The reaction is performed between the entry clone (*attL*) and a destination vector (*attR*). In practice, 1 µL of entry clone (150 ng) is incubated 1 h at RT in presence of 1 µL of destination vector (150 ng), 1 µL of LR clonase™ (Invitrogen), 1 µL of

5X LR clonase™ buffer (Invitrogen) and TE buffer to a final volume of 5 µL. After incubation, 1 µL of proteinase K (Invitrogen) at 2 µg/µL is added and incubated 10 min at 37°C. The reaction mixture can directly be used for transformation of competent agrobactaria (§ II.2.8.9).

TE buffer: 1 mM EDTA, 10 mM Tris-HCl pH 8.0

II.2.8.6 Traditional cloning by restriction enzymes and ligase

Restriction enzyme DNA digestions are performed following manufacturer recommendations (§ II.2.8.2). After digestion, DNA fragments of interest are eluted from agarose gels after electrophoresis (§ II.2.3), using the "Extract II" kit (Macherey-Nagel). DNA fragments of interest obtained by restriction enzyme reactions are cloned into digested vectors using the "Rapid DNA Ligation Kit" (Fermentas) as described above (§ II.2.8.3).

II.2.8.7 Transformation of *E. coli* by electroporation

For transformation of bacteria by electroporation, 1 µL of ligation (§ II.2.8.3) is added to 40 µL of competent bacteria prepared as described below and incubated 1 min on ice. Electroporation is done in a BioRad electroporator (2.5 kV, 25 µF, 200 W). Electroporated bacteria are resuspended in 1 mL of **LB medium**. After incubation at 37°C for 30 min allowing the regeneration of bacterial wall and expression of antibiotic resistance genes coded by the vector, bacteria are spread on LB medium containing 1.5% (w/v) agar and the appropriate antibiotic. As a selection marker for bacteria transformed with some recombinant vectors, IPTG (0.08 mM) and Xgal (10 µg/mL) are used for blue/white selection on Petri plates. After one night at 37°C, colonies containing the recombinant plasmid are white.

Preparation of competent bacteria for electroporation: *E. coli* (DH5α line), are spread on **LB medium** containing 1.5% (w/v) agar and nalidixic acid (30 µg/mL) as antibiotic. Colonies are obtained after an overnight culture at 37°C. A preculture is started at 37°C from a single colony in 20 mL of LB medium with nalidixic acid (30 µg/mL). Five mL of this overnight preculture is added to 500 mL of LB medium. Bacteria are cultivated at 37°C until obtaining an absorbance of 0.5-0.8 at 600 nm. Then, the culture is incubated on ice during 30 min and centrifuged twice for 5 min at 5,000 g with two resuspensions in sterile cold water (first with 500 mL and then with 250 mL). After a third centrifugation of 5 min at 5,000 g, the pellet of bacteria is taken in 40 mL of 10% glycerol pre-cold at 4°C, centrifuged and concentrated in 4 mL of 10% glycerol pre-cold at 4°C. The suspension of

bacteria is then aliquoted in 40 µL fractions, frozen in liquid nitrogen and conserved at -80°C. This stock can be used for about 6 months.

LB medium: 10 g/L bacto-trypone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5

II.2.8.8 Transformation of *E. coli* by heat shock

For transformation of bacteria by heat shock, 5 µL of ligation is added to 150 µL of competent bacteria prepared as described below and incubated 30 min on ice. The heat shock is induced by an incubation of 45 sec at 42°C. After addition of 1 mL **LB medium**, the whole is incubated 1 h at 37°C allowing the regeneration of bacterial wall and expression of antibiotic resistance genes coded by the vector. Bacteria are spread on LB medium containing 1.5% (w/v) agar and the appropriate antibiotic. As a selection marker for bacteria transformed with some recombinant vectors, IPTG (0.08 mM) and Xgal (10 µg/mL) are used for blue/white selection on Petri plates. After one night at 37°C, colonies containing the recombinant plasmid are white.

LB medium: 10 g/L bacto-trypone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5

Preparation of competent bacteria for heat shock: *E. coli* (DH5α line), are spread on **LB medium** containing 1.5% (w/v) agar and nalidixic acid (30 µg/mL) as antibiotic. Colonies are obtained after an overnight culture at 37°C. A preculture is started at 37°C from a single colony in 20 mL of LB medium with nalidixic acid (30 µg/mL). Two mL of this overnight preculture is added to 200 mL of LB medium. Bacteria are cultivated at 37°C until obtaining an absorbance of 0.5-0.8 at 600 nm. Then, the culture is centrifuged 10 min at 5,000 g. The pellet is resuspended in 60 mL of **buffer I** and incubated 15 min on ice. After centrifugation 10 min at 5,000 g, the pellet of bacteria is taken in 8 mL of **buffer II**. The suspension of bacteria is then aliquoted in 150 µL fractions, frozen in liquid nitrogen and conserved at -80°C.

LB medium: 10 g/L bacto-trypone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5

Buffer I: 100 mM $RbCl_2$, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 500 µM $LiCl_2$, 15% (v/v) glycerol, 35 mM potassium acetate, pH 5.8

Buffer II: 10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 7.5% (v/v) glycerol, pH 7

II.2.8.9 Transformation of *A. tumefaciens* by electroporation

For transformation of *A. tumefaciens* (LBA 4404 or GV 3101 strain) by electroporation, 1 µL of ligation is added to 40 µL of competent bacteria prepared as cited below. Electroporation is done in a BioRad Electroporator (2.5 kV, 25 µF, 200 W). Treated bacteria

are resuspended in 1 mL of **LB medium**. After incubation at 28°C for one hour allowing the regeneration of bacterial wall and expression of antibiotic resistance genes coded by the vector, bacteria are spread on LB medium containing 1.5% (w/v) agar, 25 µg/mL of rifampicine and 50 µg/mL of spectinomycin. Bacteria are incubated at 28°C for about 48 hours until colonies are formed.

LB medium: 10 g/L bacto-trypone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5

Preparation of competent agro-bacteria for electroporation: *A. tumefaciens* (LBA 4404 or GV 3101 strain), are spread on **LB medium** containing 1.5% (w/v) agar and 25 µg/mL rifampicine. Colonies are obtained after two days of culture at 28°C. A preculture is started from only single colony in 20 mL of **LB medium** containing rifampicine (25 µg/mL) and 2 mM $MgSO_4$. Five mL of this preculture is inoculated in 500 mL of LB medium containing rifampicine (25 µg/mL) and 2 mM $MgSO_4$. Bacteria are cultivated at 28°C for about 36 hours until obtaining an absorbance of 1 at 600 nm. Then the cells are put on ice for 30 min and centrifuged twice at 4,000 rpm for 5 min at 4°C with two resuspensions in 400 mL of sterile cold water. Finally, the pellet of bacteria is resuspended in 20 mL of 10% glycerol, centrifuged and concentrated in 2 mL of 10% glycerol. The suspension of bacteria is then aliquoted in 50 µL fractions, frozen in liquid nitrogen and conserved at -80°C.

LB medium: 10 g/L bacto-trypone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5

II.2.9 Sequencing of DNA

The constructs prepared are verified by sequencing using an automatic sequencer (Applied Biosystems, IBMP sequencing platform). The sequencing method used derived from that of Sanger *et al*. (1992). The alignment of sequences is done with the MacVector® software. We used primers m13Fw (5' CGCCAGGGTTTTCCCAGTCGAC 3') and m13Rv (5' TCACACAGGAAACAGCTATGAC 3').

II.3 Specific techniques for RNA

II.3.1 Extraction of total RNA

To extract total RNA from whole plants, the tissues are ground to a fine powder with liquid nitrogen after adding 1 mL of Trizol reagent (Invitrogen) per 100 mg sample. After grinding, 200 µL of chloroform per mL of Trizol reagent are added and the mixture is transferred into a tube to vortex for 2 min. An incubation at room temperature for 5 min is followed by a centrifugation at 16,000 *g* for 15 min at 4°C and the upper phase of the sample is mixed with 500 µL of isopropanol per mL of Trizol reagent. After incubating

10 min at room temperature, the tube is centrifuged at 16,000 *g* for 5 min at 4°C. The pellet is then washed with 70% (v/v) ethanol, centrifuged for 25 min at 16,000 *g* at 4°C and dissolved in 30-100 µL of autoclaved, distilled and RNase-free water.

II.3.2 RNA extraction from BY2 cell culture or from isolated mitochondria

Two mL of cell cultures or isolated mitochondria corresponding to 1 mg of proteins are spun down at 14,000 g for 5 min. Obtained pellet is resuspended in 1 mL of Trizol (Sigma TRI-reagent®) solution and leaved at RT for 5 min. To remove insoluble material from homogenates, samples are centrifuged at 12,000 g for 10 min at 4°C. Then 200 µL of chloroform is added to the supernatant and the tube is leaved for 10 min at room temperature. Aqueous phase containing RNAs are separated by centrifugation at 12,000 g for 15 min at 4°C. RNAs are precipitated by adding of 600 µL of isopropanol to the aqueous phase. After centrifugation at 12,000 g for 10 min, the pellet is washed by 75% (v/v) ethanol. After drying on ice for 5-10 min, RNAs are dissolved in sterile water.

II.3.3 Elimination of DNA from RNA samples

Before running reverse transcription (§ II.3.6), RNA samples are treated with RNase free DNase to eliminate contamination by genomic DNA. One µg of RNAs are resuspended in **reaction buffer** and 1 unit of DNase 1, RNase free enzyme (Thermo Scientific) is added. Upon incubation at 37°C for 45-60 min, phenol-chloroform extraction is performed to eliminate the enzyme. Alternatively ethanol precipitation can also work.

Two successive DNase treatments are necessary to eliminate the totality of DNA.

10X reaction buffer: 100 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$ and 1 mM $CaCl_2$

II.3.4 *In vitro* transcription of DNA

The RiboMAX^{TM} kit (Promega) is used for the reactions of DNA *in vitro* transcription under the control of the T7 RNA polymerase promoter. Transcription is run for 2 hours at 37°C in a reaction volume of 20 µL containing 1-2 µg of linearized DNA (PCR product or plasmid DNA), 4 µL of **5X T7 transcription buffer**, 6 µL of rNTP (25 mM each) and 2 µL of the T7 Enzyme Mix.

To generate radioactive transcripts, only the amount of rNTP varies: 2 µL of rNTP (ATP, GTP and CTP at 25 mM and UTP at 2.5 mM) and 40 µCi of [α^{32}P]UTP (3000 Ci/mmol) are introduced into the reaction.

Alternatively, transcription is run in the presence of 3 mM guanosine, so as to generate 5' ends without a phosphate. The transcripts are subsequently labeled at the 5' end with [γ^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase.

In all cases, after synthesis, 3 units of RNase-free DNase are added and the volume is completed to 50 µL. An incubation at 37°C for 15 min is required to digest the DNA. The reaction mixture is passed through Sephadex G-50 column to eliminate the unincorporated radioactive nucleotides (§ II.1.4).

5X T7 transcription buffer: 400 mM HEPES-KOH pH 7.5, 120 mM $MgCl_2$, 10 mM spermidine, 200 mM DTT

II.3.5 Fractionation of RNA by polyacrylamide gel electrophoresis

RNA samples, completed with one volume of **STOP buffer**, are loaded on 8% (w/v) polyacrylamide gels (acrylamide/bisacrylamide ratio: 19/1) in **1X TEB buffer** containing 7 M urea. The electrophoresis is run at 150 V using 1X TEB buffer. Upon migration, RNAs are visualized under UV light after soaking the gel in an ethidium bromide solution (0.5 g/mL) for 10 min. For radioactive RNAs, the gel is incubated under shaking for 30 min in a **fixing solution** and then dried for one hour at 80°C under vacuum. The dried gel is submitted to autoradiography.

1X TEB buffer: 90 mM Tris, 2.5 mM EDTA, 90 mM boric acid

STOP buffer: 95% deionized formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol

Fixing solution: 10% (v/v) acetic acid, 10% (v/v) ethanol

II.3.6 Reverse transcription (RT)

Reverse transcription is performed using four different conditions developed below.

II.3.6.1 RT using Super Script™ III Reverse Transcriptase

The protocol recommended by the manufacturer (Invitrogen) is followed. For the reaction, 300 ng to 500 ng of total RNA are added to 250 ng of random primers along with 1 µL of dNTP mix (10 mM each). Then the volume is completed at 13 µL by adding sterile distilled water. The mixture is incubated at 65°C for 5 min and immediately chilled on ice for at least 1 min. Four µL of **First-Strand Buffer**, 1 µL of a 100 mM DTT solution, 40 units of RNase OUT (Invitrogen) and 200 units of **Super Script™ III Reverse Transcriptase** are then added. The samples are incubated for 5 min at 25°C, followed by one hour at 55°C. By

incubating at 70°C for 15 min, the reaction is inactivated. The samples are directly used for PCR (§ II.2.5) or qPCR (§ II.2.6) reactions.

First-Strand Buffer: 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$ **Super Script™ III Reverse Transcriptase, 200 U/µL in:** 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% (v/v) Nonidet P-40, 50% (w/v) glycerol

II.3.6.2 RT using RevertAid™ H Minus First Strand cDNA Synthesis Kit

The protocol recommended by the manufacturer (Fermentas) is followed. For the reaction, 300 ng to 500 ng of total RNA are added to 250 ng of random hexamer primers. Then the volume is completed at 12 µL by adding sterile distilled water. The mixture is incubated at 65°C for 5 min and immediately chilled on ice for at least 1 min. Four µL of **5 x reaction buffer**, 1 µL of a 100 mM DTT solution, 1 µL of 10 mM dNTP mix (10 mM each) and 20 units of RiboLock™ RNase Inhibitor (Fermentas) are then added along with 200 units of RevertAid™ H Minus Reverse Transcriptase (Fermentas). The samples are incubated for 5 min at 25°C, followed by one hour at 42°C. By incubating at 70°C for 5 min, the reaction is inactivated. The samples are directly used for PCR (§ II.2.5) or qPCR (§ II.2.6) reactions.

5X reaction buffer: 250 mM Tris-HCl pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT

II.3.6.3 RT using RevertAid™ Premium First Strand cDNA Synthesis Kit

The protocol recommended by the manufacturer (Fermentas) is followed. For the reaction, 300 ng to 500 ng of total RNA are added to 250 ng of random hexamer primers. Then the volume is completed at 12 µL by adding sterile distilled water. The mixture is incubated at 65°C for 5 min and immediately chilled on ice for at least 1 min. Four µL of **5 x reaction buffer**, 1 µL of a 100 mM DTT solution, 1 µL of 10 mM dNTP mix (10 mM each) and 20 units of RiboLock™ RNase Inhibitor (Fermentas) are then added along with 200 units of RevertAid™ Premium Reverse Transcriptase (Fermentas). The samples are incubated for 10 min at 25°C, followed by 30 min at 50°C. By incubating at 85°C for 5 min, the reaction is inactivated. The samples are directly used for PCR (§ II.2.5) or qPCR (§ II.2.6) reactions.

5X reaction buffer: 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT.

II.3.6.4 RT using GoScript™ Reverse Transcriptase Kit

The protocol recommended by the manufacturer (Promega) is followed. For the reaction, 0.5 to 1 µg of total RNA are added to 250 ng of random hexamer primers. Then the volume is completed at 5 µL by adding sterile distilled water. The mixture is incubated at 70°C for 5 min and immediately chilled on ice for at least 1 min. Four µL of GoScript™ 5x reaction

buffer (Promega), 3.2 µL of a 25 mM $MgCl_2$, 1 µL of PCR nucleotide mix (final concentration 0.5 mM each dNTP) and 20 units of Recombinant RNasin® Ribonuclease Inhibitor (Promega) are then added along with 160 units of GoScript™ Reverse Transcriptase (Promega). The samples are incubated for 5 min at 25°C, followed by one hour at 42°C. By incubating at 70°C for 15 min, the reaction is inactivated. The samples are directly used for PCR (§ II.2.5) or qPCR (§ II.2.6) reactions.

II.3.7 *In vitro* ribozyme activity test

Ten µL of reaction mixture comprise 0.5 µL of 1 M Tris-HCl pH 7.5, 1 µL of 150 nM target RNA, 1 µL of [α^{32}P]-labeled target RNA (30,000 cpm) (§ II.3.4) and 1 µL of 1.5 µM ribozyme (§ II.3.4). The reaction mixture is incubated for 2 min at 75°C for denaturation and then allowed to cool down slowly to 25°C over a period of 3-4 hours to renature the target and ribozyme and allow the formation of the complex. The reaction is carried out adding different amounts of $MgCl_2$ to have final concentration of 0-50 mM. The reaction mixture is subsequently incubated for 1-2 hours at room temperature. After this, one volume of **STOP buffer** is added and the samples are run on an 8% (w/v) polyacrylamide gel (§ II.3.5). Finally, the gel is put under autoradiography.

STOP buffer: 95% deionized formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol

II.3.8 Northern blotting and hybridization with a radioactive probe

II.3.8.1 RNA fractionation on agarose gel

For northern blotting, RNA samples are fractionated by electrophoresis on 1.2% (w/v) denaturing agarose gels containing 5% (v/v) formaldehyde solution and **1X MOPS solution**. The gel is placed in **migration buffer** and loaded with 1-5 µg of RNA supplemented with **loading buffer**. Electrophoresis is run at 100 V and RNAs are visualized under UV light by the ethidium bromide (0.5 µg/mL) dissolved in the gel.

20X MOPS Solution: 0.4 M MOPS pH 7.0, 0.1 M sodium acetate, 20 mM EDTA

Migration buffer: 1X MOPS, 5% (v/v) formaldehyde solution

Loading buffer: 50% (v/v) formamide, 20% (v/v) formaldehyde solution, 1X MOPS, 10% (v/v) glycerol, 25 ng/µL ethidium bromide, 0.05% (w/v) bromophenol blue

II.3.8.2 RNA transfer onto nitrocellulose and fixing

After electrophoresis, the gel is washed 2-3 times with distilled water for 5-10 min each time and then equilibrated with **10X SSC transfer buffer**. RNAs are transferred by

capillarity from the gel onto a Hybond-N^+ (Amersham) nitrocellulose membrane. For this, the gel is put upside down on a thick blotting paper soaked in 10X SSC transfer buffer. The nitrocellulose membrane, followed by 3 sheets of thin blotting papers presoaked in 10X SSC transfer buffer are stacked. A layer of paper towels followed by a glass plate and some weight are finally added. The transfer runs overnight. The next day, to fix the RNAs, the nitrocellulose membrane is put on a Whatmann paper soaked with 2X SSC and UV cross-linked in Stratalinker® (120 kilojoules).

10X SSC transfer buffer: 1.5 M NaCl, 0.15 M sodium citrate

II.3.8.3 Preparation of the labeled probe by random priming

To prepare the labeled probe, the DECAprime kit (Ambion) is used. Twenty five ng of DNA in 11.5 µL of nuclease-free water are added to 2.5 µL of decamer solution. The denaturation is done by incubation of the mixture at 100°C for 4 min and immediate chilling on ice. Then 5 µL of 5X reaction buffer without dCTP, 50 µCi of [α^{32}P]dCTP (3000 Ci/mmole) and 1 µL of exo Klenow enzyme are added. Details on the composition of the 5X reaction buffer are not given by the manufacturer. The tube is incubated at 37°C for 10 min. To stop the reaction, 1 µL of 0.5 M EDTA is added. The volume is brought to 50 µL with nuclease-free water and the mixture is passed through a Sephadex G-50 column to eliminate the unincorporated radioactive nucleotides (§ II.1.4).

II.3.8.4 Hybridization

The membrane carrying the RNAs is placed in a hybridization tube and 10 mL of **hybridization solution** are added. Pre-hybridization is run for 1 hour at 65°C in a hybridization oven with constant rotation. The radioactively labeled probe (§ II.3.8.3), preheated to 95°C and chilled on ice, is then introduced into the hybridization tube and left to hybridize overnight at 65°C. The probe solution is eliminated and the membrane is washed in the hybridization tube, first with 10 mL of **washing buffer 1** (preheated to 65°C) for 15 min at 65°C and then with **washing buffer 2**. The membrane is finally put under autoradiography to reveal the hybridization pattern.

Hybridization solution: 5X SSC, 0.1% (w/v) SDS, 1/20 volume of liquid block (Amersham)

Washing buffer 1: 1X SSC, 0.1% SDS

Washing buffer 2: 0.1X SSC, 0.1% SDS

II.3.9 Northern Blotting using digoxigenin-labeled probe

II.3.9.1 RNA fractionation on agarose gel and transfer onto nitrocellulose

For northern blotting, RNA samples are fractionated by electrophoresis on 1.5% (w/v) agarose gels. The gel is placed in **0.5 x TAE buffer** and loaded with 1-5 µg of RNA supplemented with **loading buffer**. Electrophoresis is run at 50-75 V.

The RNA transfer onto the nitrocellulose is run like described in § II.3.8.2.

0.5 x TAE buffer: 0.5 mM EDTA, 20 mM Tris-Acetate pH 8.0

Loading buffer: 50% (w/v) glycerol, 1% (w/v) SDS, 1 mM EDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue

II.3.9.2 Preparation of the digoxigenin-labeled probe

The probes are PCR products that are digoxigenin-labelled using **PCR DIG Probe Synthesis kit** (Roche). Two successive PCR reactions are carried out with same primers for both reactions to prepare the labelled DIG probes. First PCR is run as explained in § II.2.5. In second PCR, for a final volume of 25 µL, 1 µL of previously performed PCR product is added to 2.5 µL of PCR buffer (Roche), 2.5 µL of PCR DIG Probe Synthesis Mix, 1 µM of each primer and 0.38 µL of Enzyme Mix in **storage buffer**. The reaction is carried out for 35 cycles in a Gene Cycler automator (BioRad) with each step of 40 seconds instead of 45 seconds.

Storage buffer: 20 mM Tris-HCl, pH 7.5 (25^{o}C), 100 mM KCl, 1 mM dithiothreitol (DDT), 0.1 mM EDTA, 0.5% Tween 20 (v/v), 0.5% Nonidet P40 (v/v), 50% glycerol (v/v)

10 x PCR DIG Probe Synthesis Mix: 2 mM each of dATP, dCTP, dGTP, 1.3 mM dTTP, 0.7 mM DIG-11-dUTP, alkali-labile, pH 7.0

II.3.9.3 Hybridization

The membrane carrying the RNAs is placed in a hybridization tube and 4 mL of DIG easy Hybridization Granules solution (Roche) is added. Pre-hybridization is run for 1-4 hours at 42°C in a hybridization oven with constant rotation. The DIG labeled probe, preheated to 95°C and chilled on ice, is then introduced into the hybridization tube and left to hybridize overnight at 42°C. The probe solution is eliminated and the membrane is washed twice in the hybridization tube, first with 10 mL of **washing buffer 1** for 5 min and then twice with **washing buffer 2** (preheated to 68°C) at 68°C for 15 min. The membrane is then washed thrice with **washing buffer** 3 for 5 min.

Washing buffer 1: 2X SSC, 0.1% SDS

Washing buffer 2: 0.5X SSC, 0.1% SDS

Washing buffer 3: Dig buffer with 0.3% Tween-20

Dig buffer: 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5

II.3.9.4 Development

The membrane is blocked in **blocking buffer** for 30-60 min at room temperature. Then it is incubated for 30-60 min with anti-DIG-AP (1:10,000 diluted in blocking buffer) and washed with **washing buffer 3** for 15 min at room temperature. An incubation in **detect buffer** for 5 min is followed by application of CSPD for 5 min. The membrane is finally put under autoradiography to reveal the hybridization pattern.

Dig buffer: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5

Washing buffer 3: Dig buffer with 0.3% Tween-20

Blocking buffer: Dig buffer with 1 x **blocking solution**

10 x blocking solution: blocking reagent 10 % (w/v) in **Dig buffer**

Detect buffer: 0.1 M Tris HCl, 0.1 mM NaCl, pH 9.5

II.4 Protein quantification

The concentration of a protein solution is estimated using Bio-Rad Protein Assay reagent (Bio-Rad). The reagent contains Coomassie blue which has a brown color at low pH. The pKa of Coomassie blue changes when it interacts with a protein and the color gets blue. Protein concentration is estimated by the measure of the absorbance at 595 nm in the presence of reagent (200 µL of reagent + 800 µL of sample) and comparing the result to a standard curve established with known BSA concentrations.

II.5 Techniques related to mitochondria

II.5.1 Isolation of mitochondria from hydroponic seedling cultures of *A. thaliana*

Mitochondria are isolated from hydroponic seedling cultures of *A. thaliana* (§ I.3) mostly according to Sweetlove *et al*., 2007. Four cultures of 100 mL are used to extract mitochondria. All the steps of extraction are performed at 4°C. The 8 days old seedlings are filtered and then ground in a mortar with a pestle in the presence of 30 mL of **grinding buffer**. The homogenate is filtered through a sheet of pre-moistened Miracloth placed on a canvas of 50 µm mesh. The remaining solid material is milled again in 20 mL of grinding buffer and filtered. The mortar is then rinsed with 50 mL of grinding buffer. The homogenate thus obtained is taken in 50 mL falcon tubes and centrifuge for 5 min at 1600 g to eliminate cellular debris. The supernatant is transferred into new tubes and centrifuged

5 min at 2000 g in order to remove a maximum of cellular debris. The supernatant is then centrifuged at high speed (17,200 g) for 20 min. The resulting pellet, containing the mitochondria and a number of contaminants (thylakoids, peroxisome *etc.*), is taken up in 1 mL of **washing buffer with BSA**. The suspension is deposited on 30 mL of PVP-40 continuous gradient (0-4.4% w/v) and centrifuged for 40 min at 40,000 g without brake. This gradient is made from a **light solution** with no PVP-40 and a **heavy solution** containing 4.4% (w/v) PVP-40. Mitochondria are in the heavy part, at the bottom of the gradient tube. Mitochondria taken from the bottom of the tube are divided into six tubes and diluted with 30 mL of washing buffer with BSA. After centrifugation at 31,000 g for 15 min, mitochondria are washed again in 30 mL of **washing buffer without BSA**. After centrifugation for 15 min at 31,000 g, mitochondria are placed in an Eppendorf tube and washed again with washing buffer without BSA. Mitochondria are then recovered by centrifugation at 11000 g for 8 min and taken up in 1 mL of washing buffer without BSA. Mitochondria are centrifuged once more at 11,000 g for 8 min and the resulting pellets are frozen in liquid nitrogen.

Grinding buffer: 0.3 M sucrose, 25 mM tetrasodium pyrophosphate, 2 mM EDTA, 10 mM KH_2PO_4, 1% (w/v) PVP-40, 1% (w/v) BSA, 20 mM sodium ascorbate, 20 mM L-cysteine, pH 7.5

Washing buffer with BSA: 0.3 M sucrose, 10 mM MOPS, 0.2% (w/v) BSA, pH 7.2

Washing buffer without BSA: 0.3 M sucrose, 10 mM MOPS, pH 7.2

Heavy solution: 17.5 mL 2X washing buffer with BSA, 9.8 mL Percoll, 7.7 mL 20% (w/v) PVP-40

Light solution: 17.5 mL 2X washing buffer with BSA, 9.8 mL Percoll, 7.7 mL H_2O

II.5.2 Isolation of mitochondria from *A. thaliana* cell suspension cultures

To isolate mitochondria, 10 flasks of cultures are used (§ I.2). Mitochondria are isolated according to a modified protocol described by Klein *et al*., 1998. Briefly, protoplasts derived (§ II.5.5) from cells are centrifuged at 800 g for 10 min to pellet protoplasts and washed twice with **washing buffer 1**. The pellet is taken in **extraction buffer** to a volume of 2 mL/g of fresh weight material, cells are disrupted and cell debris are removed by filtering the homogenate through a 30 µm mesh nylon membrane. After centrifugation of the filtrate at 2000 g for 15 min, the supernatant is recovered and centrifuged at 11 000 g for 15 min to pellet mitochondria. The mitochondrial pellet is re-suspended in 2–3 ml of the **washing buffer** by Dounce homogenization, are purified by centrifugation through

45/21/13.5% v/v percoll step gradient for 20 min at 14 000 g. Intact mitochondria collected from the 13.5/21% interphase, are diluted 10 times with the washing buffer and pelleted by centrifugation at 12 400 g for 10 min. This washing step is repeated twice.

Washing buffer 1: 450 mM mannitol, 3.6 mM MES, pH 5.5

Extraction buffer: 30 mM $Na_4O_7P_2$, 2 mM EDTA, 0.8% w/v polyvinylpyrrolidone K 25, 0.05% w/v cysteine, 5 mM glycine, 0.3% w/v BSA, 2 mM β-mercaptoethanol, 300 mM sucrose, pH 7.5

Washing buffer 2: 300 mM sucrose, 10 mM K_1HPO_4/K_2HPO_4 pH 7.5, 1 mM EDTA, 5 mM glycine, 0.1% w/v BSA

II.5.3 Isolation of mitochondria from potato tubers

Mitochondria are isolated from potato (*S. tuberosum*) tubers mostly according to (Neuburger *et al.*, 1982). Tubers are homogenized in a juice extractor and the suspension is mixed with one half volume of **extraction buffer**. Following filtration through a nylon net (100 µm mesh), crude mitochondria are recovered by two cycles of low- and high-speed centrifugations (1,500 and 16,000 *g*) with intermediate resuspension in **washing buffer**. The resuspended second pellet is loaded on a 30% (v/v) **Percoll solution** and centrifuged for 1 hour at 40,000 *g*. Mitochondria are recovered in the bottom third of the Percoll gradient established during centrifugation and washed twice with washing buffer.

Extraction buffer: 0.9 M sucrose, 90 mM sodium pyrophosphate, 6 mM EDTA, 0.9% (w/v) BSA, 2.4% (w/v) polyvinylpyrrolidone 25, 9 mM cysteine, 15 mM glycine, 6 mM

2-mercaptoethanol, pH 7.5

Washing buffer: 0.3 M sucrose, 1 mM EDTA, 0.1% (w/v) BSA, 5 mM glycine, 10 mM potassium phosphate, pH 7.5

Percoll solution: 30% of percoll (v/v) in washing buffer

II.5.4 Respiratory control of mitochondria

The mitochondrial respiratory control ratio, *i.e*. the ratio between the respiration rate (measured as oxygen consumption) of the mitochondria upon addition of 200 µM ADP ('active state' or state III) and the respiration rate in the absence of ADP or when the added ADP is entirely converted to ATP ('resting state' or state IV), is determined using 10 mM succinate as a respiration substrate (Douce, 1985). A ratio of 4-5 reflects a good functional state of mitochondria. The test is run in an oxygraph chamber. Mitochondria are resuspended with **respiration buffer** (50 µg of protein/mL).

Respiration buffer: 10 mM KH_2PO_4, 0.3 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 0.1% (p/v) BSA, pH 7,2

II.5.5 Preparation of protoplasts

To prepare protoplasts from plants, leaf surfaces are superficially wounded with celite using a brush but this step is omitted if cell cultures are being used. An enzymatic digestion is then performed in 2 mL/leaf **protoplast medium** 3 h at RT under very slow agitation.

Protoplast medium: Murashige and Skoog salts 4.3 g/L, MS basalt salt mix duchefa 0.5 g/L, sucrose 20 g/L, mannitol 80 g/L, macerozyme R-10 0.4 g/L, cellulase "Onuzuka R" 2 g/L)

II.5.6 Confocal imaging

Microscopic observations are performed on a Zeiss LSM 700 laser scanning confocal. The fluorochrome MitoTracker Orange (Molecular Probes) is used to monitor the integrity of mitochondria. It is the 4-chloromethyltetramethylrosamine which is a vital dye penetrating specifically into mitochondria having a membrane potential. It fluoresces in red (λm: 574 nm) when excited at 551 nm. This dye is added to isolated protoplasts at a final concentration of 0.5 pM. Image processing is done on the LSM 700 software version 5.5 (Zeiss).

II.6 Stable plant transformation

II.6.1 Stable transformation of BY2 cells by *A. tumefaciens*

II.6.1.1 Preparation of agrobacteria

Transformed agrobacteria LBA4404, are selected by cultivation during two days on **LB medium** containing 5 g/L sucrose, 25 µg/mL rifampicine and 50 µg/mL spectinomycin. Before cocultivation with tobacco cultured cells, agrobacteria are centrifuged for 5 min at 3000 g and resuspended in fresh LB medium to give an absorbance of 0.7 at 600nm.

LB medium: 10 g/L bacto-trypone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5

II.6.1.2 Transformation of BY2 tobacco cells by cocultivation

Twenty mL of three-day old BY2 tobacco cell culture and 400 µL of agrobacteria suspension are transferred in a Petri dish. To favor transformation of plant cells by *A. tumefaciens*, 200 µM of acetosyringone are added. Coculture is incubated with gentile agitation for 3 days at 28°C. To eliminate the majority of bacteria, plant cells are washed with **BY2 medium** several times.

BY2 medium: 4.3 g/L micro and macroelements M0221 (DUCHEFA), 1 mg/L thiamine, 200 mg/L KH_2PO_4, 0.2 mg/L 2,4D, 100 mg/L myoinositol, 3% (w/v)sucrose, pH 5.8

II.6.1.3 Selection of transformants

Two mL of BY2 cells are plated onto **BY2 agar medium** containing 500 µg/mL of carbenicillin to eliminate the rest of agrobacteria and 20 µg/mL of hygromycin to select the transformed cells. First calli are obtained 2-3 weeks after transformation. Obtained calli are transferred to BY2 Agar containing 20 µg/mL of hygromycin and cultivated at 25°C in darkness. They are transferred on fresh medium once a month. These calluses can be transferred into a liquid BY2 medium and maintained as cell cultures keeping the selection pressure with 20 µg/mL of hygromycin.

BY2 agar medium: 4.3 g/L micro and macroelements M0221 (DUCHEFA), 1 mg/L thiamine, 200 mg/L KH_2PO_4, 0.2 mg/L 2,4D, 100 mg/L myoinositol, 3% (w/v) sucrose, 10 g/L agar, pH 5.8

II.6.2 Stable transformation of *A. thaliana* plants by *A. tumefaciens*

II.6.2.1 Preparation of agrobacteria

Transformed agrobacteria GV-3101, are selected by cultivation at 28°C during two days on **LB medium** containing 5 g/L sucrose, 25 µg/mL rifampicine and 50 µg/mL spectinomycin. A colony of transformed agrobacteria previously is grown for 12 to 20 h at 28°C in 50 mL of LB medium to give an absorbance of 0.8 at 600nm. After centrifugation at 1500 g for 10 min, the bacteria are resuspended in 50 mL of 10 mM $MgCl_2$. A second centrifugation at 1500 g for 10 min allows to retrieve bacteria and resuspend them in 500 mL of **agro-infiltration medium**.

LB medium: 10 g/L bacto-trypone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5

Agro-infiltration medium: 2.4 g/L micro and macroelements M0255 (DUCHEFA), 5% sucrose, 0.5 mL/L Silvet-77, 200 µM acetosyringone

II.6.2.2 Transformation of *A. thaliana* plants by floral dip

The plant flowers are dipped for 90 seconds in agro-infiltration medium with bacteria (§ II.6.2.1). The plants are then placed in small greenhouses and put under obscurity for 48 hours. Then they are put in green houses (25°C, 16 h light and 8 h dark) till harvesting of seeds.

II.6.2.3 Harvesting and selection of transformed seeds

Six to ten weeks later, it is possible to harvest the seeds. These are then sterilized. For this, about 100 seeds are placed in a 2 mL Eppendorf tube and 1 mL of **sterilization solution** is added. After incubation for 15 min with continuous stirring, the supernatant is removed and seeds are washed 5 times with absolute ethanol. The seeds are then dried overnight in a laminar flow hood. The next morning, they are placed on a **selection medium**.

Sterilization solution: 70% (v/v) ethanol and 4% (v/v) Tween 20

Selection medium: 4.3 g/L micro and macroelements M0255 (DUCHEFA), 10 g/L agar, 1% (w/v) sucrose, 500 µg/mL carbenicillin, 20 µg/mL hygromycin, pH 5.7

II.6.2.4 Selection and regeneration of transformed plants

Seeds on plates (§ II.6.2.3) are stratified in the dark at 4°C for 2-4 days to synchronize the germination time. They are then placed in *in vitro* growth chamber (25°C, 16 h light and 8 h dark) for 2-4 weeks. All seeds germinate but only transformed seedlings continue to grow. Because of hygromycin, the growth of non-transformed seedlings stops at the two-leaf stage. The selected seedlings are transferred again to **selection medium** and then transplanted into soil when they are large enough. Two weeks in growth chamber (25°C, 12 h light and 12 h dark) are needed to limit losses due to their adaptation. When plants are large enough, they are analyzed to select the correctly transformed plants. For this, DNA of 4-5 leaves is extracted (§ II.2.1) and analyzed by PCR (§ II.2.5) and sequencing (§ II.2.9). Six-ten weeks later, the seeds are harvested from the plants containing the correct construct and germinated again on selection medium. All the seeds continue to grow if the plant line is homozygous. For heterozygous plant line, another cycle of selection is performed.

Selection medium: 4.3 g/L micro and macroelements M0255 (DUCHEFA), 10 g/L agar, 1% (w/v) sucrose, 500 µg/mL carbenicillin, 20 µg/mL hygromycin, pH 5.7

II.6.3 Stable transformation of *N. tabacum* plants by *A. tumefaciens*

II.6.3.1 Preparation of sterile *N. tabacum* plants and transformation

Transformed agrobacteria LBA4404 are prepared as described in § II.6.1.1. Leaf discs of 1 cm^2 are cut from *N. tabacum* plants grown *in vitro* on **germination medium.** They are slightly incised in order to increase the area of infection for transformed bacteria. They are then immersed for 15 minutes in previously prepared agrobacterium solution and dried on sterile filter paper. The leaf discs are then cultured *in vitro*, with the face upside down in contact with the **coculture medium** for 3-4 days at 28°C (12 h light and 12 h dark).

Germination medium: 4.3 g/L micro and macroelements M0238 (DUCHEFA), 10 g/L agar, 1% (w/v) sucrose, pH 5.7

Coculture medium: 4.3 g/L micro and macroelements M0238 (DUCHEFA), 825 mg/L NH_4NO_3, 2 mg/mL glycine, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L putrescine, 0.1 mg/L thiamine, 3% sucrose, 0,8% bacto-agar, 2 mg/mL BAP, 0.05 mg/L ANA, pH 5,8

II.6.3.2 Obtaining and selection of transformed callus from leave disks

The leaf discs are then transferred to a **selection medium 1** at 25°C (12 h light and 12 h dark). Every two to three weeks, the leaf discs are planted on this medium, upper surface in contact with the medium. After one to two months, calli appear on the edges, they are transferred to individual pots with selection medium when young leaves are formed.

Selection medium 1: 4.3 g/L micro and macroelements M0238 (DUCHEFA), 825 mg/L NH_4NO_3, 2 mg/mL glycine, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L putrescine, 0.1 mg/L thiamine, 3% sucrose, 0,8% bacto-agar, 350 mg/L cefotaxime, 20 mg/L hygromycin, pH 5,8

II.6.3.3 Regeneration of whole tobacco transformed plants

When the roots have emerged and the leaf apparatus is composed of 1 to 3 internodes, the plants are planted in a soil rich in minerals. Three to four weeks in growth chambers (25°C, 12 h light and 12 h dark) are needed for acclimatization and to avoid losses. They are then placed in a greenhouse with controlled atmosphere. According to growth, they are transplanted into larger and larger pots until flowering.

II.6.3.4 Harvesting and selection of transformed seeds

Four to six months later, it is possible to harvest the seeds. These are then sterilized. For this, about 100 seeds are placed in a 2 mL Eppendorf tube and 1 mL of 70% (v/v) ethanol is added for 15 seconds. The ethanol is removed and 1 mL of **sterilization solution** is added. The seeds are put with continuous stirring for 15 min. Then washed 5 times in sterile water and placed on a **selection medium 2**.

Sterilization solution: 4% (w/v) Sodium Hypochlorate and 4% (v/v) Tween 20

Selection medium 2: 4.3 g/L micro and macroelements M0238 (DUCHEFA), 10 g/L agar, 1% (w/v) sucrose, 350 mg/L cefotaxime, 20 µg/mL hygromycin, pH 5.7

II.6.3.5 Selection and regeneration of transformed plants

Seeds grown on plates are stratified in the dark at 4°C for 2-4 days to synchronize the germination time. They are then placed in *in vitro* growth chamber (25°C, 12 h light and 12 h dark) for 2-4 weeks. All seeds germinate but only transformed seedlings continue to grow. Because of hygromycin, the growth of non-transformed seedlings stops at the two-leaf stage. The selected seedlings are transplanted into soil. Two weeks in growth chamber (25°C, 12 h light and 12 h dark) are needed to limit losses due to their adaptation. When plants are large enough, they are analyzed to select the correctly transformed plants. For this, DNA of 4-5 leaves is extracted (§ II.2.1) and analyzed by PCR (§ II.2.5) and sequencing (§ II.2.9). Four to six months later, the seeds are harvested from correctly transformed plants.

Selection medium 2: 4.3 g/L micro and macroelements M0238 (DUCHEFA), 10 g/L agar, 1% (w/v) sucrose, 350 mg/L cefotaxime, 20 µg/mL hygromycin, pH 5.7

II.7 Preparation of *in vivo* constructs

We generated PCR products containing ribozymes using specific direct primer and cHDVforpER8 as reverse primer (Table 4). The direct primer contained, from the 5' end to the 3' end, a *Hind*III restriction site, the complete sequence coding for the ribozyme, the linker and the 5' end of the PKTLS. The reverse primer contained, from the 5' end to the 3' end, an *Eco*RI restriction site and a sequence complementary to the 3' end of the *cis*-ribozyme of the HDV. The template for the PCR was a previous construct in the pER8 vector containing the sequence coding for the PKTLS linked to the HDV *cis*-ribozyme (pER8-PKTLS-cHDV). The PCR product was cloned in pUCAP plasmid between *Eco*RI and *Hind*III sites. After verification of correct sequence, the insert was cleaved from the recombinant pUCAP using the unique restriction sites *Asc*I and *Pac*I and finally cloned as described before into the *Asc*I and *Pac*I sites of the inducible transcription unit of the pER8 vector, generating the pER8-Rz-L-PKTLS-cHDV plasmid which was then used for agro-transformation of *N. tabacum* BY2 cell suspensions and *N. tabacum* or *A. thaliana* plants.

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SYNTHESE EN FRANCAIS

I. Introduction

Dans une cellule végétale, il y a trois génomes : nucléaire, mitochondrial et chloroplastique. Tous trois opèrent d'une façon coordonnée pour assurer les fonctions générales de la cellule, dont la photosynthèse et la respiration, sources d'énergie de la plante. En plus de la production d'énergie, les mitochondries jouent un rôle central dans beaucoup d'autres fonctions, telles que la régulation de l'état redox ou la mort cellulaire programmée par apoptose, et dans différentes voies métaboliques essentielles. Les processus génétiques mitochondriaux sont eux-mêmes complexes chez les plantes et ils influencent différents caractères agronomiques importants, en particulier la fertilité, la vigueur et la compatibilité croisée. Manipuler génétiquement les mitochondries est donc d'un intérêt à la fois fondamental et appliqué. Cependant, l'étude du système génétique mitochondrial complexe des plantes supérieures et de ses mécanismes de régulation n'a pas pu bénéficier des approches transgéniques. En effet, les méthodologies conventionnelles ne permettent pas la transformation génétique de ces organites cellulaires dans les organismes autres que la levure et l'algue unicellulaire *Chlamydomonas reinhardtii*.

Une stratégie alternative a été développée au laboratoire. Elle prend avantage de l'existence d'un processus naturel de transport cellulaire dont la fonction est d'assurer l'import de certains ARN de transfert (ARNt) du cytosol dans les mitochondries. En effet, dans de nombreux organismes, le jeu d'ARNt codé par le génome mitochondrial n'est pas suffisamment complet pour permettre la synthèse protéique. Les mitochondries des plantes supérieures doivent ainsi importer entre un tiers et la moitié de leurs ARNt du cytosol et en particulier les ARNt spécifiques de la valine (Salinas *et al.*, 2008). A partir de ces données, le laboratoire d'accueil a développé une stratégie qui utilise un mime d'ARNt valine de plante pour adresser dans les mitochondries des ARN d'intérêt exprimés à partir de transgènes nucléaires. La séquence à importer est associée à l'extrémité 5' du mime d'ARNt qui joue le rôle de "navette" de transport mitochondrial. Le mime d'ARNt utilisé est d'origine virale. Il s'agit de la structure "TLS" (pour "tRNA-like structure") formée par les 82 derniers nucléotides de l'extrémité 3' de l'ARN génomique du virus de la mosaïque jaune du navet ("*Turnip yellow mosaic virus"* ou "TYMV") (Matsuda and Dreher, 2004). La séquence comprise entre les nucléotides 83 et 109 en amont de la partie TLS adopte elle-même une structure en pseudo-noeud qui contribue à la reconnaissance de la structure TLS par la valyl-ARNt synthétase (Matsuda and Dreher, 2004). La reconnaissance par l'aminoacyl-ARNt synthétase spécifique est importante pour l'import des ARNt dans les

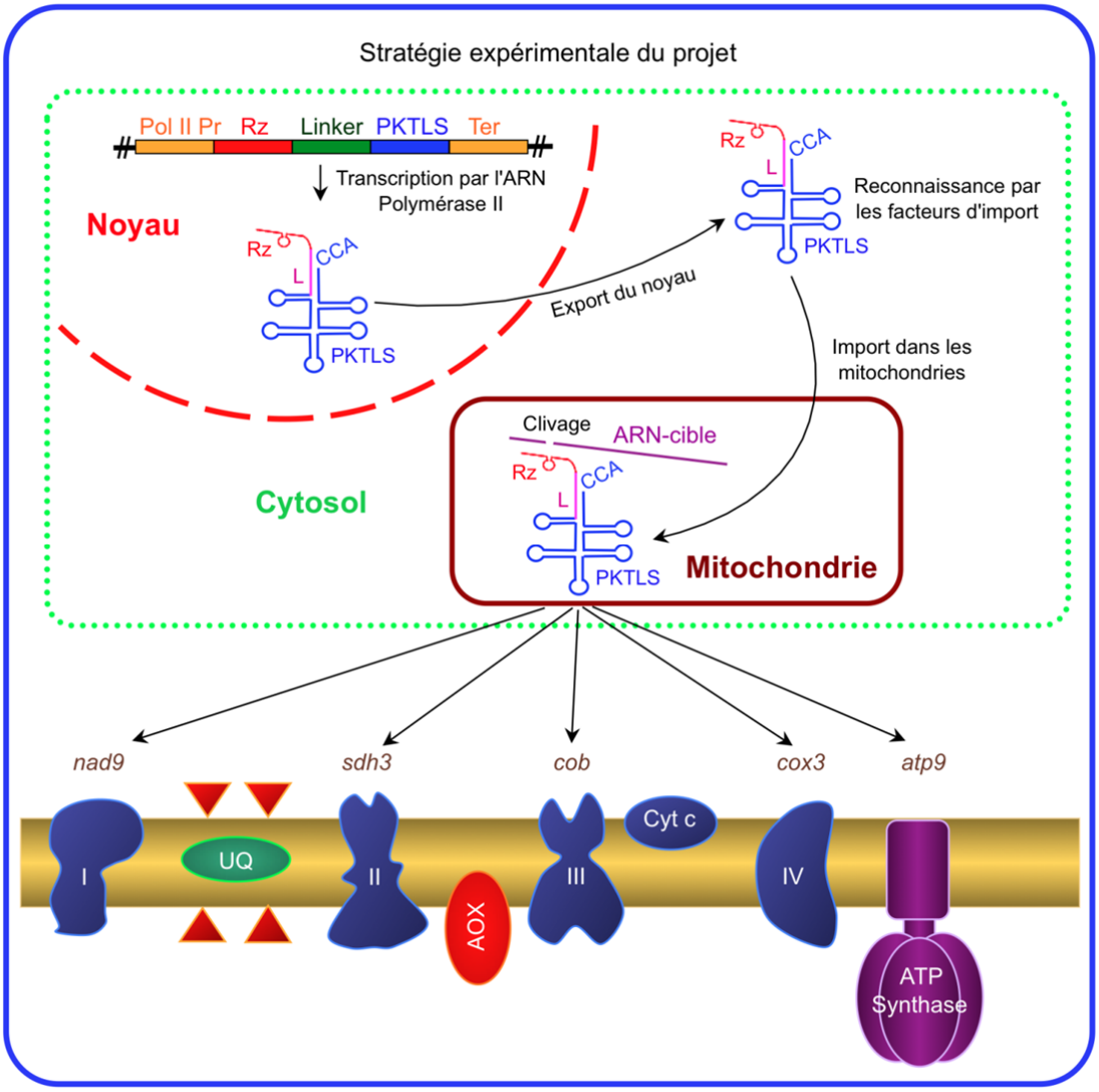

Figure 61. Stratégie expérimentale développée.
L'ARN composite *trans*-ribozyme-PKTLS est transcrit dans le noyau, à partir d'un transgène, et transporté dans les mitochondries à travers le cytosol grâce à la reconnaissance de la partie PKTLS par le mécanisme naturel d'import des ARNt. Pol II Pr : promoteur pour l'ARN polymérase II; Rz : séquence du *trans*-ribozyme; L : séquence de liaison; PKTLS : mime d'ARNt; Ter : terminateur. Cinq ARNm mitochondriaux ont été choisis comme cibles pour des *trans*-ribozymes spécifiques : *nad9*, *sdh3*, *cob*, *cox3* et *atp9*.

mitochondries chez les plantes (Salinas *et al.*, 2008). C'est donc la combinaison de la TLS et de la structure en pseudo-noeud qui est utilisée comme navette d'adressage mitochondrial. Elle a été appelée "PKTLS". Notre laboratoire a démontré que cette stratégie moléculaire permet d'importer des ARN d'intérêt dans les mitochondries de suspensions cellulaires ou de plantes entières après transformation nucléaire stable avec des constructions de gènes appropriées (Val *et al.*, 2011). L'utilisation d'un *trans*-ribozyme spécifique comme ARN "passager" associé au mime d'ARNt PKTLS (Figure 61) a ensuite permis d'obtenir, pour la première fois chez un eucaryote multicellulaire, l'invalidation dirigée d'un ARN dans les mitochondries (Val *et al.*, 2011).

Le développement d'une telle stratégie de manipulation dirigée du transcriptome dans les organites ouvre la possibilité d'étudier de façon directe les mécanismes de régulation génétique mitochondriaux et les processus de coordination avec le noyau et les plastes. En l'absence de méthodologies de transformation des mitochondries, peu d'études ont été consacrées à ces questions dont l'importance fonctionnelle est fondamentale (Giegé *et al.*, 2005; Giraud *et al.*, 2009). En effet, tous les complexes de la chaîne de phosphorylation oxydative sont formés de sous-unités codées par le génome nucléaire et de sous-unités exprimées à partir de gènes mitochondriaux. Des mécanismes génétiques intégrés semblent donc indispensables pour assurer la fonctionnalité des organites. Selon la vision la plus répandue actuellement, le contrôle génétique dans les mitochondries des cellules végétales se ferait principalement au niveau post-transcriptionnel. Le domaine reste cependant ouvert et l'idée qu'il pourrait exister des mécanismes de régulation mitochondriaux et des voies de signalisation rétrograde médiés par les ARN doit être considérée. Mon travail de doctorat a été consacré à cette problématique que j'ai abordée en manipulant les ARN mitochondriaux dans les cellules végétales à l'aide de *trans*-ribozymes spécifiques exprimés par des transgènes nucléaires.

II. Résultats et Discussion

Les premières expériences ont été réalisées en important dans les mitochondries de suspensions cellulaires de *Nicotiana tabacum* et de plantes d'*Arabidopsis thaliana* un *trans*-ribozyme dirigé contre l'ARN messager (ARNm) *atp9* qui code pour la sous-unité 9 de l'ATP synthétase. Quatre autres ARNm mitochondriaux ont ensuite été choisis comme cibles pour des *trans*-ribozymes spécifiques : *nad9* (sous-unité 9 du complexe I), *sdh3* (sous-unité 3 du complexe II), *cob* (cytochrome b, complexe III) et *cox3* (sous-unité 3 du

complexe IV). Les différents ARN composites *trans*-ribozyme-PKTLS ont été exprimés dans des suspensions cellulaires de *N. tabacum* et dans des plantes de *N. tabacum* ou d'*A. thaliana*, *sdh3* n'étant pas un gène mitochondrial dans ce dernier cas.

II.1. Analyse *in vitro* de l'activité des ribozymes

Les *trans*-ribozymes ont été élaborés sur le type "tête de marteau" avec des adaptations destinées à favoriser leur activité dans les conditions physiologiques de faible concentration en magnésium (Val *et al.*, 2011). Ils ont été associés au mime d'ARNt PKTLS par l'intermédiaire d'une séquence de liaison spécifique (linker) ayant une structure la plus neutre possible. L'activité des ARN catalytiques peut être influencée par de nombreux facteurs structuraux. Avant d'introduire l'approche *in vivo*, la fonctionnalité des différents systèmes ribozyme/cible a donc dans un premier temps été analysée *in vitro*. En effet, au cours d'expériences antérieures du laboratoire, le clivage d'ARN-cibles par un *trans*-ribozyme n'avait pas toujours été couronné de succès. Il s'agissait notamment de s'assurer que la combinaison des ribozymes avec le mime d'ARNt PKTLS ne détruisait pas leur activité catalytique.

Pour chaque système, l'ARN-cible et l'ARN composite ribozyme/linker/PKTLS ont été synthétisés *in vitro* par transcription de matrices ADN adéquates avec l'ARN polymérase du phage T7. L'activité de clivage *in vitro* a ensuite été étudiée avec différents rapports molaires ribozyme/cible et dans différentes conditions de réaction, avec une attention particulière pour la dépendance vis-à-vis de la concentration en magnésium. Les résultats de ces expériences ont montré que les systèmes d'ARN catalytiques que nous avions élaborés étaient efficaces avec des rapports cible/ribozyme réalistes et à faible concentration de magnésium. Beaucoup d'entre eux étaient même actifs, voire tout à fait efficaces, en l'absence de magnésium ajouté à la réaction. Des *trans*-ribozymes à tête de marteau élaborés de façon appropriée peuvent donc développer une activité catalytique élevée à des concentrations physiologiques de magnesium. De plus, l'activité de clivage en conditions de magnesium faible n'était clairement pas compromise par la combinaison avec le linker et le mime d'ARNt PKTLS. Les différents ARN composites ribozyme/linker/PKTLS étaient donc susceptibles de fonctionner dans les conditions physiologiques mitochondriales, ce qui validait la stratégie moléculaire et ouvrait la voie pour les expériences *in vivo*.

II.2. Invalidation des ARN-cibles mitochondriaux *in vivo*

Après démonstration des activités *in vitro*, des constructions de gènes codant pour les ARN composites ribozyme/linker/PKTLS ont été assemblées dans le vecteur d'expression pER8 sous le contrôle d'un promoteur inductible par l'oestradiol. Les constructions ont été utilisées pour transformer des cellules de *N. tabacum*, ainsi que des plantes d'*A. thaliana* et de *N. tabacum*. Les cellules et les plantes transformées (cultivées en conditions de jours longs) ont été traitées avec de l'oestradiol, puis récoltées jour par jour sur un cycle de 5 à 19 jours. Des ARN totaux et mitochondriaux ont été isolés à partir des différents échantillons et analysés par RT–PCR et "northern blot", ce qui a permis de démontrer l'expression induite et l'import mitochondrial des ARN composites ribozyme/linker/PKTLS. L'expression des ribozymes chimériques dans les cellules et les plantes transformées traitées à l'oestradiol a ensuite été analysée plus en détail par RT–PCR quantitative (RT-qPCR) et à différents stades de développement. Le taux d'ARN ribozyme/linker/PKTLS atteignait généralement un plateau au second et troisième jour suivant un traitement unique à l'oestradiol, avant de décroître progressivement, en particulier à l'approche de la phase stationnaire de croissance pour les cultures cellulaires. Pour les plantes, l'expression des transgènes a pu être maintenue de façon continue en répétant le traitement à l'oestradiol tous les trois jours.

Les différents échantillons ont dans un deuxième temps servi de matrice de RT-qPCR pour déterminer le niveau des ARN-cibles visés par les *trans*-ribozymes dans les mitochondries. Les niveaux de contrôle ont été établis avec des ARN provenant de cellules ou de plantes transformées non-induites et avec des ARN isolés à partir de cellules ou de plantes non transformées mais traitées à l'oestradiol. Nous avons ainsi démontré que, par rapport aux échantillons de contrôle, le niveau des ARNm-cibles *nad9* et *atp9* pouvait être réduit dans des proportions allant jusqu'à 75% au troisième jour d'expression des ribozymes correspondants dans les cellules ou les plantes transformées. Une invalidation moins efficace a été observée dans le cas des ARNm-cibles *sdh3* et *cox3*, tandis que l'invalidation de l'ARNm *cob* n'était pas significative. A un stade précoce de développement des plantes, une chute des ARNm-cibles a été observée un jour après le début de l'expression des ribozymes, suivie d'une remontée à un niveau quasi normal au jour suivant, puis d'une invalidation définitive et généralement plus importante à partir du troisième jour. Un tel profil évoque la possibilité d'une réaction transitoire de "défense" des mitochondries vis-à-vis de l'invalidation de l'un de leurs ARNm essentiels. Afin de confirmer les résultats avec une seconde méthode, nous avons effectué des analyses de "northern blot" pour les

transformants exprimant un ribozyme contre l'ARNm *nad9*. Le profil obtenu avec une sonde spécifique de cet ARNm était parfaitement en accord avec les résultats de RT-qPCR, avec une forte invalidation de la cible mitochondriale au premier jour d'expression du ribozyme, un rebond jusqu'au niveau initial le deuxième jour et un retour de l'invalidation au troisième et quatrième jour. L'ensemble de ces expériences a permis d'établir l'import mitochondrial des ribozymes chimériques exprimés à partir de transgènes nucléaires, suivi par l'invalidation spécifique de leurs ARN-cibles. Notre approche moléculaire est apparue très efficace pour invalider des ARN mitochondriaux dans des plantes entières, montrant un potentiel important pour l'investigation des mécanismes de régulation dans les organites. L'effet de rebond observé avec tous les systèmes ribozyme/cible constituait une première indication dans cette direction.

Plus d'évidences en faveur de mécanismes de contrôle des ARNm dans les mitochondries ont été obtenues en analysant l'invalidation des ARN-cibles en relation avec le stade de développement des plantes ou les conditions de culture. Les résultats avec de très jeunes plantes ont soulevé l'hypothèse que, lorsque la demande d'activité mitochondriale est élevée, la chute du taux de l'ARN-cible pourrait être perçue et peut-être promouvoir une synthèse accrue. D'après les données obtenues avec des plantes plus âgées, il semble à l'inverse que, lorsque la demande est faible, une invalidation drastique d'un ARNm mitochondrial peut être maintenue de façon continue sur une longue période.

Finalement, nous avons testé le comportement de notre système dans différentes conditions de culture, avec notamment des pousses d'*A. thaliana* cultivées dans des conditions hydroponiques particulières où la germination et le développement se font directement en suspension dans un milieu liquide. Les expériences ont été développées avec les transformants exprimant le ribozyme contre l'ARNm *atp9*. Les analyses de RT-qPCR effectuées sur ce matériel ont donné des résultats similaires à ceux obtenus pour les très jeunes plantes cultivées sur milieu solide. L'ARN ribozyme/linker/PKTLS a été exprimé efficacement mais le taux de l'ARN-cible *atp9* n'a pas été modifié significativement par rapport à celui des plantes de contrôle. Ces expériences ont montré une solide dépendance de l'efficacité, de la cinétique et du maintien de l'invalidation de l'ARN-cible par le ribozyme vis-à-vis du stade de développement des plantes et de leur état physiologique. Des observations très similaires ont été faites avec les ribozymes chimériques dirigés contre l'ARNm mitochondrial *nad9*, montrant la reproductibilité de ces données avec des systèmes

ribozyme-cible différents. L'ensemble des résultats obtenus n'en démontre pas moins que l'expression nucléaire et l'adressage mitochondrial d'ARN catalytiques permet d'invalider de façon très efficace des ARN majeurs dans les mitochondries des cellules végétales et des plantes entières et de manipuler spécifiquement le transcriptome.

II.3. Régulation mitochondriale et réponse rétrograde

Ayant établi l'invalidation spécifique *in vivo* d'ARN-cibles mitochondriaux à l'aide de *trans*-ribozymes importés, la question de l'existence de processus de régulation et de coordination des ARN a été abordée en analysant la réponse des mitochondries et du reste de la cellule à la chute d'un transcrit individuel. Nous avons ainsi étendu l'analyse par RT-qPCR à l'ensemble du transcriptome mitochondrial connu. Nous avons caractérisé en particulier le comportement de la plupart des ARNm mitochondriaux codant pour des sous-unités des complexes de la chaîne de phosphorylation oxydative (OXPHOS) dans les plantes d'*A. thaliana* transformées exprimant les ribozymes chimériques dirigés contre les ARNm *nad9* et *atp9*. Nous avons également sélectionné plusieurs ARNm d'origine nucléaire, notamment les ARN *aox1a* et *aox1b*. Ces ARNm codent pour l'oxydase alternative, une protéine enzymatique qui peut détourner le chemin des électrons dans la chaîne respiratoire et réduire l'oxygène. L'expression de cette protéine a été associée au dysfonctionnement de la phosphorylation oxydative, à des situations de stress thermique ou hydrique, ainsi qu'à l'attaque de pathogènes. En analysant les ARN *aox1a* et *aox1b,* il s'agissait de déterminer si un dialogue entre les systèmes génétiques nucléaire et mitochondrial pouvait s'établir en réponse à l'invalidation d'un ARN mitochondrial. Pour chacun des ARN sélectionnés, nous avons vérifié qu'il n'y avait pas de ressemblance de séquence avec *nad9* ou *atp9* pouvant donner lieu à des clivages indésirables par les ribozymes exprimés. Les résultats ont montré des modifications importantes, souvent mais non exclusivement négatives, dans le niveau de la plupart des ARN mitochondriaux en réponse à l'invalidation dirigée des transcrits *nad9* et *atp9*.

Pour les plantes exprimant un ribozyme contre *nad9*, nous avons testé 32 ARN autres que *nad9*, 29 d'origine mitochondriale et 3 d'origine nucléaire. Des 29 ARN mitochondriaux analysés, 12 ont montré une chute substantielle de plus de 30%. La diminution de toute une série d'ARNm codant pour des protéines des complexes de la chaîne de phosphorylation oxydative, tels que *nad1b, nad1c, nad4, nad5a, nad5b, cob, cox1, atp1, atp4* et *atp9*, a suggéré qu'il existe un système de régulation qui coordonnerait l'expression des gènes

mitochondriaux codant pour des sous-unités d'un même complexe ou de différents complexes. Cependant, nous avons noté que les ARNm correspondant à un même complexe ne diminuaient pas nécessairement tous. Ceci était le cas par exemple pour le complexe I, pour lequel les ARNm *nad1b, nad1c, nad4, nad5a* et *nad5b* diminuaient, alors que *nad2b, nad6* and *nad7* n'étaient pas affectés significativement. En ce qui concerne les plantes exprimant un ribozyme contre *atp9*, nous avons testé 23 ARN autres que *atp9*, 18 d'origine mitochondriale et 5 d'origine nucléaire. Les résultats ont confirmé l'essentiel des données obtenues avec l'invalidation de *nad9*. Des 18 ARN mitochondriaux analysés, tous ont montré une chute substantielle, à l'exception d'*atp4* qui augmentait de 80%. Nous avons d'autre part observé des variations très importantes du niveau des ARNm nucléaires *aox1a* et *aox1b* après invalidation de *nad9* ou *atp9* dans les mitochondries.

Comme le niveau de nombreux ARN mitochondriaux diminuait après l'invalidation dirigée de *nad9* ou *atp9*, nous avons vérifié que ceci n'était pas dû à un démantèlement général des mitochondries. Des analyses complémentaires par qPCR ont montré que l'invalidation de l'ARNm *nad9* n'était pas associé avec une diminution du contenu des cellules en ADN mitochondrial. Nous avons également déterminé le profil des organites par microscopie confocale et nous avons observé des populations similaires de mitochondries dans des protoplastes de plantes de contrôle et de plantes exprimant un ribozyme contre *nad9*.

Pour consolider les données et générer une vue globale de ces processus de régulation, nous avons entamé des analyses par hybridation sur puces à ADN (puces CATMA V.6, URGV, Evry). Les premiers résultats ont confirmé les données obtenues par RT-qPCR et northern blot et ont montré une réponse importante à l'échelle de l'ensemble du transcriptome. L'invalidation de l'ARNm *nad9* et son effet, souvent négatif, sur le comportement de nombreux autres ARN mitochondriaux, dont *nad1b, nad1c, nad2b, nad4, nad5, nad9, cob, cox1, atp1, atp4, ccb203, ccb206, ccb452, rps3* et *rrn18*, ont été retrouvés. De plus, le taux de près d'une trentaine de transcrits chloroplastiques était affecté, négativement pour la plupart mais positivement dans quelques cas. Enfin, l'expression de plus de 2500 gènes nucléaires était significativement modifiée en réponse à l'invalidation dirigée d'un ARNm majeur (*nad9*) dans les mitochondries. L'effet était varié, certains ARN montrant une chute drastique, d'autres une augmentation importante ou seulement des changements modérés. Ces analyses par puces à ADN ont ainsi permis d'identifier d'autres ARN intéressants qui

pourraient aider à comprendre les voies de régulation entre les organites et le noyau ou à l'intérieur des mitochondries.

Nous avons porté une attention particulière aux gènes codant pour les protéines PPR ("pentatricopeptide repeat"). Beaucoup de ces protéines sont prédites pour être adressées dans les mitochondries ou les chloroplastes (Lurin *et al.*, 2004). Les protéines PPR se lient aux ARN dans les organites et recrutent différents facteurs pour l'édition, la maturation ou la traduction (Sosso *et al.*, 2012; Zehrmann *et al.*, 2012; Zhu *et al.*, 2012). Certaines publications impliquent un rôle des protéines PPR dans la régulation des gènes (Hammani *et al.*, 2011a, 2011b; Saha *et al.*, 2007; Schmitz-Linneweber and Small, 2008), ce qui en fait des candidats potentiels pour la suite de nos études. Avec les puces à ADN, nous avons effectivement fait des observations intéressantes pour un ensemble de 16 ARNm codant pour des protéines PPR. Une augmentation significative a été mise en évidence pour la plupart de ces ARNm, tandis que l'un d'entre eux, correspondant au gène AT1G69290 qui code pour une protéine PPR adressée dans les mitochondries, a montré une diminution drastique (68%). GUN I, codée par le gène AT2G31400, est une autre protéine PPR importante impliquée dans différentes voies de régulation rétrograde. Elle est considérée comme un commutateur majeur de régulation car elle semble constituer un point de convergence entre différentes voies de signalisation reliant les mitochondries, les chloroplastes et le noyau. Dans nos analyses de puces à ADN, l'ARNm GUN I a montré une augmentation substantielle (113%). Finalement, les ARNm codant pour des facteurs de transcription de la famille TCP ou pour les NAD(P)H déshydrogénases mitochondriales ont également montré des résultats intéressants pour la suite de nos analyses.

Ces observations impliquent clairement que l'invalidation d'un ARN mitochondrial enclenche des mécanismes de régulation et renforcent l'idée d'un contrôle du niveau des différents ARN dans les mitochondries et d'une coordination inter-compartiments. En particulier, un dialogue rétrograde semble exister entre le génome mitochondrial et le génome nucléaire. Les données présentées dans ce travail ne permettent pas encore de déterminer si cette régulation est le résultat d'une signalisation génétique ou métabolique mais elles pointent en direction d'une synchronisation de l'expression.

II.4. Premières analyses sur les mécanismes de régulation

Nos résultats soulèvent à présent la question des mécanismes qui sous-tendent les processus de régulation que nous avons mis en évidence. L'hypothèse la plus directe serait celle d'un changement rapide des taux de transcription dans les organites. Nous avons donc commencé des analyses de transcription de type "run-on" avec des mitochondries isolées à partir de plantes de contrôle ou de plantes exprimant l'ARN composite ribozyme/linker/PKTLS dirigé contre l'ARNm *nad9*.

Dans les mitochondries des plantes, la polyadénylation est un processus transitoire qui déclenche la dégradation (Holec *et al.*, 2008). Nous avons utilisé cette propriété pour tester si la diminution du niveau de nombreux ARN mitochondriaux en relation avec l'invalidation de *nad9* ou *atp9* était due à une vitesse accrue de dégradation. Nous avons développé pour cela des analyses de RT-qPCR dans lesquelles les réactions de transcription inverse étaient amorcées avec de l'oligo(dT) au lieu des héxamères au hasard introduits lors des expériences précédentes, afin d'amplifier les intermédiaires de dégradation. Nous avons sondé les formes polyadénylées d'une série d'ARN mitochondriaux, en particulier ceux ayant montré une forte diminution en relation avec l'invalidation de *nad9*. Les résultats n'ont pas révélé d'augmentation du niveau des formes polyadénylées détectables en parallèle avec les cinétiques d'invalidation de *nad9*. Nous n'avons donc à ce stade aucune évidence en faveur d'une dégradation plus importante des ARN testés.

III. Conclusion et perspectives

Tandis qu'il a été considéré jusqu'à présent que les processus de régulation dans les mitochondries des plantes se déroulent essentiellement au niveau post-transcriptionnel, les résultats de mon projet de thèse impliquent que les niveaux des ARN mitochondriaux sont coordonnés et que modifier ces niveaux provoque une réponse rétrograde qui se répercute sur les transcriptomes nucléaire et chloroplastique. De plus, ces mécanismes se sont avérés être en relation avec le développement des plantes et les conditions de culture.

Dans une perspective appliquée, certaines de ces manipulations pourraient provoquer un phénotype mâle-stérile, un caractère agronomique recherché. Dans nos premiers essais d'induction des transgènes à long terme, l'expression du ribozyme composite dirigé contre l'ARNm *nad9* a effectivemment semblé arrêter les plantes au stade de la floraison. Une analyse plus détaillée des inflorescences et du pollen permettra de mieux caractériser ce

phénotype. Ces études seront poursuivies également avec des plantes d'*A. thaliana* exprimant les ribozymes chimériques de façon constitutive.

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Résumé

Le système génétique complexe des mitochondries de plantes supérieures n'a pu être étudié par des approches transgéniques car les méthodes conventionnelles ne permettent pas de transformer ces organites. Une approche alternative a été développée au laboratoire, grâce à l'existence d'un processus naturel assurant l'import d'ARN de transfert (ARNt) du cytosol dans les mitochondries. Il a été montré qu'un mime d'ARNt peut servir *in vivo* de navette pour importer dans les mitochondries de plante des ARN-passagers exprimés à partir de transgènes nucléaires. L'utilisation d'un *trans*-ribozyme comme séquence-passagère a permis d'obtenir l'invalidation spécifique d'un ARN messager (ARNm) majeur dans les mitochondries de cellules végétales transformées. Nous avons mis en oeuvre cette stratégie pour développer des études de régulation mitochondriale. Cinq ARNm mitochondriaux (*nad9, sdh3, cob, cox3* et *atp9*) ont été choisis comme cibles pour des *trans*-ribozymes spécifiques à tête de marteau. Après validation de l'activité de ces ribozymes *in vitro*, les vecteurs d'expression portant les transgènes correspondants ont servi pour transformer des cultures cellulaires de *Nicotiana tabacum*, des plantes d'*Arabidopsis thaliana* (pour *nad9, cob, cox3* et *atp9*) et des plantes de *N. tabacum* (pour *sdh3*). L'invalidation spécifique des ARN mitochondriaux ciblés par les ribozymes a été établie *in vivo*. La réponse, en termes de régulation, à l'invalidation des cibles individuelles a été analysée au niveau de l'ensemble du transcriptome. Alors qu'il a été généralement considéré jusqu'à présent que les processus de régulation mitochondriaux chez les plantes se passent essentiellement au stade post-transcriptionnel, nos résultats sont fortement en faveur de mécanismes de coordination des ARNm dans les mitochondries et entre les organites et le noyau.

Abstract

The complex genetic system of higher plant mitochondria could not be studied by transgenic approaches because conventional methods do not permit genetic transformation of these organelles. An alternative approach has been developed in the laboratory, thanks to the existence of a natural process of transfer RNA (tRNA) import from the cytosol into mitochondria. It was shown that a tRNA mimic can be used *in vivo* as a shuttle for importing into plant mitochondria passenger RNAs expressed from nuclear transgenes. Taking a *trans*-cleaving ribozyme as a passenger sequence allowed to obtain the specific knockdown of a major messenger RNA (mRNA) in the mitochondria of transformed plant cells. We used this strategy to develop mitochondrial regulation studies. Five mitochondrial mRNAs (*nad9, sdh3, cob, cox3* and *atp9*) were chosen as targets for specific *trans*-cleaving hammerhead ribozymes. After validating the *in vitro* activity of these ribozymes, the corresponding expression constructs served to transform *Nicotiana tabacum* cells, *Arabidopsis thaliana* plants (for *nad9, cob, cox3* and *atp9*) and *N. tabacum* plants (for *sdh3*). Specific *in vivo* ribozyme-mediated knockdown of the targeted mitochondrial RNAs was established. The regulation response to the knockdown of the individual targets was analyzed at the whole transcriptome level. Whereas it has been generally considered so far that mitochondrial regulation processes in plants essentially occur at the post-transcriptional stage, our results strongly support mRNA coordination mechanisms within the organelles and between the organelles and the nucleus.