UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE Sciences de la vie et de la santé

Institut de Biologie Moléculaire des Plantes

THÈSE présentée par :

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soutenue le : **07 juillet 2017**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Aspects Moléculaires et Cellulaires de la Biologie/Biologie végétale

APPROCHES BIOTECHNOLOGIQUES DE L'EXPRESSION ET DE LA DIVERSITE DU GENOME MITOCHONDRIAL DES PLANTES

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Acknowledgements

I would bow my head before ALLAH. The almighty, the omnipresent, the merciful, the most gracious, the compassionate, the beneficent, who is the entire source of all knowledge and wisdom endowed to mankind and who blessed me with the ability to do this work. I could not ever have done this work without the belief I have in ALLAH.

This thesis represents not only my work at the keyboard; it is the result of hard work of four years at IBMP specifically within the GenEx department.

First and foremost I wish to thank my supervisors, **Dr. Andre DIETRICH** and **Dr. Jose Manuel GUALBERTO** for their interest, inspiring guidance, constructive criticism. Without their constant help, deep interest and vigilant guidance, the completion of this work would not have been possible. I am indebted to them for this accommodative attitude, patience, sympathetic behavior and administrative measures in providing me all the necessities required to complete this work.

My humble and respectable thanks to all the members of the PhD jury: Prof. Claire REMACLE, Prof. Katarzyna ROLLE, Prof. Patrick VERNET and Dr. Nina ENTELIS, who kindly accepted to evaluate my PhD work.

I have great honor to express my most sincere gratitude and heartfelt thanks to all my lab members: Frederique WEBER-LOTFI, Daria MILESHINA, Monique LE RET, Clementine WALLET, Nicolas CHEVIGNY, Arnaud FERTET, Deborah SCHATZ, Pierre MERCIER and Adnan Khan NAIZI for their experimental and technical support during my PhD manipulations. I am truthfully grateful to Adnan Khan NAIZI and Frederique WEBER-LOTFI for their deep kindness and support during my PhD work on experiments and input in the thesis write up. I would like to acknowledge Abdelmalek ALIOUA, who always helped me in the statistics, sequencing and qPCR. My special thanks to Pierre MERCIER for doing different joint experiments. My sincere gratitude is also extended to all members of GenEx department.

I am obliged to all gardeners for taking care of my plants in the green house during my experiments. Also thanks to Jerome MUTTERER for sharing his computer for doing statistical calculations.

I owe a humble and respectable gratitude to all my teachers in my life whose sincere efforts enable me to reach at this destiny. I am humbled to thank my Pakistani students in France: Azhar Ayaz, Madah, Ikram, Waseem, Zahid, Naeem, Asad, Abid, and Hira and many more for their sincerity and support in making my stay easier and lively in Strasbourg, France. Many friends from Pakistan also encouraged me during this especially Asma.

I am thankful to Bahauddin Zakariya University, Multan, and Higher Education Commission (HEC), Pakistan for my financial support to continue my PhD in France.

I would like to thank my parents Mr. & Mrs. Muhammad Anwar who provide me all the inspiration and courage to complete my work. Special thanks to my Brothers and Sisters especially Gauri and Iqra (my cute nephew) for their immense support. In the last but not least, I would like to extend thanks to all friends and colleagues for their appreciation, encouragement and moral support during my research work.

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INTRODUCTION

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MATERIALS AND METHODS

References

ABBREVIATIONS

A and mA	Ampere and milliampere
ADP	Adenosine diphosphate
ATP, dATP	Adenosine triphosphate, Deoxyadenosine triphosphate
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
BIR	Break induced repair
bp, kb	Base pair, kilobases
BSA	Bovine serum albumin
°C	Degree (s) Celsius
CaMV	Cauliflower Mosaic Virus
cm, µm and nm	Centimeter, micrometer and nanometer
CMS	Cytoplasmic male sterility\
cpDNA	Chloroplast DNA
CRR	Chloroplast retrograde regulation
Ct	Threshold cycle
DR	Direct repair
DSB	Double strand break
DSBR	Double strand break repair
DNAPs	Organellar DNA polymerases
DNA/cDNA/mtDNA	Deoxyribonucleic acid / Complementary/Mitochondrial
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetate
eGFP	Enhanced green fluorescent protein
mtExo	Mitochondrial exosome
mtTFAM/mtTFBM	Mitochondrial transcription factor A/B
g, mg, µg and ng	Gram, milligram, microgram and nanogram
GUN	Genomes uncoupled
h and min	Hour and minute
His	Histidine
HJ	Holiday Junction
HR	Homologous recombination
HSPs	Heat shock proteins
HDV	Hepatitis Delta Virus
HSPs/LSP	Heavy/light strand promoter
IMS	Mitochondrial intermembrane space
IR	Intermediate size repeat
L, mL and µL	Liter, milliliter and microliter
LB	Luria Bertani medium
LRs	Large repeated sequences
mmol and µmole	Millimole and micromole
M and mM	Molar and millimolar
MCS	Multiple cloning site

MELAS	Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis and Stroke-like episodes
MITOMAP	Human mitochondrial genome database
MRR	Mitochondrial retrograde regulation
MMBIR	Microhomology mediated break-induced replication
MMEJ	Microhomology-mediated end-joining
MMR	Mismatch repair
mtDNA	Mitochondrial DNA
MTS	Mitochondrial targeting presequence
NCS	Non-chromosomal stripe
NER	Nucleotide excision repair
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
nm	Nanometer
nt	Nucleotide
NTP	Nucleotide triphosphate
O_H / O_L	Origins of replication of heavy / light-strand
OGDRAW	Organellar Genome DRAW
ODB	Organellar DNA binding proteins
ORF	Open reading frame
OSB	Organellar single-stranded DNA binding proteins
OXPHOS	Oxidative phosphorylation
PAP	3’-phosphoadenosine 5’-phosphate
PCD	Programed cell death
PCR / qPCR	Polymerase Chain Reaction / quantitative PCR
PEGE	Pulse filed gel electrophoresis
PET	Photosynthetic electron transport
PGE	Plastid gene expression
RITOLS	Ribonucleotide incorporation throughout the lagging strand
PKTLS	Pseudoknot tRNA-Like Structure
POLRMT	Mitochondrial RNA polymerase
PPR	Pentratricopeptide repeat
RpoT/RpoTm/RpoTp/RpoTmp	Nuclear-encoded RNA polymerase / mitochondria- / plastid- / dual-targeted
RRR	Replication, repair and recombination
PRL1	Pleiotropic response locus 1
PS	Passenger sequence
PSI and PSII	Photosystem I and Photosystem II
PQ	Plastoquinone
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
RT	Reverse transcription

SDSA	Synthesis-dependent strand annealing
sec and ms	Second and millisecond
SSG	Single strand gap
SSA	Single strand annealing
SSB	Single strand binding
ssDNA	Single stranded DNA
SSS	Sub-stoichiometric shifting
TCR	Transcription coupled repair
T-DNA	Transfer DNA
TLS	tRNA-Like Structure
TYMV	Turnip Yellow Mosaic Virus
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

INTRODUCTION

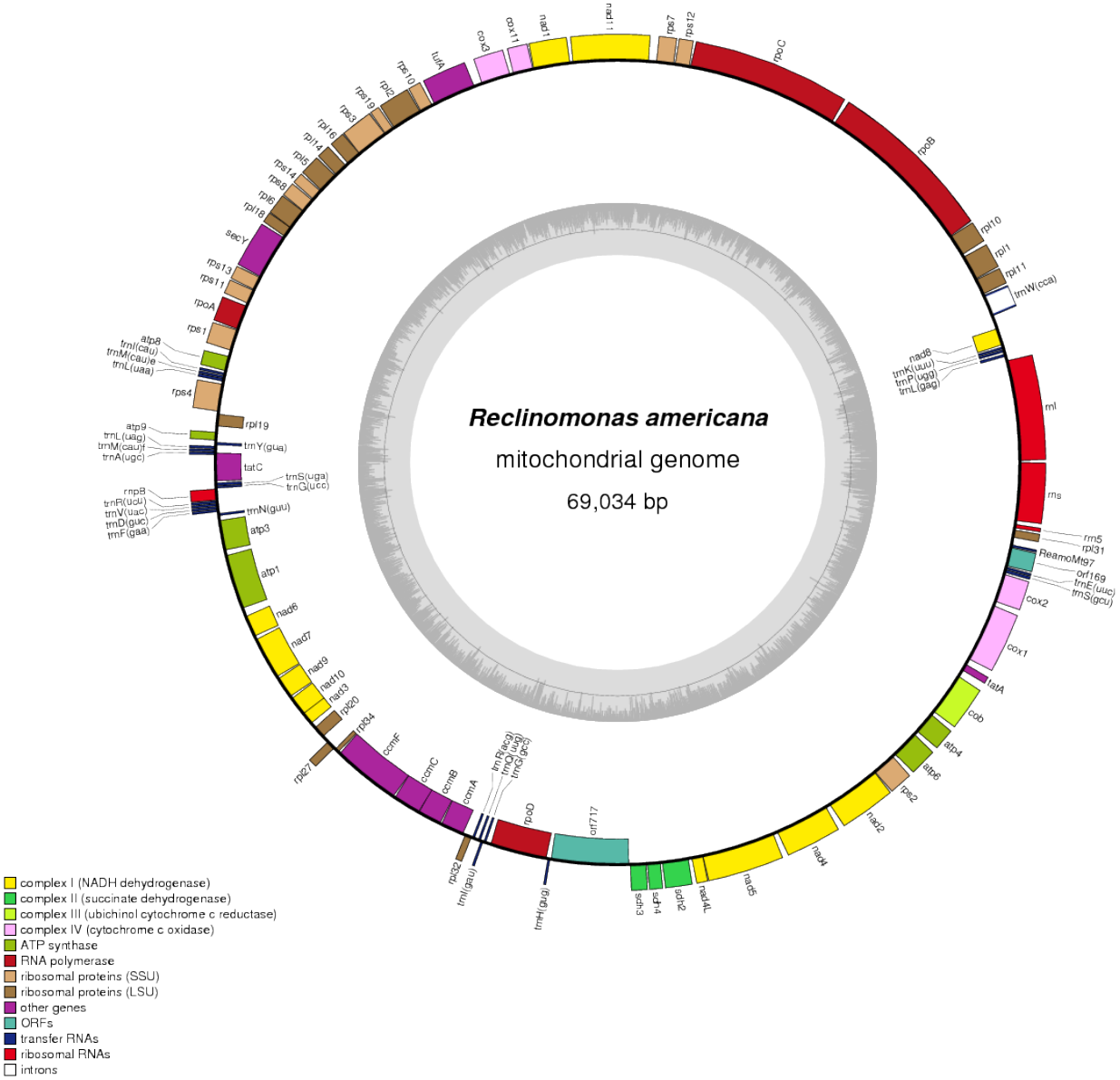


Figure 1: Physical map of the *Reclinomonas americana* mitochondrial genome. Obtained from the Organellar Genome DRAW website (ogdraw.mpimp-golm.mpg.de); Lohse, Drechsel et al. 2007 . The GC content is represented by the grey circle in the middle. GenBank: NC_001823.

I. Organization of the mitochondrial genome

It is considered that mitochondria evolved from free-living organisms resembling α-proteobacteria, following a single endosymbiotic event over 1.5 billion years ago (Gray, Burger et al. 2001; Margulis, Chapman et al. 2006). Nuclear genes encode the vast majority of the mitochondrial proteins but mitochondria have kept their own DNA, which replicates independently. Now the complete mitochondrial genomes of many eukaryotes have been sequenced. Mitochondria ensure vital functions such as energy production, regulation of the redox status, metabolic pathways and programmed cell death. Still, regardless of the many conserved biochemical functions, one of the most striking features of mitochondria, in both plants and animals, is their remarkably dissimilar genomes. Mitochondrial genomes of different organisms vary in size, gene content and organization.

I.1 The mitochondrial genome of protists

Protists are considered to be among those organisms that developed mitochondria, thus, data got from protist mitochondrial genomes can help in better understanding their origin. The length of protist mtDNA is on average 40 kb, further, 70% of its nucleotides are A+T. Protists have both linear and circular genome but the circular genome is predominant. Among eukaryotes, they have the highest number of genes in the mitochondrial genome. In gene alignment, the mtDNA of protists stays nearer towards plants as compared to animal or fungi (Lang, Burger et al. 1997; Burger, Gray et al. 2013). The mtDNA of the Jakobid protozoan *Reclinomonas americana* has 97 genes, which are encoding proteins and stable RNAs. The genome of *R. americana* can be represented as a unique circular map (Figure 1), with a high density of genes that are found on both strands. Further sequenced mtDNAs encompass only part of the *Reclinomonas* gene content. It is proposed that the set of genes of the *R. americana* mitochondrial genome is the closest to that of the pro-mitochondrial genome. During evolution, mitochondria lost most of their genes, due to reduction or migration into the nucleus, but this happened differently in different evolutionary lines. The extra number of genes in *R. americana* is considered to play an important role in mitochondrial biogenesis (Odintsova and Iurina 2002; Burger, Gray et al. 2013). *R. americana* has 18 unique mitochondrial genes. These 18 genes have still not been reported in any other mtDNA. Among them, some participate in the mitochondrial oxidative phosphorylation (OXPHOS) chain, but others play important roles in other functions, *e.g. tufA* (a

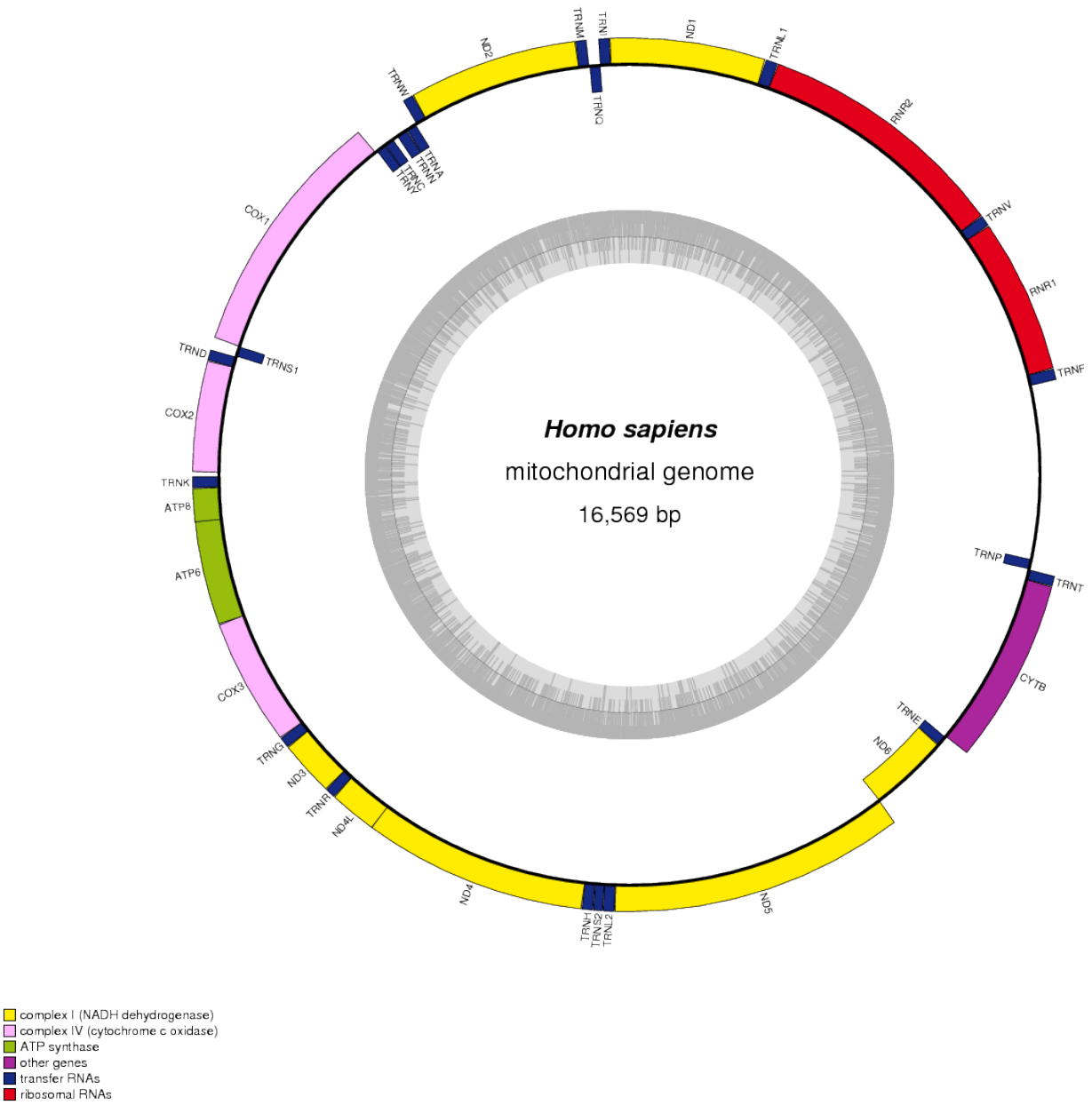


Figure 2: Physical map of the *Homo sapiens* mitochondrial genome. Obtained from the Organellar Genome DRAW website (ogdraw.mpimp-golm.mpg.de); Lohse, Drechsel et al. 2007. The GC content is represented by the grey circle in the middle. GenBank: NC_012920.

translation factor) and *secY* (involved in protein translocation). The transfer RNAs (tRNAs) and the ribosomal RNAs (rRNAs) encoded in the mitochondrial genome of protists remain relatively alike their suggested eubacterial ancestors. While a small number of protist mtDNAs codes for a minimal tRNA set that is enough to read all codons, the majority of them has only a few tRNA genes. For this reason, the rest of the tRNAs must be imported from the cytosol and is encoded by the nucleus. Protist mitochondrial genes rarely have introns; however the mitochondrial genomes of certain photosynthetic green, as well as red algae contain introns, which are also predominant in plant mitochondrial genomes. Such resemblances between photosynthetic protists and plants can propose a common pedigree for their mitochondrial genomes (Lang, Gray et al. 1999).

I.2 The mammalian mitochondrial genetic system

The mitochondrial genome of mammals is represented as a closed, circular DNA molecule. The structure as well as the organization of the genes in the mtDNA is greatly conserved among mammals (Moustafa, Uchida et al. 2015). Human mitochondria have a 16.6 kb genome, which encodes 13 proteins, 2 rRNAs and 22 tRNAs (Figure 2) (Gustafsson, Falkenberg et al. 2016). The OXPHOS system is made of about 90 proteins, which have a double genetic basis. These proteins are encoded either by nuclear genes, translated on cytosolic ribosomes then imported into mitochondria, or encoded by the mtDNA then translated on mitochondrial ribosomes. The components encoded by the mtDNA are considered to be vital, as they are needed to build up the OXPHOS complexes in the mitochondrion and produce cellular ATP.

The strands of the mammalian mtDNA molecule are depicted as heavy (H) and light (L) strand, due to their diverse buoyant densities in a cesium chloride gradient. This variation results from the base composition of each strand: the H-strand is guanine-rich, while the L-strand is guanine poor. The non-coding region (D-loop), which is known as the “control region”, comprises the main controlling components for the replication and expression of the mitochondrial genome in mammals. The D-loop region sizes 880 bp to 1400 bp, depending on the species. Due to repeated sequences, the length of the D-loop can be extended significantly in several species. The control region contains the promoters for the transcription of each strand. These promoters are termed light-strand promoter (LSP) or heavy-strand promoter (HSP), depending on which mtDNA strand functions as the template for transcription. Upon transcription of each strand, large

polycistronic transcripts are formed, that will be further processed to generate the mature tRNAs, rRNAs and mRNAs letting to the expression of the mitochondrial information (Shadel and Clayton 1997; Gustafsson, Falkenberg et al. 2016). Transcription termination sequences prevent transcription from proceeding into the control region. L-strand transcription is taking place from one single promoter (LSP). Whereas H-strand transcription is started from two precise and differentially regulated sites that are named as HSP1 and HSP2. The transcription start site of HSP1 is found nearly 100 bp upstream of HSP2, which generates a transcript that covers the two rRNA genes and terminates at the 3'-end of the 16S rRNA-encoding gene. The HSP2 transcription start site is found near the 5'-end of the 12S rRNA gene, which generates a polycistronic transcript from almost the whole H-strand, covering the two rRNA genes and 12 mRNA encoding genes. The polycistronic precursor RNAs are then further processed to generate the individual tRNAs, rRNAs and mRNAs. Excision of the tRNAs from the polycistronic transcripts is used to create the mature mRNAs and rRNAs. This approach of RNA processing is called the 'tRNA punctuation model'. RNA production from the light strand is well-ordered through the LSP promoter, which produces transcripts encoding one protein (ND6), eight tRNAs, and produces the RNA primer that is required for the initiation of mtDNA replication from the origin of H-strand DNA replication (O_H) also located in the D-loop (Campbell, Kolesar et al. 2012). Replication of the mtDNA is different from that of the genomic DNA, but the exact mechanism is still a matter of discussion.

In the past, mammalian mitochondrial genome replication was investigated by usage of cesium chloride-purified mtDNA visualized by electron microscopy (McKinney and Oliveira 2013). The strand-displacement model is used since the last 35 years to account for the mammalian mtDNA replication. In this model, the process of replication is started at the O_H in the D-loop and DNA synthesis continues in one direction to produce a new H-strand (leading strand). At this time, there is no synthesis of the second strand (lagging strand). As the synthesis of the H-strand continues along the template, the mtSSB single-stranded DNA (ssDNA)-binding protein covers the parental H-strand. In this way, mtSSB inhibits the chances of random initiation of RNA primer synthesis on the displaced strand. At about two-thirds of the distance around the genome, the replication origin of the lagging strand (O_L) is reached, so that the replication of the lagging strand starts and the process of replication proceeds continuously. Elongation of the lagging strand continues along the previously displaced single-stranded template in the opposite

direction. So, replication of both strands is linked, because synthesis of the leading strand is necessary for the synthesis of the lagging strand. In this way, both strands are synthesized in an asymmetric manner until two daughter molecules are formed. In the strand displacement model, initiation and termination sites are not similar for the two strands. Continuously, replication beginning and finish of the leading strand will take place at O_H, while for the lagging strand it will be at O_L. Further, the factors that are necessary for the production of the two daughter strands are not the same. Synthesis from O_H uses a double-stranded DNA (dsDNA) as a template for initiation and for this it depends on the TWINKLE factor for unwinding of the DNA. On the other hand, synthesis from O_L initiates directly on ssDNA and therefore TWINKLE is not needed (Clayton 2000; Clayton 2003; McKinney and Oliveira 2013). In this case, the process is unidirectional, continuous, asymmetric and asynchronous.

Two further models have been proposed for the replication of the mammalian mtDNA, the model of ribonucleotide incorporation throughout the lagging strand (RITOLS) and the model of strand-coupled mtDNA replication. Both of these models are principally built on mtDNA replication intermediates that were observed by using neutral two-dimensional agarose gel electrophoresis (called as 2-D-AGE).

In the ribonucleotide incorporation throughout the lagging strand (RITOLS) model, replication takes place in the same manner, except that the displaced parental H-strand is coated with RNA fragments (Yasukawa, Reyes et al. 2006). For adding of RNA intermediates there are two proposed theories. First, that the RNA intermediates in this model are processed transcripts (comprising tRNA and rRNA) that are continuously attached with the lagging-strand template, so that the replication fork proceeds and they remain attached until they separate, are degraded or even they can be processed further in the lagging-strand DNA synthesis (Wanrooij, Fuste et al. 2008; Gustafsson, Falkenberg et al. 2016). According to the “bottlace theory”, the RNAs created during transcription are annealed to the displaced strand and are then processed into small fragments. But in the bottlace theory, the different enzymes that are required for the hybridization of RNA intermediates to DNA are not defined. Furthermore, it is uncertain how highly structured molecules like tRNAs and rRNAs can be melted to anneal to ssDNA.

According to the strand-coupled model, the displaced H-strand is covered with the mitochondrial single-stranded DNA binding protein (mtSSB). Initiation occurs bidirectionally but is stopped

upon reaching in the initiation zone O_H (Yasukawa, Reyes et al. 2006). Synthesis of the leading strand is continuous in the 5' → 3' direction, by DNA polymerase γ (Pol γ). But the lagging strand must be synthesized in short fragments, primed by RNA (Okazaki fragments). The processing of Okazaki fragments takes place in the same way as it happens in the nuclear replication, through specific helicases and flap-endonucleases. PIF1 is a helicase that unwinds short flaps, having the 5' RNA primers for every Okazaki fragment (Futami, Shimamoto et al. 2007; Li, Lin et al. 2016). After this, the single strand binding protein SSBP1 binds to the DNA in the flap to modulate the association of the flap endonucleases DNA2 and FEN1, which cleave the 5' of the flap and the DNA polymerase fills the gaps (Liu, Qian et al. 2008; Wanrooij, Fuste et al. 2008). Each fragment is joined by DNA ligase III to form a continuous strand.

It is considered that mitochondria have almost 1,200 diverse proteins and many of them are needed for the expression of the mtDNA. The expression of mtDNA involves different levels of regulation such as replication, transcription, processing of primary transcripts, mtDNA maintenance, RNA modification, RNA stability, coordination of translation, translation by mitochondrial ribosomes, and insertion of many proteins into the mitochondrial inner membrane (Clayton 2000; Gustafsson, Falkenberg et al. 2016).

I.3 The plant mitochondrial genome

Plant mitochondria have a complex and peculiar genetic system. The plant mitochondrial genome has a number of features that distinguish it from the animal and fungal mitochondrial genomes and from those of other living beings (Yurina and Odintsova 2016). The mtDNA of higher plants and photosynthetic algae vary extremely in size, as well as in structural organization. The mitochondrial genome of animal cells has a small circular and compact structure. But the mitochondrial genome of photosynthetic eukaryotes can have a circular or a linear structure: *e.g.* the genomes of green algae can be linear like in *Chlamydomonas reinhardtii* (Allen, Fauron et al. 2007; Smith, Hua et al. 2010; Lohse, Drechsel et al. 2013). The mitochondrial genome of *Euglena gracilis* is a mixture of various linear DNA molecules (Kolesnikov and Gerasimov 2012). However, such a structure could not be found in the bryophyte *Marchantia polymorpha* (Oda, Yamato et al. 1992; Oldenburg and Bendich 1998) by electron microscopy. The mitochondrial genomes of a few plant species are larger than bacterial genomes in the genus *Silene* (Sloan, Alverson et al. 2012). Conversely, the mitochondrial

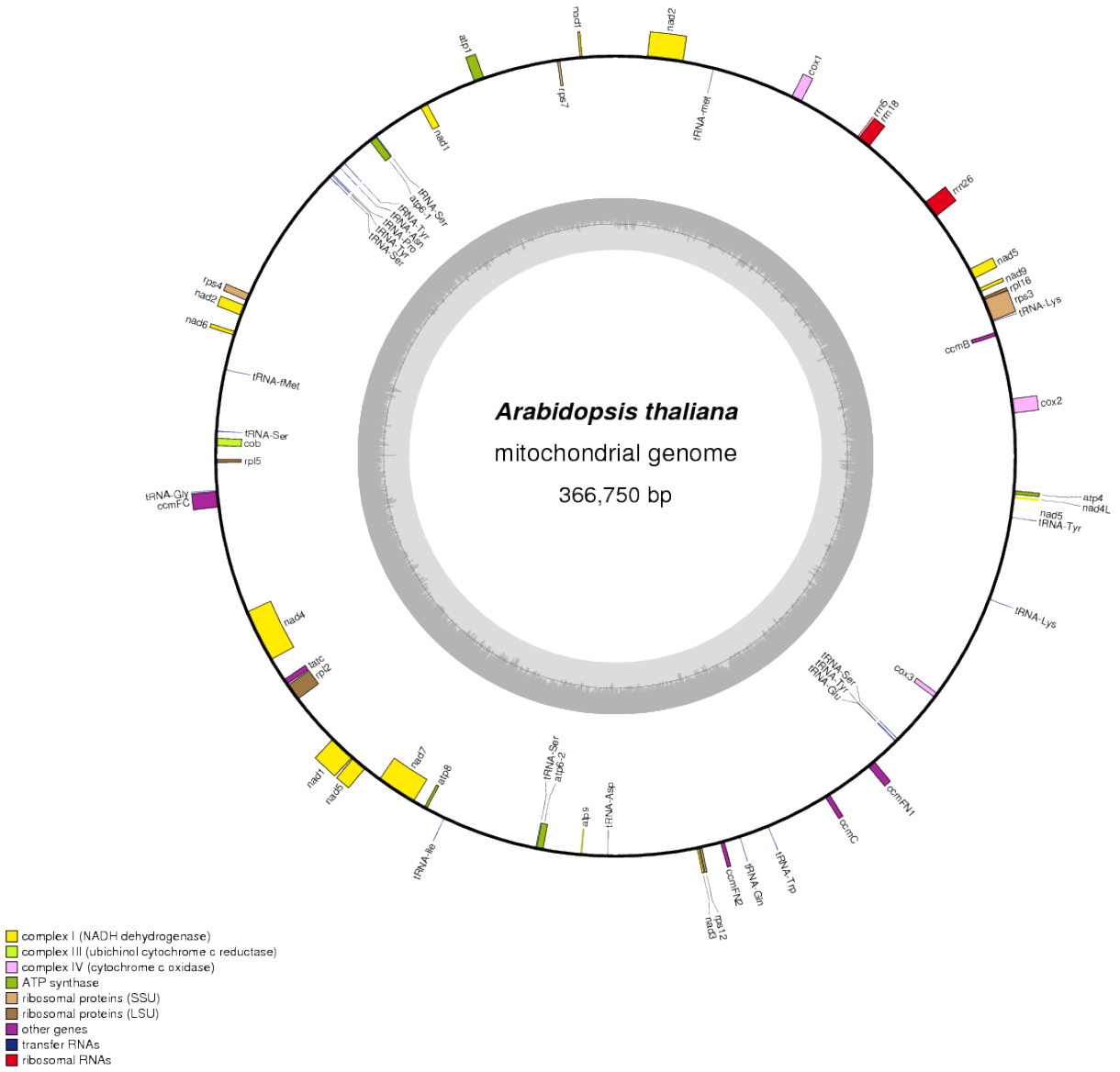


Figure 3: Physical map of the *Arabidopsis thaliana* mitochondrial genome. Obtained from the Organellar Genome DRAW website (ogdraw.mpimp-golm.mpg.de);Lohse, Drechsel et al. 2007. The GC content is represented by the grey circle in the middle. GenBank: JF729201.

genome of the liverwort *Marchantia pomlymorpha* comprises 186 kb (Ohyama, Takemura et al. 2009). The common mtDNA size in angiosperms is usually in the range of 200 to 700 kb. The number of mitochondrial protein genes fluctuates from 32 to 67, reflecting gene losses and transfers to the nuclear genome during evolution. The size of the first completely sequenced mtDNA, that of *Arabidopsis thaliana* (ecotype C24), is 367 kb. It encodes 32 proteins, 3 rRNA (5S, 18S, and 26S), and 22 tRNAs (Figure 3). This means roughly a doubling of the protein gene number for a 22 fold larger mtDNA than that of mammals. Also, the big differences that can occur between the plant mtDNA sizes is not reflecting the gene content but rather the large amount of non-coding DNA in these genomes (*i.e.* dispersed repeats, introns, intergenic spacers and extraneous DNA sequences). As stated above, plant mitochondria have a large amount of non-coding sequences (up to 90%), In the multicellular green alga *Volvox carteri*, most of the non-coding sequences consist of short palindromic (inverted) repeats (11-77 bp) spread over the intergenic and intronic parts of the mtDNA (Yurina and Odintsova 2016). At the point when palindromes form hairpin structure, the length of the hairpin stem is 4-7 bp, while the loop has 3-5 bp and regularly comprises the 5′-TAAA-3′ or 5′-TTTA-3′ sequences. Often, a short palindromic repeat is embedded into another palindrome; subsequently, extra complex bigger repeats are shaped. The maximal length of such repeats is 633 bp Some of them can form tRNA like structures (Smith and Lee 2009).

I.4 Structure of the plant mitochondrial DNA

Historically, the mitochondrial genome of angiosperms was described as a circular molecule of double-stranded DNA, having a full set of mitochondrial genes and called “master chromosome” (Mower, Case et al. 2012). The size of mitochondrial genomes of the master chromosomes is 221 kb in rape and 739 kb in maize lines of CMS-C (Yurina and Odintsova 2016). As these values incorporate the arrangements of non-coding sequences and duplications, the gene density of plant mtDNA is very low (Sugiyama, Watase et al. 2005; Kubo and Newton 2008). Every master chromosome contains one or several pairs of large repeated sequences, in inverse or direct orientation, with dynamic recombination between them. This results in different isomeric sub-forms of the master chromosome, depending on the orientation of the copies of repeated sequences (Sugiyama, Watase et al. 2005; Yurina and Odintsova 2016). Thus, the mitochondrial genomes mapped in angiosperms display as a main circular master chromosome, with a complete set of mtDNA genes, plus a pool of different subgenomic rings formed due to homologues

recombination between repeated sequences (Sugiyama, Watase et al. 2005; Kubo and Newton 2008; Alverson, Rice et al. 2011; Sloan, Alverson et al. 2012; Wang, Chu et al. 2014). The structure of the subgenomic forms of the mtDNA may fluctuate significantly in various angiosperm species. For instance, the mitochondrial genome of tobacco comprises the master chromosome (430 bp) and six subgenomic forms. The master chromosome has 36 protein-coding genes, three rRNAs genes and 21 tRNAs genes (Sugiyama, Watase et al. 2005). The mitochondrial genome of *Raphanus sativus* can be presented as the master chromosome and two small circular subgenomic forms. The *Eruca sativa* mitochondrial genome may be divided into six master circles and four subgenomic molecules *via* three pairwise large repeats (Wang, Chu et al. 2014). The mitochondrial genome of cucumber (*Cucumis sativus*, Cucurbitaceous) likewise has three components. It contains one big master chromosome (1556 kb) plus two small independent chromosomes of 45 and 84 kb. A complete set of mitochondrial genes is located on the main chromosome. Functional genes are not found on the small chromosomes. Repeated sequences of significant size similar to master chromosome sequences are not present on the small chromosomes (Alverson, Rice et al. 2011; Sloan 2013). These are independent, although they can sometimes recombine with the rest of the genome.

The mitochondria of numerous plant species also contain different sorts of plasmids replicating independently from the main chromosome. The occurrence of self- replicating plasmids with commonly unknown functions also increases the level of complexity in the mitochondrial genomes by recombination of repeats (Yurina and Odintsova 2016). For instance, maize CMS-S mitochondria additionally contain free linear DNA plasmids bearing terminal inverted repeats (TIRs). These plasmids recombine with TIR-homologous sequences inside the main mitochondrial genome and create linear ends (Matera, Monroe et al. 2011).

I.5 Evolution of the plant mtDNA by recombination

As mentioned, mitochondrial genomes of photosynthetic eukaryotes are characterized by a high structural variability and a gene arrangement differing considerably not only between evolutionary distant but also between closely related genera (Woloszynska, Kmiec et al. 2006; Wang, Chu et al. 2014). Intraspecies changes in mtDNA length and in the order of gene arrangement appear frequently in angiosperms (Sugiyama, Watase et al. 2005; Kubo and Newton 2008). For example, intraspecies diversity of the mitochondrial genome is observed in the two

plant species *Beta Vulgaris* and *Silene vulgaris* related to gynodioecy (having only female flowers on some plants and bisexual flowers on the other plants of the same species). The plants having such kind of mtDNA are responsible for male sterility commonly happening in natural populations (McCauley 2013; Sloan 2013). Awesome interspecies contrasts in mtDNA structural organization are seen in angiosperms (*e.g. Nicotiana tabacum, A. thaliana, B. vulgaris, Oryza sativa, and Brassica napus*). The repeated sequences have no homology to each other in these angiosperms. The sequences of intergenic spacers and the organization of genes are very much different, which shows a common reorganization of the mitochondrial genome structure during the evolution of higher plants (Sugiyama, Watase et al. 2005). For example, *A. thaliana* and *B. vulgaris* (sugar beet) have only 21% of similar nucleotide sequences in the mitochondrial genomes (Kubo and Newton 2008). Similarly, significant interspecies changes in the mitochondrial genome size and structural organization are observed in green algae.

The mtDNA of vascular plants usually has recombining repeated sequences, which are a cause of rearrangements. Due to this, the plant mitochondrial genome can exist as a population of different structures. Homologous recombination between long repeats is particularly common, resulting in interconvertible genomic structures. It has been demonstrated that recombination includes 6.5 and 4.2 kb mtDNA repeats in *A. thaliana.* Sequence evaluations in *A. thaliana* accessions Ler, Col-0 and C24 revealed the loss of one copy of the 4.2 kb large repeat in Ler (Forner, Weber et al. 2005; Gualberto, Mileshina et al. 2014). Homologous recombination between medium length repeats (50-600 bp) happens infrequently but prompts complex genomic rearrangements. Thus, medium length repeats are considered to ensure a key role in the evolution of angiosperm mitochondrial genomes (Kubo and Newton 2008). Recombination with the contribution of short homologous nucleotide repeats comprising of a few nucleotides may prompt the arrangement of chimeric genes (Yurina and Odintsova 2016). Some mtDNAs appeared to lack one copy of the common large repeated sequences. The circular mapping mitochondrial genome of white mustard (*Brassica hirta*) was viewed as an exception among angiosperms since it had no large repeats with high recombination activity. Anyhow, it was recently demonstrated that the sequenced mitochondrial genomes of grape (*Vitis vinifera*), pumpkin (*Cucurbita pepo*), mung bean (*Vigna radiata*), duckweed (*Spirodela polyrhiza*), and bamboo (*Bambusa oldhamii*) do not have repeats of more than 1 kb either. Hence the proposal that long repeats ensure no critical role in mitochondrial genome functioning. The complex

structural organization of the different mitochondrial chromosomes of plants makes it hard to investigate the mechanisms of inheritance and replication of these mtDNA (Sloan 2013).

I.6 Heteroplasmy in the evolution of the mtDNA

When more than one mitochondrial genotype is found in an individual this condition is called heteroplasmy. Normally, one mitochondrial genotype, which is called mitotype, is predominant, while the second exists in low extent (Woloszynska and Trojanowski 2009). The predominant mitotype is responsible for the determination of the phenotype of the organism. In the start of the twenty-first century, heteroplasmy was seen as a normal condition in plant mitochondria (Kmiec, Woloszynska et al. 2006). Heteroplasmy is very much reported in the studies on cytoplasmic male sterility (CMS). The principal confirmation of heteroplasmy in plants was accounted for in maize normal and sterile (S and T) cytoplasms. In angiosperms, heteroplasmy has been detected in the natural population as the effect of biparental inheritance of the mitochondrial genome (Mogensen 1996). In gynodioecious plants, mitochondrial heteroplasmy appears to be a typical characteristics of natural populations (Welch, Darnell et al. 2006). Recently, by using quantitative Real Time PCR for the *atp1* and *cox1* genes, 61 out of 408 individuals representative for 22 normal populations of *Silene vulgaris* showed mitochondrial heteroplasmy, pointing out that the phenomenon is very much spread in this species (Pearl, Welch et al. 2009).

The most common reason for the heteroplasmy in plant mitochondria is recombination happening *via* short repeats. In heteroplasmic plants, two parental forms of the short recombination repeats are found in the main mitochondrial genome (Vitart, De Paepe et al. 1992; Hartmann, Recipon et al. 1994; Kanazawa, Tsutsumi et al. 1994; Albert, Lelandais et al. 2003; Woloszynska and Trojanowski 2009; Gualberto, Mileshina et al. 2014). But substoichiometric molecules contain both or only one of the corresponding crossover products (Hartmann, Recipon et al. 1994; Kanazawa, Tsutsumi et al. 1994; Bellaoui, Martin-Canadell et al. 1998). This situation can be explained through the phenomenon of recombination taking place between the short repeats that are found in the main mitochondrial genome (Janska, Sarria et al. 1998; Arrieta-Montiel, Lyznik et al. 2001). Recombination between short repeats can be followed by the loss of one of the products due to its replication deficit or selective removal in mtDNA segregation (Arrieta-Montiel, Lyznik et al. 2001). The studies show that some sublimons have substantial resemblance to the main genome molecules, but contain kilobases of sequences that

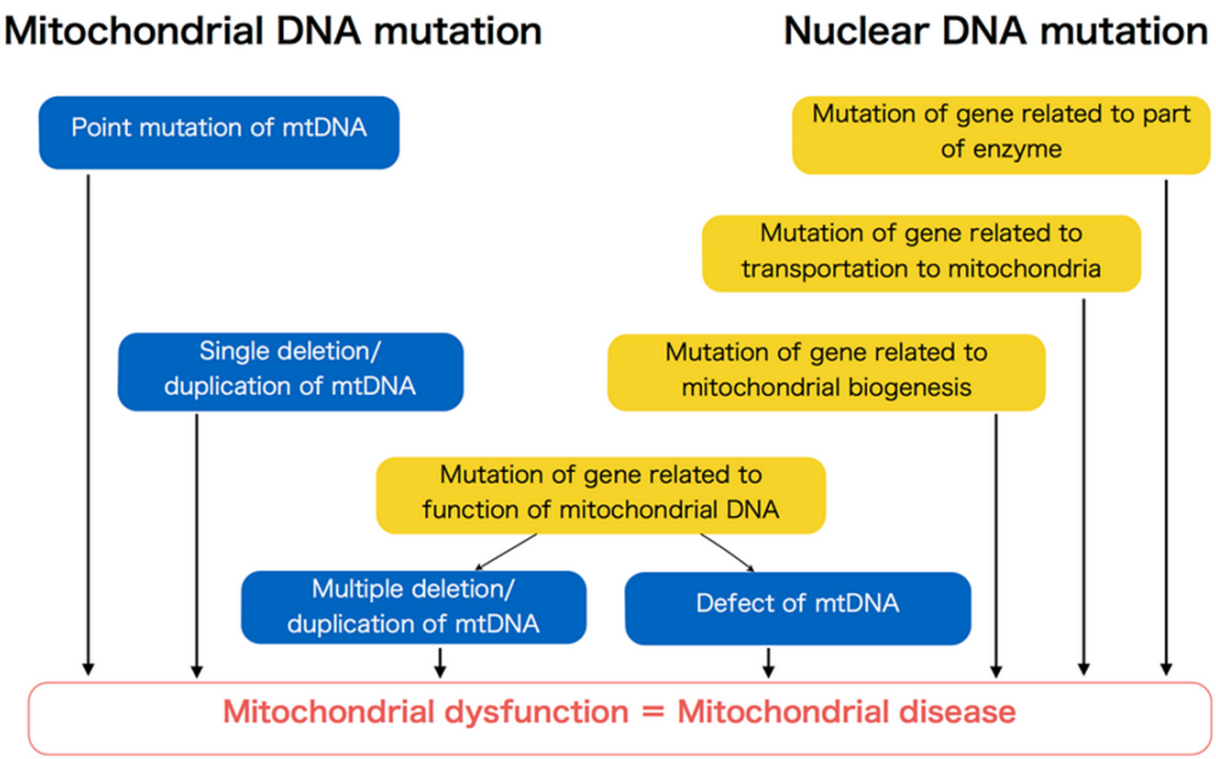


Figure 4: Classification of mitochondrial diseases. Different proteins required for the main functions of a mitochondrion are synthesized from the mtDNA. mtDNA abnormalities decrease mitochondrial function and prevent energy supply, eventually causing mitochondrial diseases (adapted from: Mt-handbook, Genetic Counseling Unit, National Center of Neurology and Psychiatry, http://koinobori-mito.jp/index.php?pathinfo=koinobori-mito.jp/index.php&pathinfo=en/mito-disease-list).

are not similar to the main genome. This cannot be explained only by one recombination event involving small repeats, but likely goes through further sequence rearrangements happening along rapid evolution. Such sequence rearrangements can occur through secondary recombination events and also insertions/deletions or DNA transfer between organelles. The resultant sublimons differ significantly from the original mitotype. But understanding the process is possible only when we know about the common progenitor mtDNA or the molecular intermediates in this chain of rearrangements that occurs in the mitochondrial genomes of phylogenetically similar species (Arrieta-Montiel, Lyznik et al. 2001).

II. Importance of the mitochondrial genome

II.1 Mitochondrial disorders in humans

In the cells of eukaryotic organisms, mitochondria ensure vital functions such as energy production through oxidative phosphorylation, or metabolic pathways. They are therefore essential for cell survival (Greaves and Taylor 2006; Berkowitz, De Clercq et al. 2016). Any mutation in the mitochondrial genome may be expected to result in neurodegenerative diseases in human beings or to contribute to diseases such as Alzheimer's disease or some types of cancer and diabetes (Schon, DiMauro et al. 2012). On the other hand, many mitochondrial diseases can be caused by nuclear gene disorders because most of the proteins that are essential for mitochondrial metabolism and maintenance are nuclear-encoded (Figure 4). Changes in nuclear genes coding for mitochondrial proteins have been studied in mitochondrial defects in patients and nuclear genetic problems can be responsible for abnormalities in the mitochondrial genome of patients (Greaves and Taylor 2006; Yoon, Koob et al. 2010; Legati, Reyes et al. 2016). At any age, mitochondrial syndromes can be present. In the past, it was generally considered that syndromes from nuclear DNA abnormalities would normally show up during childhood, while consequences of abnormalities in the mtDNA (primary or secondary to a nuclear DNA abnormality) would appear in later childhood or in adult life. But latest research established that a lot of mtDNA disorders can be present already during childhood, while disorders due to mutations in nuclear genes can appear in the adult life (Leonard and Schapira 2000; Koopman, Willems et al. 2012; Vafai and Mootha 2012; Koopman, Distelmaier et al. 2013; Chinnery 2015). Thousands of copies of mtDNA are present in each human cell. Naturally, these copies are the same at birth, i.e. they are homoplasmic. Conversely, following a mutation in the

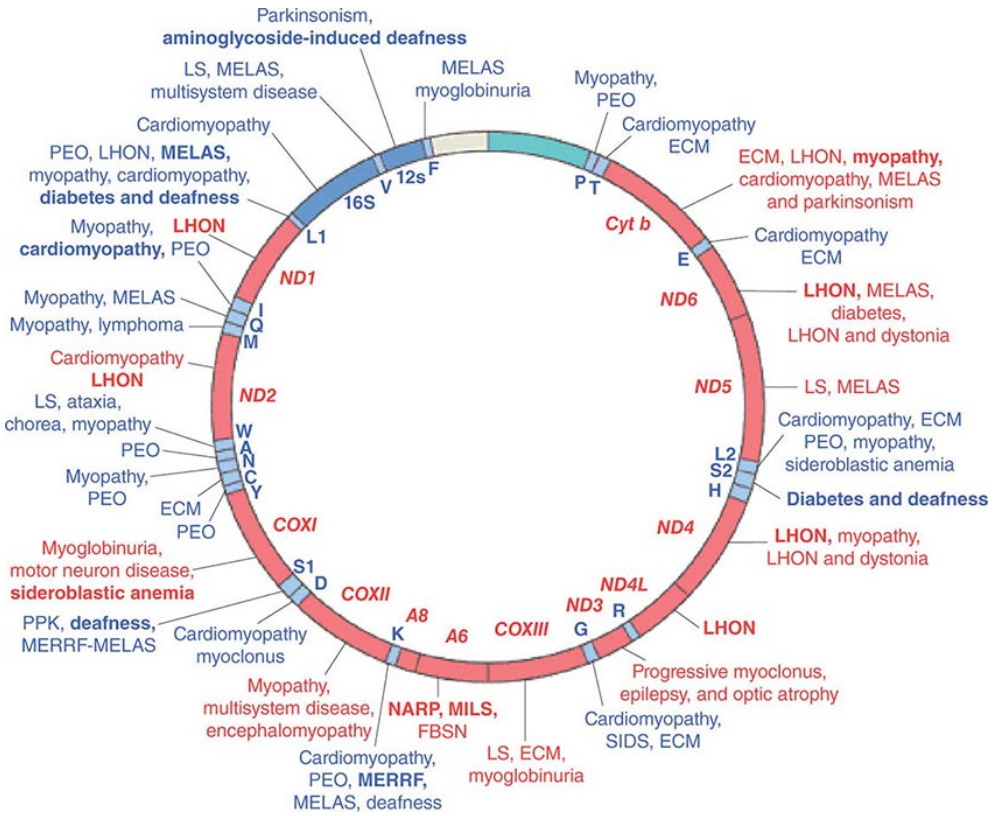


Figure 5: Mutations in the human mitochondrial genome known to cause disease.

Disorders that are frequently or prominently associated with mutations in a particular gene are shown in **boldface**. Diseases due to mutations that impair mitochondrial protein synthesis are shown in blue. Diseases due to mutations in protein-coding genes are shown in red. ECM, encephalomyopathy; FBSN, familial bilateral striatal necrosis; LHON, Leber's hereditary optic neuropathy; LS, Leigh syndrome; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibers; MILS, maternally inherited Leigh syndrome; NARP, neuropathy, ataxia, and retinitis pigmentosa; PEO, progressive external ophthalmoplegia; PPK, palmoplantar keratoderma; SIDS, sudden infant death syndrome. (adapted from Susan M. Domchek 2005)

mtDNA, individuals will be heteroplasmic and carry a mixture of mutated and wild-type mtDNAs in each cell (Holt, Harding et al. 1988; Holt, Harding et al. 1990; Sallevelt, de Die-Smulders et al. 2017). The study of patient cells and cybrids has shown that for the biochemical expression of a mitochondrial mutation the proportion of mutated mtDNA copies should pass a threshold level (Schon, Bonilla et al. 1997; Jokinen 2016). Pathogenic mtDNA mutations in humans are thus recessive in nature and the threshold level of mutant mtDNA must be exceeded before clinical symptoms start. *De novo* mutations and somatic mtDNA segregation of inherited mutations have various threshold levels determining disease start and clinical severity (Jokinen 2016). Between individuals of the same family, the fraction level of mutated mtDNA may change. Further, the level of heteroplasmy can vary between organs and tissues within the same individual, which has been shown for a number of mtDNA mutations by histocytochemical assessment of cytochrome c oxidase (complex IV) activity in individual cells (Greaves and Taylor 2006; Genova and Lenaz 2015; Gammage, Van Haute et al. 2016). During mitotic segregation, the number of mutant mtDNA copies in daughter cells can change and because of this the phenotype also changes consequently. This process illustrates how symptoms due to mutations in the mtDNA can change from one clinical phenotype into another one as patients grow older. During fertilization, the pattern of transmission of mtDNA and mtDNA point mutations or deletions does not resemble Mendelian inheritance (Jokinen 2016). A mother having an mtDNA point mutation will transmit it to all offspring, but only her daughters will pass it to their children. A disease found equally in both sexes but with no signal of paternal transmission is highly indicative of mtDNA point mutation (Dimauro and Davidzon 2005; St John, Facucho-Oliveira et al. 2010). The female germ line is responsible for the transmission of mtDNA point mutations. During oogenesis, the segregation of mutated mtDNA is an essential factor for defining the heteroplasmy level, and probably the potential disease in the resulting offspring (Jokinen 2016).

Mutations in the mtDNA can be divided into two groups: i) maternally-inherited point mutations that can be heteroplasmic or homoplasmic and affect protein, tRNA or rRNA genes and ii) large scale deletions that span many genes (Figure 5). In addition, recombination across inverted repeats can cause sequence inversions (Yang, Seluanov et al. 2013). In patients, the clinical symptoms associated with a given mtDNA mutation can vary, while a given syndrome can be due to different mutations, showing the extensive genetic heterogeneity that describes these

disorders. A good example is the MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) syndrome (Jokinen 2016), which in more than 80% of the cases is caused by the 3243A>G mutation in the $tRNA^{Leu}$(UUR) gene, yielding a variety of biochemical defects (Goto, Nonaka et al. 1990; Kobayashi, Momoi et al. 1990; El-Hattab, Emrick et al. 2016). But MELAS can also result from point mutations in other tRNA genes or even in protein–encoding genes such as MTND5 and MTND1.

The clinical phenotypes of mtDNA mutations have been reviewed for instance by (Tuppen, Blakely et al. 2010). In a patient who is suspected to suffer from an mtDNA disease, a diagnosis is often only established after completion of a large set of clinical, histochemical, biochemical and finally molecular genetic examinations (Greaves and Taylor 2006; Filosto, Lanzi et al. 2016). The latest sequencing methods can now give the complete qualitative and quantitative data about mtDNA point mutations, single and multiple deletions, level of mutation heteroplasmy and deletion breakpoints in a single experiment (Tang and Huang 2010; Aisner, Berry et al. 2016). Over 300 pathogenic mtDNA mutations have now been defined (more detail in the MITOMAP database, http://www.mitomap.org) and linked with an impressive array of clinical syndromes.

In the mitochondrial genome, the processes leading to deletions are not well established and the proposed mechanisms are still under discussion. Based on the studies on deletion formation, the diverse structures observed at deletion points suggest different models. These are slipped strand-strand mispairing, illegitimate elongation, homologous recombination, and DNA degradation and mispairing due to an error of repair of double-strand breaks. Slipped-strand mispairing and illegitimate elongation would take place during asynchronous mtDNA replication (Lewis, Uchiyama et al. 2016). Homologous recombination would occur upon repair of double-strand breaks (Tadi, Sebastian et al. 2016; Vriend, Prakash et al. 2016). The slipped mispairing model needs asynchronous mtDNA replication starting at the O_H origin of replication and occurs between two perfect or imperfect repeats. Asynchronous replication can leave the H-strand partially single-stranded and give the two repeats a chance to mispair to generate mtDNA deletions. When the upstream repeat in the parent H-strand pairs with the downstream repeat in the L strand, a single-strand loop is generated. This loop is degraded following strand breaks and a new deleted parent H strand is closed by ligation and used as a template to generate a deleted L-strand (Chen, He et al. 2011). The illegitimate elongation model also needs asynchronous

mtDNA replication, but in this case the upstream repeat of the daughter H-strand mispairs with the downstream L-strand repeat. This process generates a shortened daughter H-strand, which can be extended together with L-strand replication to make deleted mtDNA molecules (Ba, Wu et al. 2016). Both models produce some unusual secondary structural arrangements so that to bring H-strand upstream and L-strand downstream repeats together. An intra-molecular homologous recombination-based model has also been put forward (Hirano, Martí et al. 2004). In this model, deletion formation needs that homologous segments of the mtDNA align, which can be promoted by DNA supercoiling or by the electrostatic properties of the DNA. Strand break and exchange between upstream and downstream repeats then make a Holliday junction-type structure. Branch migration of the crossover, strand extension and ligation then make recombined and deleted mtDNA molecules (Tadi, Sebastian et al. 2016).

Another model proposed suggests mtDNA deletions take place as a consequence of repair of double-strand breaks (DSBs) (Krishnan, Reeve et al. 2008). The process would be initiated by single-stranded regions generated through resection of broken ends by an exonuclease activity. The single strands would then anneal with microhomologous sequences elsewhere in the genome. Repair, ligation and degradation of the remaining exposed single strands would finally generate deleted mtDNA.

II.2 Mitochondrial genome alterations in plants

Research has been performed on the different mitochondrial genomes of angiosperm species, including *Beta vulgaris, Arabidopsis thaliana, Brassica napus, Triticum aestivum, Oryza sativa , Zea mays , Vitis vinifera, Nicotiana tabacum, Vigna radiata* and *Citrullus lanatusd* (Asaf, Khan et al. 2016). In the wild beet species (*oenothera fruticosa*), one distinctive mitochondrial mutant has been found. In this mutant, the activity of cytochrome *c* oxidase is reduced by 50% as compared to the normal beet. The COX2 polypeptide lacks the last eight amino acids, due to shortening by a nonsense mutation in the *cox2 orf*. Remarkably, this mutated *cox2* exists in a homoplasmic condition, *i.e.* no normal copy of the *cox2* gene is identifiable in the genome, and a single, smaller and less stable COX2 protein is synthesized (Ducos, Touzet et al. 2001). The mutant is male sterile but the phenotype is otherwise normal. So maybe there is a parallel pathway in this mutant that is sufficient to compensate the 50% reduction of the regular cytochrome oxidase pathway (Rasmusson, Geisler et al. 2008). Deletions as consequences of

recombination *via* relatively short repeated sequences can lead to the loss of specific genes in angiosperm mitochondrial genomes. These types of mutants have visible phenotypes, which include retarded development, leaf variegation due to chlorophyll loss, and a pleiotropic effect of the mitochondrial deficiency (Kubo and Newton 2008; Gualberto, Mileshina et al. 2014; Qi, Zhao et al. 2016). A series of spontaneous growth-defective mutants that are called as non-chromosomal stripe (NCS) mutants have been identified. These kinds of mutants are heteroplasmic due to deletions in mitochondrial genes, as for example *cox2*, *nad4*, or *rps3* (Lauer, Knudsen et al. 1990; Marienfeld and Newton 1994; Newton, Mariano et al. 1996; Levsen, Bergero et al. 2016). In the tobacco *Nicotiana sylvestris*, there are homoplasmic *nad7*-deficient mutants that show male sterility and slow growth (Pineau, Mathieu et al. 2005). These mtDNA deletion mutants can be a source of information to understand the interactions between the nuclear and mitochondrial genomes (Kuzmin, Karpova et al. 2004; Dahan and Mireau 2013).

II.3 Cytoplasmic male sterility in plants

The cytoplasmic male sterility (CMS) trait is described as the impairment of pollen production in higher plants, while gene male sterility (GMS) is defined as the interaction between nuclear and mitochondrial genes. Most often, the late stages of pollen formation are impaired in CMS plants. The specific mechanism by which male sterility happens in plants varies from species to species. CMS phenotypically appears as changes in tapetum or sporogenic tissues leading as a consequence to the production of abortive pollen or to the absence of pollen. But in some species, flower morphology is likewise altered. In some cases, homeotic substitutions of floral parts are found. CMS has been observed in more than 300 plant species, as well as in interspecies hybrids (Touzet 2012; Horn, Gupta et al. 2014; Miller and Bruns 2016). The phenotype of male sterility inherits maternally. CMS plants always have a special type of mitochondria that is different from wild-type mitochondria, especially due to mtDNA rearrangements.

The main role in the nuclear genetic control of CMS is made by *Rf* (*restorer of fertility)* genes (Kazama, Itabashi et al. 2016). It is considered that in this control various other genes are also engaged, the existence of which is suggested by an indirect signal. These proposed genes have a role in CMS formation and in some cases in reversion to normal fertility. It is not excluded that the function of some, if not all of them, is not precisely directed at CMS control. Many different

types of CMS have been identified in numerous species. These types vary in the phenotypic expression, as well as in the mode of inheritance. In many cases, the diverse forms of CMS type are categorized by a specific type of mtDNA with a definite system of nuclear genetic control. CMS can be found in different forms in the same species (for example, T, C, and S types in maize *Zea mays* (Touzet 2012; Ren, Nagel et al. 2015; Gurdon, Svab et al. 2016).

CMS is extensively used in breeding, as male sterile lines help in directed crosses and in the production of unique hybrid lines benefiting from heterosis. For instance wheat (*Triticum aestivum L)* is a main food crop in the world, and plays a very important role in global food security. Hybrid breeding of maize and rice resulted in a huge increase in grain yield but in the case of wheat it is not as effective so far, although improvement of hybrid wheat heterosis for commercial grain production has been reported recently (Geyer 2016). Using CMS is one of the best methods to produce hybrid seeds and increase grain yield. But self-pollination and incomplete fertility restoration of the hybrids remain the main obstacles linked to CMS hybrid wheat. Different CMS forms can be produced by γ-irradiation and by chemical mutagenesis (Sadoch, Goc et al. 2003; Clemente, Park et al. 2016). However, the best method to get innovative CMS forms is still to search in wild populations of commonly available species. Different CMS forms are stably maintained at certain frequencies in various wild populations. According to theoretical models, two reasons can stop CMS elimination from a wild population: i) the female benefit, *i.e.* more reproduction ability of female plants versus hermaphroditic ones and ii) the choice of the fertility-restoring alleles and their matching with the CMS-inducing cytoplasm (Budar, Touzet et al. 2003). The earlier may be connected with the fact that female monoecious plants and male sterile plants do not require energy for developing male sex organs. Female plants in some species can produce more seeds than hermaphroditic ones, and these seeds have extra sustainable features (Gouyon and Couvet 1987; Thompson and Tarayre 2000). But this does not apply to all different species in which CMS was found (Gouyon and Couvet 1987; Laporte, Viard et al. 2001). In CMS lines of a few species, the development of pollen is impaired at late times of growth of male organs, *i.e.* at a time where much energy has already been used.

The proportion of female monoecious individuals and hermaphrodites may change greatly in natural populations. For example, the rate of female monoecious individuals in populations of

Thymus vulgaris varies from 5 to 95%, while in *Plantago lanceolata*, it can go from 0 to 22%. These kinds of species were described to have female benefits, manifested in a higher number and better sustainability of seeds in female monoecious plants (Ivanov and Dymshits 2007). For getting benefit in the production, it is recommended to grow different forms of CMS, but also with fertile plants. Genetically modified maize gave a better seed production upon joint cultivation of genetically modified CMS forms and fertile plants, as compared to these forms cultivated individually. Keeping CMS forms in a wild population needs additional resistance to pathogens and pests, as shown for a few CMS forms (Ivanov and Dymshits 2007). Eliminating purely cytoplasmic G-type CMS in sugar beet showed that the presence of not only CMS-inducing mtDNA, but also of some particular alleles generally called as sterility maintainers is required to maintain CMS. Pollination of male sterile plants with pollen of lines having the mixture of essential genes for CMS realization, generates predominantly or entirely male sterile offspring. Alternate alleles of these genes are not giving male sterility, but acting as restorers of fertility. Owing to this, the presence of the CMS system in a wild population of a particular species indicates the presence of restorer of fertility alleles. Further, fertility restoration in a generation not only relies on *Rf* genes but also on other genetic and non-genetic factors (Geyer 2016). All these factors are associated with the correction of defects in active pollen production resulting from the expression of mitochondrial CMS-inducing genes. Restoration can occur at any point of the genetic process. *Rf* genes are trying to suppress the effects of different factors in the CMS development or in the impaired function (Chen and Liu 2014; Naresh, Singh et al. 2016; B. Feil march 2003).

II.4 Cytoplasmic male sterility in maize

Maize is the third main grain crop after rice and wheat. For this reason, using different forms of CMS in hybrid production can significantly decrease the expenditure of seed production. In 2014 in Europe, the cereal production (wheat, barley, grain maize and corn mix) increased by 30.7%, as compared to the two previous years (http://ec.europa.eu/eurostat/statistics-explained/index.php/Agricultural_production_-_crops). It is suggested that the ratio of valuable hybrids resulting from the use of CMS will continue to rise (Weingartner, Camp et al. 2004). Recently two new hybrid lines of maize have been selected (Cooper, Gho et al. 2014). There are three different types of cytoplasm that cause male sterility in maize: T-cytoplasm or **T**exas cytoplasm (Dewey, Timothy et al. 1987), S-cytoplasm (U**S**DA) (Gabay-Laughnan, Kuzmin et al.

2009) and C-cytoplasm (Kohls, Stamp et al. 2011; Chen and Liu 2014; Chen, Zhang et al. 2016). These three CMS types are produced by different mitochondrial mutations and are distinguished by specific nuclear restorer of fertility genes that respond to the CMS trait.

The environmental conditions have very much importance for the production of hybrid seeds (Kole, Muthamilarasan et al. 2015). Functional restoration of male fertility is needed for the hybrid, while complete and stable male sterility of the maternal parent is required throughout the production of hybrid seeds, so that no self-pollination happens. Conservation of male sterility would also be essential if CMS is used for the control of transgenic pollen.

The environmental conditions have strong effects on partial restoration of male fertility in maize (Tracy, Everett et al. 1991; Gabay-Laughnan, Kuzmin et al. 2009; Weider, Stamp et al. 2009; Bueckmann, Thiele et al. 2016). It has been observed that plants look like sterile in one place, while they can vary in the level of fertility at another place (Tracy, Everett et al. 1991; Wolf 2016). Different environmental factors like temperature, photoperiod and light intensity are considered as very important. Humid and cool conditions are considered to be effective in the restoration of fertility, while dry and hot conditions would maintain sterility (Tracy, Everett et al. 1991; Bueckmann, Thiele et al. 2016). In maize, the sensitivity of S-CMS to environmental conditions is the strongest, while that of C-CMS is in between S-CMS and T-CMS (Buckmann, Thiele et al. 2014).

II.5 C-type cytoplasmic male sterility in maize

C-type CMS is a sporophytic system, in this case breakdown of tapetal cells disturbs the generation of pollen (Huang, Xiang et al. 2012; Bosacchi, Gurdon et al. 2015). The disorder of pollen in C-CMS anthers takes two different forms, located in separate anthers of the same plant (Skibbea and Schnablea 2005; Chen, Zhang et al. 2016). Rather than the mechanism, the timing of tapetal disturbance can distinguish these two forms (Lee, Gracen et al. 1979). Despite comparisons of mitochondrial sequences and studies on the level of expression of mitochondrial genes, the cause of C-CMS is still a subject of debate (Allen, Fauron et al. 2007). Indeed, in the case of C-CMS, no special chimeric transcript or protein that would be responsible for the phenotype was found. Instead, the level of ATP6 and ATP9 proteins decreases in specific tassels in C-CMS, which is not observed in the presence of a restorer gene (Sofi, Rather et al. 2007; Meyer 2009; Langewisch 2012). Both of these proteins are mitochondrially encoded components

of the ATP synthase F_0 subunit. The mitochondrial genome of C-CMS lines has two copies of the *atp9* gene. One is the regular copy of *atp9* (*atp9-1*) present in S, T and normal cytoplasm. This copy might be transcribed to a lower level in C-CMS, probably due to a mutation in the region close to the transcription start site. The second copy (*atp9-2*) is the main *atp9* transcript in C-CMS. The coding part is similar to *atp9*-1, while it has a chimeric 5' UTR. This leads to a co-transcription with the upstream *cox2* gene and a bigger transcript (4 kb instead of 1 kb). For this reason, it is considered that the *atp9-2* transcript is not translated well in C-CMS plants (Kohls 2010). At the time of flowering and pollen production, the need for ATP synthesis is high. In the tapetal cells of the anther, mitochondria may not be able to face the ATP demand with a lower ATP9 and ATP synthase level, resulting in the abortion of the developing pollen.

II.5.1.1 Restoration of fertility in C-CMS

There are three nuclear restorer genes for C-CMS, called as *Rf4*, *Rf5* and *Rf6*, that can restore fertility. This trigenic arrangement can be replicated anywhere in the genome (Kohls, Stamp et al. 2011; Bohra, Jha et al. 2016; Chen, Zhang et al. 2016). Restorer genes *Rf4* and *Rf5* were mapped to chromosomes 8 and 5 (Sisco 1991; Hu, Tang et al. 2006). In further studies, (Hu, Tang et al. 2006) mapped the *Rf-1* gene that works as an inhibitor of *Rf5* and resembles *Rf-7*. The corresponding interaction between parental and maternal features plays a key role. This is illustrated in studies by (Sotchenko, Gorbacheva et al. 2007) in a cross between C-CMS and maintainer lines having normal cytoplasm, the offspring of which was partially or fully restored. In the C-CMS system, a particular form of partial restoration was found called as "late break of sterility". In this case, pollen shedding can happen after several weeks of emergence of the silk (Kohls, Stamp et al. 2011).

II.5.1.2 T-type cytoplasmic male sterility in maize

The T-cytoplasm has been very much used in hybrid seed production before 1970. Later on, it was removed from the local market as a result of an epidemy caused by *Cochliobolus heterostrophus* race T that cruelly reduced yield (Wise, Bronson et al. 1999). After this, much research has been conducted to know the molecular mechanism essential for male sterility and toxin sensitivity. In this case, a novel mitochondrial *orf*, T-*urf13* was associated with male sterility (Wise, Pring et al. 1987). T-*urf13* encodes a 13 kDa protein that binds to the mitochondrial inner membrane as an oligomer (Forde and Leaver 1980; Wise, Pring et al. 1987;

Zhang, Li et al. 2007). When T-cytoplasm lines are treated with methomyl, a carbamate insecticide commonly called as t-toxin and that is produced by *Cochliobolus heterostrophus* race-T, mitochondria quickly swell, release small ions and molecules and finally oxidative phosphorylation is uncoupled (Levings and Siedow 1992). As a consequence, in the developing anthers, the mitochondrial perturbations in T-cytoplasm causes the early collapse of the tapetal cell layers and leads to the failure to produce pollen (Liu, Cui et al. 2001).

II.5.1.3 Restoration of fertility in T-CMS

Restoration of fertility in T-cytoplasm lines is done by the action of dominant alleles of two nuclear-encoded genes called as *rf1 and rf2* (Horn, Gupta et al. 2014). Normal functioning of pollen needs at least one restorer gene to minimize the effects of the T-URF13 protein (Wise, Bronson et al. 1999). The process of T-cytoplasm fertility restoration is sporophytic. In this case, the genotype of the sporophytic tissue, instead of gametophytic tissue, creates the ability of an individual to work normally. In this way, a plant that is heterozygous for *Rf1* and *Rf2a* is male fertile even it has only one-quarter of the pollen that has both restoring alleles (Chen and Liu 2014; Bohra, Jha et al. 2016).

II.5.1.4 S-type cytoplasmic male sterility in maize

The maize S-type cytoplasm is characterized as having two linear plasmids, S1 and S2. Both of these plasmids are similar to the plasmid-like mtDNA molecules called as R1 and R2 in fertile American races of maize having the RU cytoplasm. R2 is similar to S2. Both are about 5.4 kb in size and they vary in a single *Bgl*1 site that is missing in S2. While R1 is 7.4 kb in size, roughly 1 kb bigger than S1, and has 2.6 kb that do not match to S1 (Gabay-Laughnan, Kuzmin et al. 2009). S1 and S2 plasmids have 208 bp terminal inverted repeats (TIRs), covalently bound to a terminal protein (Kemble and Thompson 1982; Levings and Sederoff 1983; Paillard, Sederoff et al. 1985; Gabay-Laughnan, Kuzmin et al. 2009; Feng, Zheng et al. 2015).

The main mitochondrial genome of S-CMS maize has two integrated TIRs containing repeats that are very active in intragenomic recombination. TIR sequences provide the target sites for homologous recombination between the free linear S plasmids and the main mitochondrial chromosome (Levings, Kim et al. 1980; Lonsdale, Hodge et al. 1984; Schardl, Lonsdale et al. 1984; Gabay-Laughnan, Kuzmin et al. 2009; Matera, Monroe et al. 2011). Flanking genomic regions that are located upstream of the integrated TIR in the main genome are called as σ and ψ,

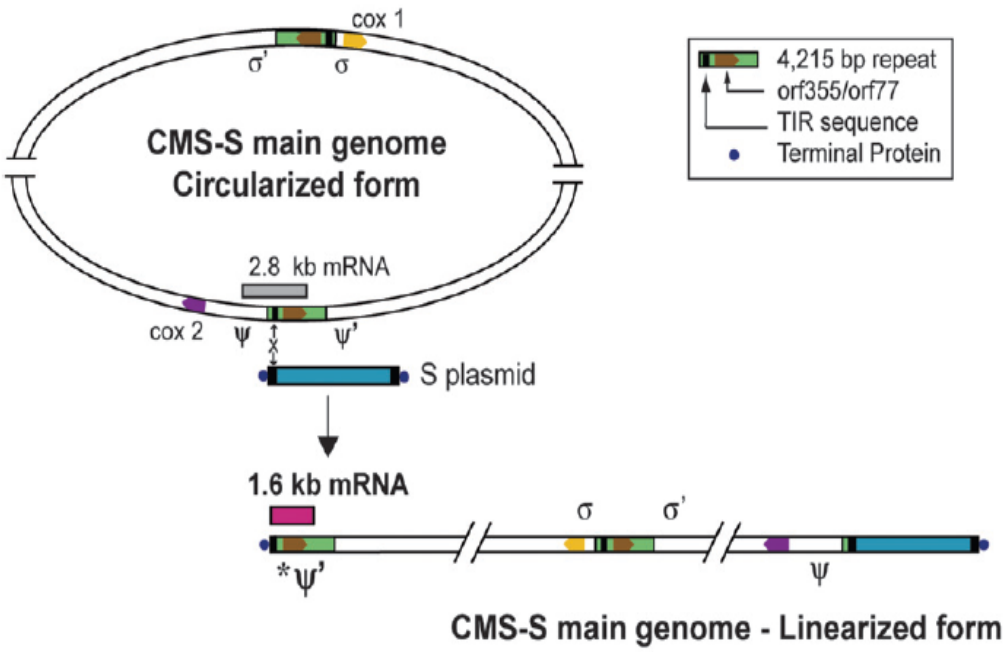


Figure 6: Graphic representation of how recombination between linear S plasmids and integrated TIR sequences next to *orf355*/*orf77* leads to linearized ends of S-CMS mitochondrial genomes. Two copies of a 4.2 kb R repeat (green) carrying *orf355*/*orf77* sequences (brown inserts) are integrated in the genome and are flanked by different regions designated as σ and σ′, ψ and ψ′. One copy is downstream of *cox1* (yellow) and the other downstream of *cox2* (purple). Free S1 and S2 (represented by "S plasmid" in blue) recombine through TIR sequences to yield linearized S-CMS genomes. Recombination and the resulting linearized genome are depicted only for the ψ copy of the 4.2 kb repeat, although recombination at the σ copy is equally likely. A 1.6 kb *orf355*/*orf77* transcript (pink) is associated with the presence of the linear ends (adapted Gabay-Laughnan, Kuzmin et al. 2009)

while the regions located downstream of each repeat are known as σ′ and ψ′ (Schardl, Lonsdale et al. 1984; Gabay-Laughnan, Kuzmin et al. 2009). The region that has all the common repeats of four junctions (σ–σ′, σ–ψ′, ψ–ψ′, and ψ–σ′) is called as R-repeat. The 4.2 kb of the R-repeat have been sequenced (Zabala, Gabay-Laughnan et al. 1997; Xiao, Zhang et al. 2006; Allen, Fauron et al. 2007) and shown to contain two chimeric open reading frames called *orf355* and *orf77* (Figure 6). These are particular to S-CMS mitochondrial genomes (Matera, Monroe et al. 2011). Through the TIRs, the S-plasmids recombine with homologues sequences in the 4.2 kb R repeat of the main genome, causing the linearization of the main mtDNA and generating a S-plasmid-like terminal structure just upstream of the *orf355*/*orf77* region (Levings, Kim et al. 1980; Schardl, Lonsdale et al. 1984). The S-male sterile phenotype is related to the expression of a 1.6 kb transcript that has both *orf355* and *orf77* (Langewisch 2012).

II.5.1.5 Restoration of fertility in S-CMS

The *Rf3* gene, a naturally occurring nuclear restorer of fertility, can fully restore the fertility of S-CMS plants. The action of the restorer gene is gametophytic, *i.e.* the restoring alleles can function in the haploid male gametophyte. In this situation, the cytoplasm with *Rf3* is normal and starch is filled, whereas in *rf3* plants pollen grains are nonfunctional and collapse. The *Rf3* restorer is found in the native inbred lines, such as Tr, Ky21, C.I.21E, and in the Pioneer Hi-Bred inbred lines CE1, BH2, JG3, and JG5 (Duvick 1956; Duvick 1965; Beckett 1971). It is considered that a rearranged mtDNA sequence is likely to be present in revertants because the nuclear restorer of fertility factor *Rf3* functions for CMS-S plants in basically all nuclear backgrounds (Newton, Mariano et al. 1996; Matera, Monroe et al. 2011; Feng, Zheng et al. 2015). It was ascertained that the mtDNA region called R-repeat is rearranged itself in all cytoplasmic revertants, irrespective of the nuclear background. This is because almost all cytoplasmic revertants do not have the 1.6 kb RNA transcribed from the R-repeat in sterile plants. Further, the nuclear restorer decreases the abundance of the main R-repeat transcripts, including the CMS-S specific 1.6 kb transcript (Zabala, Gabay-Laughnan et al. 1997). The presence and mtDNA integration of the S1 and S2 plasmids vary between revertants having different nuclear backgrounds. For example, in cytoplasmic revertants rising in M825, the S2 integrated sequences are rearranged. Nonetheless, 38-11 revertants show lower levels of integrated S2, and integrated S1 sequences are rearranged. While W182BN and H95 revertants do not have S2 sequences (Small, Earle et al. 1988). From the available literature, we can say

that specific mtDNA changes related to reversion to fertility in S-CMS are still a subject of debate.

III. Coordination between the nucleus and the organelles

As recalled above, in a plant cell, there are three genomes: nuclear, mitochondrial and chloroplastic. These need to be well coordinated with each other to ensure the core functions of the cell such as photosynthesis or respiration. In the photosynthesis process, chloroplasts change solar energy into chemical energy, whereas mitochondria generate ATP by oxidative phosphorylation (OXPHOS). In history, (Bradbeer, Atkinson et al. 1979) provided the first report related to the existence of a communication between these organelles. Coordination between organelles and the nucleus is made by complex and integrated anterograde (nucleus to organelle) and retrograde (organelle to nucleus) signaling pathways. The anterograde mechanism regulates organelle gene expression in reaction to endogenous or exogenous signals that are perceived in the nucleus. In retrograde signaling, organelles send signals to the nucleus on their functional state and activity. Both anterograde and retrograde pathways are of primary importance for the regulation and coordination of various processes in living organisms, such as developmental processes, reactions to biotic and abiotic factors, protein trafficking or alterations in chromatin structure (Woodson and Chory 2008; Jazwinski 2014). Plants have developed a multipart and intricate regulatory network for coordination of cellular activities and vary their mode of action for energy production and metabolism, depending on environmental changes. Thanks to that, they can survive in different unfavorable conditions. Mitochondria and chloroplasts, the pivotal metabolic cores, coordinate for diverse purposes as stress sensors that observe stress and give retrograde signals for a nuclear-encoded network of adaptative responses. The mitochondrion has only a small set of genes, which encode mostly components of the OXPHOS chain. Similarly, the chloroplast has only part of the genes for the components of the photosynthetic process. Mitochondria and chloroplasts are thus semi-autonomous organelles because they are dependent on a large set of nuclear genes (Andersson, Karlberg et al. 2003; Leister 2003; Leister 2012; Battersby and Richter 2013; Cao and Qin 2016). Almost all functional aspects of mitochondria and chloroplasts are rigorously regulated by nuclear gene expression. Therefore, inter-compartment communication is needed to coordinate the subcellular proteomes and maintain the right subunit stoichiometry of the organellar complexes. Mitochondria and chloroplasts send

signals back to regulate nuclear gene expression according to their functional activities (Fernandez and Strand 2008; Woodson and Chory 2008; Jazwinski 2014).

Mitochondrion-to-nucleus signaling is called as mitochondrial retrograde regulation (MRR). Signals due to variations in mitochondrial functions may be linked to metabolic pathways or to general reactive oxygen species (ROS) signaling pathways and cause changes in gene expression. However these would be still mitochondria-directed responses. Increasing data shows that MRR is an important regulatory/response system in plants, animals and fungi. The existence of mitochondrial signaling is conserved in eukaryotes, but the signaling cascades vary. Mechanistic variations are expected to be due to variations in metabolic functions and in regulation networks among animal cells and in yeast. Mitochondrial disorders related to MRR dysfunction are now expected to be a significant reason for various human diseases (Butow and Avadhani 2004; Weissig, Cheng et al. 2004; Lane 2006; Cao and Qin ; Sanz 2016). From different studies, we know that the state of the mtDNA or its level of expression may affect nuclear gene expression, and consequently mitochondrial biogenesis. Initial studies in *Neurospora crassa* found that cells exposed to mitochondrial transcription or translation inhibitors had an increased level of nuclear-encoded RNA polymerase and aminoacyl-tRNA synthetases in mitochondria (Barath and Kuntzel 1972). St-Pierre *et al.* recently showed that variation in the expression of nuclear and mitochondrial-encoded genes is coordinated at the level of translation during metabolic adaptation to fuel source changes (St-Pierre and Topisirovic 2016). These and other similar studies led to the hypothesis that mitochondrion-encoded factors, which would be transported to the nucleus, might regulate the transcription of a number of nuclear genes coding for mitochondrial proteins (Barath and Kuntzel 1972; Nagley 1991; Poyton and McEwen 1996; Lenka, Vijayasarathy et al. 1998). In particular, it has recently been put forward that mitochondria would generate non-coding RNAs to regulate nuclear genes (Dietrich, Wallet et al. 2015).

III.1 Chloroplast retrograde regulation in plants (CRR)

The current research in agriculture relating to plant reactions to environmental stress is increasing significantly in the context of the variations in climatic conditions. The chloroplast is a hub of metabolic processes, most of which are energy-intensive. It is thus an environmental stress sensor that communicates with the nuclear compartment to promote adaptative stress

responses. A tight coupling between nuclear gene expression and chloroplast-localized processes is needed. This coordination between the nucleus and the chloroplast is called chloroplast retrograde regulation (CRR) in plants. The significance of CRR is well established, however the exact signaling components are still debated (Gollan, Tikkanen et al. 2015). The latest research on *Chlamydomonas reinhardtii, A. thaliana* and maize has helped to understand CRR pathways and identify factors involved (Tourasse, Choquet et al. 2013; Belcher, Williams-Carrier et al. 2015). Pigment biosynthesis, *i.e.* intermediates of carotenoid and tetrapyrrole biosynthesis, plastid gene expression (PGE), plastid protein import, generation of reactive oxygen species (ROS) and ROS-related processes, as well as redox processes in photosynthesis are considered as major components of CRR. Sugar and hormone signaling also participate in these interactions (Gollan, Tikkanen et al. 2015; Chan, Mabbitt et al. 2016).

Chloroplast retrograde signals have two main roles: First, to ensure the proper functions of the plastid multi-protein complexes. The subunits of these are encoded by both the nuclear and the plastid genomes, hence the need for coordinated. The second role is to change the expression of nuclear genes according to environmental changes, because the environmental conditions disturb the metabolic activities and the functional state of the plastids. Therefore, CRR signaling is very much important for plant responses to abiotic stresses such as temperature or drought stress (Feng, Guo et al. 2016).

Massive gene transfer from the chloroplast ancestor to the nucleus occurred during the evolution time, that is why today most of the proteins functioning in the chloroplast are encoded by nuclear genes (Martin and Herrmann 1998; Chotewutmontri and Barkan 2016). But still fully functional higher plant chloroplasts encode 60 to 80 proteins, which comprise components of the photosynthetic machinery, of the sulfate assimilation pathways, of aromatic amino acid biosynthesis, of carotenoid biosynthesis and of fatty acid synthesis (Chan, Crisp et al. 2010). Conversely, numerous components of photosynthesis and chloroplast biogenesis are encoded by the nucleus, which thus controls the development of photosynthetic complexes (Singh and Matthews 1994; Jarvis 2008; Zerges 2016). The phosphonucleotide 3'-phosphoadenosine 5'-phosphate (PAP) accumulates during stress and transfers from the chloroplast to the nucleus, so that it can activate the stress responsive genes (Estavillo, Crisp et al. 2011; Chan, Phua et al. 2016). A recent publication on retrograde signaling mutants shows that PAP controls nuclear gene expression in response to stress, can inhibit cell death and compromises plant innate

immunity *via* inhibition of the RNA-processing 5′-3′ exoribonucleases. The activity of SAL1 can be inhibited in the chloroplast during drought and excess light, subsequently activating PAP changes and modulating nuclear gene expression, as proven by the changes in the expression of *ELIP2* and *APX2* in the *alx8* mutant (Barajas-Lopez Jde, Blanco et al. 2013; Chan, Phua et al. 2016). Different proteins such as ABA-INSENSITIVE 4 (ABI4) (Eckstein, Krzeszowiec et al. 2016; Kmiecik, Leonardelli et al. 2016), Pleiotropic Response Locus1 (PRL1) (Lee 2016) and Golden2-Like (GLK) (Nagatoshi, Mitsuda et al. 2016) have been reported to act as pleiotropic regulators coordinating the modulation of nuclear transcription upon retrograde plastid signals.

Deep search performed to find the different factors that are involved in this chloroplast-to-nucleus signaling during chloroplast biogenesis, *i.e.* the process in which a mature chloroplast is developed, highlighted a set of plastid metabolic intermediates (Bobik and Burch-Smith 2015; Martin, Leivar et al. 2016). Proteins with functions in both the chloroplast and the nucleus have been proposed to be involved in CRR. For the adaptation of plants in different changing physiological conditions, these signals are expected to work in changing the expression of nuclear genes (Belcher, Williams-Carrier et al. 2015). Presently, we have two major models of retrograde signaling, biogenic and operational control (Kleine and Leister 2016). In the biogenic control, coordination is vital, so that the cell does not lose valuable assets such as polypeptides that are energy-intensive to produce. In this way, the incomplete chloroplast and the nucleus just have to coordinate to create a complete chloroplast, with the plastid giving commands on the genes to the nucleus (Pogson, Woo et al. 2008). Photosynthesis-associated nuclear genes (PhANGs) are considered to be the best targets for studying retrograde signaling, however plastid genes are also involved in tuning the expression of nuclear genes during stress (Bobik and Burch-Smith 2015). A meta-analysis of microarray studies on systems where high levels of retrograde signaling occurred has recognized an essential module of 39 nuclear genes that were focused on regulation in response to all signals inspected (Glasser, Haberer et al. 2014).

As the chloroplast matures, communication between chloroplast and nucleus becomes more visible. At this time, the chloroplast and the nucleus are required to communicate to keep the functioning of the chloroplast at maximum levels (Belcher, Williams-Carrier et al. 2015). That is, photosynthesis and other metabolic processes have to be coordinated according to changes in metabolites and variations in environmental conditions. This process of communication from the chloroplast to the nucleus, with the subsequent variations in nuclear gene expression, is known as

'operational control' and needs to have the metabolic processes running "optimally" in mature chloroplasts (Pogson, Woo et al. 2008).

Nuclear-encoded photosynthesis genes are thoroughly down- or unregulated in reaction to variations in the redox status of the chloroplast (Fey, Wagner et al. 2005). The herbicide norflurazon decreases photosynthesis and causes down regulation of nuclear genes (Oelmuller, Levitan et al. 1986; Kakizaki, Matsumura et al. 2009). Modification of chloroplast-localized sulfate assimilation (Mugford, Yoshimoto et al. 2009) by mutagenesis was also shown to affect nuclear gene expression. *De novo* fatty acid biosynthesis in the chloroplast yields acyl chains that are then moved to various cellular compartments. These fluctuations of acyl chains is strictly controlled by nuclear genes, so as to match the ratio of fatty acid biosynthesis (Ohlrogge and Jaworski 1997).

Tetrapyrrole signaling is the most studied retrograde signaling pathway. Mg-protoporphyrin IX (Mg-protoPIX) was proposed to interact with gene products of the *GENOMES UNCOUPLED (GUN)* locus that have been considered as regulators of retrograde signaling (Koussevitzky, Nott et al. 2007; Zhang 2007; Brzezowski, Sharifi et al. 2016). When photosynthesis is repressed by herbicide treatment in the *gun* mutants, transcription of nuclear genes is uncoupled from plastid development, showing failure of retrograde signaling (Susek, Ausubel et al. 1993). The participation of the main enzymes of the tetrapyrrole biosynthesis pathway in the *gun* phenotype led to the acceptance of tetrapyrroles, particularly Mg-protoporphyrin IX (Mg-ProtoPIX), as retrograde signals (Brzezowski, Sharifi et al. 2016; Chan, Phua et al. 2016). Different changes in nuclear gene expression due to stress-induced alteration of biosynthesis and integration of the chlorophyll biosynthetic intermediate Mg-ProtoPIX and of its methyl ester (Mg-ProtoPIX-ME) have been characterized ((Brzezowski, Sharifi et al. 2016; Sun and Guo 2016) and references therein). By using the fluorescence properties of tetrapyrrole under confocal laser scanning spectroscopy, Mg-ProtoPIX was shown to accumulate in the chloroplasts and subsequently in the cytosol in stress situations (Ankele, Kindgren et al. 2007). This suggests that the signaling metabolite Mg-ProtoPIX is transferred from the chloroplast to the cytosol, transferring the plastid signal to the nucleus. But later on, the role of Mg-ProtoPIX/Mg-ProtoPIX-ME as a plastid signal was challenged, as no relationship between the accumulation of Mg-ProtoPIX and retrograde signaling was detected in further studies (Moulin, McCormac et al. 2008; Monshausen 2012). So, in spite of considerable promise, the role of Mg-protoPIX as a retrograde signal

remains to be consolidated. Heme was recently proposed to be a strong candidate for participating in chloroplast-to-nucleus signaling (Chan, Phua et al. 2016). Interestingly, heme is considered as a mitochondrial retrograde signal in yeast (Leister 2012).

Initially chloroplast retrograde signaling studies were conducted on two barley chloroplast ribosome-deficient mutants, whose deficiency in plastid functions causes down-regulation of nuclear-encoded plastid proteins (Bradbeer, Atkinson et al. 1979). After this, major research for the understating of retrograde signaling has been done in plastid development and chlorophyll biosynthesis through the communication with nuclear genes that encode plastid-localized chlorophyll-binding proteins. Studies were developed in young mustard seedlings, *A. thaliana*, pea, or barley, using treatment with lincomycin, chloramphenicol, or streptomycin, the inhibitors of plastid protein synthesis that lead to photobleaching (Nott, Jung et al. 2006; Inaba, Yazu et al. 2011; Odintsova 2011; Liao, Hsieh et al. 2016). When chloroplast development was blocked by the inhibitors, the expression of hundreds of nuclear genes functional in photosynthesis and chloroplast biogenesis was strongly decreased (Koussevitzky, Nott et al. 2007). Conversely, genetic screens showed that the *A. thaliana gun* mutants continue to express the nuclear genes smoothly after photobleaching (Susek, Ausubel et al. 1993). This study provides evidence for heme/chlorophyll biosynthesis coordination in the plastid as a way of retrograde signaling (Woodson, Perez-Ruiz et al. 2011). The exact biochemical activity of the GUN1 protein is not well established. It is a chloroplast-localized, RNA-binding pentatricopeptide repeat (PPR) protein that might regulate gene expression post-transcriptionally by altering RNA editing, splicing, or stability (Woodson, Perez-Ruiz et al. 2013). So, GUN1 might regulate one or a small set of chloroplast RNA(s) that can participate in retrograde signaling. A downstream factor could be the ABI4 transcription factor that can bind a conserved promoter motif located in many of the regulated genes (Koussevitzky, Nott et al. 2007).

Studies were carried out on mutants lacking a major plastid protein import component of the TOC (translocon at the outer envelope membrane of chloroplasts), *i.e.* TOC159 (Kakizaki, Matsumura et al. 2009), or the plastid protein import2 (ppi2) receptor (Bischof, Baerenfaller et al. 2011) to understand CRR pathways. *toc159* mutants showed a severe albino phenotype, pointing to a defect in chloroplast biogenesis. Further, in these mutants a significant reduction in the level of many, but not all, nuclear-encoded photosynthesis-associated proteins was also noted. On this basis, it was suggested that TOC159 is a main receptor needed for the massive

accumulation of photosynthesis-associated proteins (Jarvis and Kessler 2014). Conversely, the *ppi2* mutation also affects the accumulation of proteins that are not involved in photosynthesis (Bischof, Baerenfaller et al. 2011).

As an obligatory result of aerobic metabolism, plants permanently produce a variety of reactive oxygen species (ROS), including hydrogen peroxide, superoxide radicals, singlet oxygen, and hydroxyl radicals (Bailey-Serres and Mittler 2006; Suzuki, Koussevitzky et al. 2012; Mignolet-Spruyt, Xu et al. 2016; Sharma, Lalgudi et al. 2016). The chloroplast is a main generator of ROS through photosynthesis. In the beginning, it was considered that these chemically reactive molecules are only toxic byproducts of the metabolism of plant cells. Therefore they should be removed by antioxidative enzymes and antioxidants (Bailey-Serres and Mittler 2006; Noctor and Foyer 2016). It is well established that chloroplasts possess a large range of ROS-scavenging mechanisms (Asada 2006). Nonetheless, now it is clear that ROS are also involved in signaling processes monitoring biotic and abiotic stresses (Maruta, Noshi et al. 2012; Foyer and Noctor 2013; Noctor, Mhamdi et al. 2014). Due to coordination between ROS and metabolism, changes in cellular homeostasis might cause a change in the level of ROS in the plastid compartment, thus changing the redox status of the plant cells. Different types of abiotic stresses hinder excitation, energy transfer and electron transport in photosystem II (PSII). But control of excitation energy transmission and of electron transport goes with the formation of ROS (Suzuki, Koussevitzky et al. 2012). Several forms of ROS are generated in different ways, such as the singlet state $^1O_2$ is made through the excitation energy transfer, while the superoxide anion O_2^- and H_2O_2 are produced during electron transport (Pospisil and Prasad 2014). It is thought that ROS may diffuse away from the point of their production and subsequently elicit a set of signaling processes in a wide range of biotic and abiotic stress conditions (Suzuki, Koussevitzky et al. 2012; Choudhury, Panda et al. 2013). ROS and photosynthesis redox imbalance are considered to activate a range of retrograde signaling paths throughout stress conditions and thus to play an essential role in the acclimation of plants (Galvez-Valdivieso and Mullineaux 2010; Suzuki, Koussevitzky et al. 2012). The redox status of the chloroplast can be characterized by observing the state of the plastoquinone, ascorbate, and glutathione pools (Foyer, Bloom et al. 2009; Suzuki, Koussevitzky et al. 2012; Foyer and Noctor 2013; Petrov, Hille et al. 2015). ROS production can also occur in heat stress conditions (Choudhury, Panda et al. 2013). At a particular level of ROS, signaling pathways start to reprogram the expression of nuclear genes

encoding chloroplast and mitochondrion-targeted proteins. Bioinformatic analysis of transcriptomes, proteomes and metabolomes showed that ROS are commonly linked to particular responses (Ma, Rahmat et al. 2013). ROS are very short lived, with the exception of H_2O_2. It is thus assumed that activation of the signal is controlled by the reaction of ROS with nearby secondary messengers that can cross the chloroplast envelope and initiate direct signaling from outside of the organelles (Moller, Jensen et al. 2007; Pluchino, Choudhary et al. 2016). ROS are relatively general signaling molecules because many stress-related processes, such as programmed cell death or pathogen defense, are also related to ROS production (Petrov, Hille et al. 2015; Hirt 2016). In chloroplasts, β-cyclocitral (β-CC) is an oxidative product of carotenoids that can initiate singlet oxygen signaling (Sun and Guo 2016). So, ROS are assumed to initiate signaling cascades within the chloroplast, which then pass the envelope by unknown means (Pfannschmidt 2010).

A chain of redox components is involved in photosynthetic electron transport (PET) that is electrochemically connected in each step (Foyer and Noctor 2016). The process of photosynthesis takes place in light. The redox state of the PET components gives signals related to environmental light conditions. In higher plants, it is considered that the redox state of the PET components can affect nuclear gene expression, mostly at the level of transcription. Plastoquinone (PQ) is an intrinsic membrane electron carrier that can connect photosystem II with the cytochrome b_6/f complex and is involved in both linear and cyclic electron transport. The PQ pool is one of the major components involved in PET-derived retrograde signaling. In low light, most of the PET components like PQ are in an oxidized state. In higher light conditions, PQ and other PET components are in a reduced state due to higher excitation pressure (Nott, Jung et al. 2006). Manipulation of the redox status of the PQ pool has been performed by using different herbicides like 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5,-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB). Electron transport can be blocked by these herbicides before (DCMU) or after (DBMIB) the PQ pool, mimicking the effect of low or high light intensities, respectively (Morales-Flores, Olivares-Palomares et al. 2015). Nuclear genes that are induced by high light are also induced upon DBMIB treatment in the absence of high light. DCMU treatment prevents high light induced gene expression (Gray, Sullivan et al. 2003). This suggests that a regulatory function of the redox state of the PQ pool exists in plants.

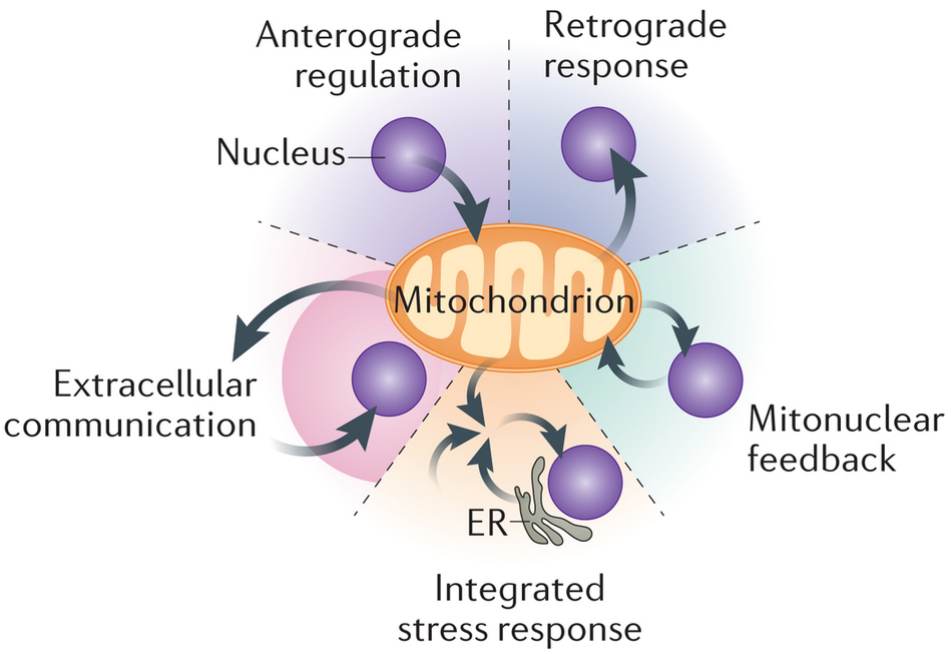


Figure 7: Communication between mitochondria and the nucleus. Mitochondria and the nucleus communicate closely with each other. Signals sent from the nucleus to mitochondria constitute anterograde regulation, whereas those sent by mitochondria to the nucleus are defined as the retrograde response (adapted from Quiros, Mottis et al. 2016)

III.2 Mitochondrial Retrograde Regulation (MRR)

Variations in the functional status of the mitochondria and subsequent changes in nuclear gene expression are interconnected by mitochondrial retrograde regulation (MRR) (Figure 7). MRR is mostly studied in yeast and in animals. It is a new field of study in plants, where the involved mechanisms and the different components are not well defined. MRR in plants may be related to heat shock, pathogen attack and programmed cell death, metabolic signaling, ROS production or O_2 sensing (Rhoads and Subbaiah 2007; Woodson and Chory 2008; Camejo, Guzman-Cedeno et al. 2016). Different phenotypes have been linked to defects in mitochondrial functions in plants (Rhoads and Subbaiah 2007; Schwarzlander and Finkemeier 2013; Wallet, Le Ret et al. 2015). Plant mitochondrial mutants commonly show both cytoplasmic male sterility and altered nuclear gene expression (Gualberto, Mileshina et al. 2014). Such mutants have been identified in different plant species, *e.g.* nonchromosomal stripe (NCS) mutants in maize (De Clercq 2013). These types of mutants have variegated leaves, because they have dysfunctional mitochondria with high levels of heteroplasmic mutated mtDNA in the affected pale stripes and mitochondria that work regularly in the normal looking green sectors. The normal looking sectors have higher levels of non-mutated mtDNA and show less effect on chloroplast development. While the affected sectors have mutations in the mtDNA and show abnormal chloroplast development (Newton, Gabay-Laughnan et al. 2004). NCS mutants in maize have a distinctive pattern of induction of the nuclear genes encoding the mitochondrial alternative oxidase (AOX) (Karpova, Kuzmin et al. 2002; De Clercq 2013).

Expression of nucleus-encoded heat shock proteins (HSPs) usually follows alteration of plant mitochondrial functions. MRR related to heat stress changes the expression of HSPs in *A. thaliana* (Bita and Gerats 2013). Heat stress can cause male sterility in crop plants and the impairment of pollen is considered to cause yield reduction (Bhandari, Siddique et al. 2016). For example in barley and in *A. thaliana*, when anthers developed at high temperature (30-35◦C), cell proliferation stopped, vacuoles were increased, the development of chloroplasts was affected and mitochondrial abnormalities were observed (Sakata, Oshino et al. 2010). In these examples, changes in the expression of nuclear genes are started by mitochondrial perturbations and the responses are assumed to be produced by MRR. This MRR process related to heat stress is similar to the coordination between endoplasmic reticulum and nucleus found in animals and in plants and that is called as "unfolded protein response" (Patil and Walter 2001; Martinez and

Chrispeels 2003; Liu and Howell 2016). A related signaling pathway from mitochondria to the nucleus started by unfolded proteins in mitochondria has also been identified in animals (Zhao, Wang et al. 2002). The original mechanism of this recently discovered mitochondrial HSP- and heat-stress-related MRR in *A. thaliana* has yet to be determined.

Plants suffer from a range of biotic stresses such as fungal, bacterial, or viral infections. This results in excessive loss. But plants can develop specific defense pathways, depending on the sort of pathogen they are confronted to (Garcia-Brugger, Lamotte et al. 2006; Pandey, Rajendran et al. 2016). Jasmonic acid (JA) and ethylene-dependent reactions appear to be started through necrotrophs, while salicylic acid (SA) response is stimulated by biotrophic pathogens. During pathogen attack, different types of compounds produced by pathogens, called as elicitors, can disrupt mitochondrial functions and induce nuclear gene expression (Rodriguez-Salus, Bektas et al. 2016). A good example of an elicitor is harpin, a glycine-rich and heat-stable protein that is produced by bacterial pathogens. Exposure to harpin causes an interruption of mitochondrial ATP production in *A. thaliana* and in tobacco cells, two species that are hosts for bacterial pathogens generating harpin (Xie and Chen 2000; Balandin and Castresana 2002; Krause and Durner 2004). Harpin induces the expression of HSP genes and genes encoding plant defense proteins, but also of genes encoding the mitochondrial AOX isoforms (Millar, Whelan et al. 2011). In addition, it enhances the roles of mitogen-activated protein kinases that depend on increased ROS level (Samajova, Plihal et al. 2013). Another example of an elicitor is the victorin peptide that causes the disruption of the inner mitochondrial membrane potential, the production of mitochondrial ROS and programmed cell death (Curtis and Wolpert 2002; Yao, Tada et al. 2002; Chapa-Oliver and Mejia-Teniente 2016). Secondary metabolites of plant pathogens can also inhibit the mitochondrial electron transport chain. How changes in the expression of nuclear genes was triggered in these examples has not been identified. Nevertheless, the mitochondrial dysfunction suggests that mitochondria directed nuclear gene expression variations.

As a variety of mitochondrial effectors cause changes in the expression of the alternative oxidase pathway, the regulation of AOX genes can be used as a model for the study of MRR in plants (Rhoads and Subbaiah 2007; Rhoads 2011). The expression pattern of the AOX genes has thus been studied in mitochondrial mutants or upon treatment with compounds disrupting mitochondrial activities such as hormones, salicylic acid and other signaling molecules (Zaninotto, La Camera et al. 2006; Xia, Zhou et al. 2015). The AOX pathway participates in the

hypersensitive response (HR), a form of programmed cell death that occurs at the site of infection to stop pathogen spread (Zaninotto, La Camera et al. 2006; Boualem, Dogimont et al. 2016; González, Brito et al. 2016). For instance, tobacco plants infected with *Tobacco mosaic virus* develop an HR response and increase the expression of AOX to have less lesions in the plant (Ordog, Higgins et al. 2002; Walters 2010). Furthermore, the lower size and number of the lesions after *Tobacco mosaic virus* inoculation observed in wild type plants was also found in mitochondrial mutants with a modified redox status, as well as a higher expression of AOX and of antioxidant enzymes (Dutilleul, Garmier et al. 2003). Other studies linked salicylic acid and AOX to the HR in tobacco, suggesting that salicylic acid worked as an uncoupler of the mitochondrial electron transport chain (ETC) at lower concentration and as an inhibitor of electron transfer at higher concentration (Norman, Howell et al. 2004; Vanlerberghe 2013). Adding more amount of salicylic acid caused the expression of AOX in tobacco, which may be linked to MRR started by inhibition of the mitochondrial ETC (Rhoads and McIntosh 1993). If the efficiency of salicylic acid inhibition of the mitochondrial ETC and the MRR response affecting nuclear genes are related, then the profiles of the genes induced must be similar. This is valid for some target genes, but not others. Therefore, the overlap in the mechanisms is not certain.

In MRR, ROS are also involved. The best studied case for MRR in plants has been the treatment by the Complex III inhibitor antimycin A (AA) (Watanabe, Yamori et al. 2016). AA treatment is known to induce retrograde marker genes, as for instance AOX1a in *A. thaliana* (Dojcinovic, Krosting et al. 2005; Ng, Ivanova et al. 2013). When plant cells are treated with AA, an increased ROS production can be observed in the mitochondria, as detected with 2',7'-dichlorofluorescein, suggesting that the ROS generated by AA may work as signaling intermediates. The role of mitochondrial ROS as a signaling factor was further supported when AA-induced retrograde signaling was decreased in tobacco upon overexpression of AOX and ROS decrease (Maxwell, Wang et al. 1999). Further proof for the role of mitochondrial ROS in retrograde signaling arises from the similarities between the MRR response and the transcriptional responses to various ROS activities and abiotic stresses such as H_2O_2 and salt. This is studied by chemical induction or genetic defect in mitochondrial components (Van Aken, Pecenkova et al. 2007; Meyer, Tomaz et al. 2009). For instance, mutants for the *NDUFS4* gene encoding the 18 kDa subunit of Complex I carry higher basal superoxide levels when measured

by NBT staining (Meyer, Tomaz et al. 2009). Recently it was shown that various AA-affected transcripts depending on the ANAC017 core transcription factor also respond to H_2O_2 application (Ng, Ivanova et al. 2013). Remarkably, 87% of the transcript variations triggered by H_2O_2 were also affected in *anac017* mutants. This indicates that ANAC017 might be a basic controller of H_2O_2 reactions and that at least one path for ROS creation can be through mitochondria. Yet to come is to understand by which intermediates mitochondrial ROS signals would cause an MRR response.

III.3 Cross-talk between mitochondria and chloroplasts

Chloroplasts and mitochondria are metabolically codependent, as photosynthesis offers substrates used for mitochondrial respiration and chloroplasts depend on a variety of compounds produced by mitochondria. Additionally, respiration by mitochondria keeps photosynthetic efficiency at its maximum in the chloroplast (Raghavendra and Padmasree 2003). Interaction between mitochondria and chloroplasts has been investigated by using unicellular organisms such as the alga *Chlamydomonas reinhardtii.* Genetic studies have provided information on the cross-talk between mitochondria and chloroplasts and gave convincing evidence that the inter-organellar communication involves intracellular metabolic pools (Raghavendra and Padmasree 2003). During stress and in developmental processes, redox interactions are a key to understand the coordination between intracellular compartments. During photosynthesis, respiration and photorespiration, ROS and other redox factors are generated by chloroplasts, mitochondria and peroxisomes. The chloroplastic and mitochondrial redox status is monitored and proper retrograde signaling is sent to the nucleus (Kleine and Leister 2016), but ROS and redox signals are also involved in coordination between chloroplasts and mitochondria (Huang, Van Aken et al. 2016).

The multifunction ABI4 transcription factor is a component of abscisic acid, sugar and developmental signaling in plants (Berkowitz, De Clercq et al. 2016). Characterization of *abi4* mutants showed that ABI4 is involved also in MRR (Zhang, Yuan et al. 2016). It works for instance as a promoter-binding, negative regulator of the AOX1a gene, potentially during MRR. Analysis of gene expression control by ABI4 showed that it is involved in both MRR and CRR pathways (Giraud, Van Aken et al. 2009; Estavillo, Crisp et al. 2011).

The existence of suppressor genes in the chloroplast that act on a mitochondrial mutation was reported in the green alga *Chlamydomonas reinhardtii* (Bennoun and Delosme 1999). In the barley albostrians mutant, lack of chloroplast activity causes a compensatory rise in mitochondrial gene copy number and mitochondrial transcript levels (Hedtke, Wagner et al. 1999). The coordination between mitochondria and chloroplasts was also studied using the inhibitors norflurazon (NF) and lincomycin (Lin) to block the development of chloroplasts and study the effects on mitochondrial gene expression, RNA editing and splicing (Liao, Hsieh et al. 2016). The editing of most mitochondrial transcripts was not affected, but editing of the *nad4*-107, *nad6*-103, and *ccmFc*-1172 sites decreased slightly when seedlings were treated with NF and Lin. Steady-state mRNA levels of several mitochondrial genes also increased significantly. In addition, blocking the development of chloroplasts with NF and Lin affected the expression of nuclear genes encoding subunits of the mitochondrial electron transport chain complexes. So, impairment of chloroplasts may coordinately up-regulate the expression of nuclear and mitochondrial genes encoding subunits of respiratory complexes (Liao, Hsieh et al. 2016).

As to reciprocal effects on chloroplast properties in potato and in barley mutated for genes coding for mitochondrial proteins such as glycine decarboxylase, in both species the mutants have a lower glycine decarboxylase activity and a decreased photorespiration, causing an over-reduced state and a higher energy balance in the chloroplast (Igamberdiev, Bykova et al. 2001). The rate of photosynthesis is decreased in male sterile mutants of *Nicotiana sylvestris* lacking the *nad7* gene or in mutants defective in the processing of the *nad4* transcript, both coding for a subunit of mitochondrial Complex I, which emphasizes mitochondrial control on photosynthesis (Sabar, De Paepe et al. 2000). In the same mutants, nuclear genes encoding non-phosphorylating respiratory enzymes were activated, so as to maintain normal respiration levels. In the *prors1-1* mutant of *A. thaliana*, defective in the organellar Prolyl-tRNA synthetase targeted to both mitochondria and chloroplasts, nuclear photosynthetic genes were very much down regulated, supporting the implication of CRR and MRR mechanisms, but also of cross-talk between mitochondria and chloroplasts, in reprogramming the expression of nuclear genes (Pesaresi, Schneider et al. 2007). Communication between the organelles in plants recently appeared to involve specialized membrane contact sites (MCS) (Perez-Sancho, Tilsner et al. 2016). Further recent reports investigate the coordination between mitochondria and chloroplasts, and explore the possible involvement of organellar gene expression (Ng, De Clercq et al. 2014; Shinde,

Villamor et al. 2016; Dahal and Vanlerberghe 2017). The nature of the signals that might mediate these processes and how such signals would be perceived and integrated in the organelles is not established (Woodson and Chory 2008; Woodson and Chory 2012).

IV. Manipulation of mitochondrial genetics

In view of the important role of these organelles in eukaryotic cells, their contribution in disease mechanisms and their status for agronomical traits, a number of laboratories have tried to manipulate mitochondrial genetics. However, at present, conventional transformation methodologies do not allow to manipulate the mitochondrial genome. Regular transformation of the mitochondrion has only been successful in the unicellular organisms *Saccharomyces cerevisiae*, *Candida glabrata*, and *Chlamydomonas reinhardtii*, using conventional biolistics (Bonnefoy and Fox 2007; Zhou, Liu et al. 2010; Niazi, Mileshina et al. 2013; Mileshina, Niazi et al. 2015). Diverse alternative strategies have thus been explored aiming to introduce DNA constructs or RNAs of interest into mitochondria *in vitro* or *in vivo*.

IV.1 Transfection of mitochondria *in vivo*

The first mitochondrial transformation was done in the yeast *S. cerevisiae* using microprojectile bombardment (biolistics) for transfection and taking advantage of the highly active homologous recombination machinery of the organelles for integration of the exogenous DNA into the mtDNA (Armaleo, Ye et al. 1990; Bonnefoy and Fox 2007). *S. cerevisiae rho*^{0} strains that lack mtDNA are commonly used for initial biolistic transfection with a plasmid carrying a sequence destined to complement a mitochondrial deletion. The transfected cells are then crossed with a *rho*$^-$ strain carrying the deletion, thus generating *rho*$^+$ mitochondrial transformants by complementation of the deletion with the exogenous sequence (Bonnefoy and Fox 2000; Bonnefoy, Bsat et al. 2001). In yeast, the availability of convenient selectable markers makes the isolation of mitochondrial transformants a regular approach (Bonnefoy and Fox 2007). In *C. reinhardtii* mitochondrial transformation has also been successful by using biolistics for transfection and cloned gene constructs or PCR fragments as transforming molecules (Remacle, Cardol et al. 2006). Different respiratory-deficient strains (*dum* strains) lacking an mtDNA region with the left telomere and some gene coding sequences (*cob*, *nad4* or *nad5*) could be saved by mitochondrial transformation with linear or circular constructs able to reconstitute the complete genome by homologous recombination, followed by selection in the dark for recovery

of functional respiration (Randolph-Anderson, Boynton et al. 1993; Remacle, Cardol et al. 2006). High transformation efficiency was attained (100-250 transformant/µg DNA) by using linearized plasmid DNA. Myxothiazol-resistant transformants were created by introducing the *mud2* mutation at codon 129 in the *cob* gene. Afterwards, a deletion of 23 codons was made in the *nad4* gene, with deleterious effects on Complex 1 assembly and activity, as well as on total cellular respiration (Remacle, Cardol et al. 2006; Larosa and Remacle 2013). Still, after two month selection in the dark, some transformants remained heteroplasmic, which suggested that the process of mtDNA segregation can be slow.

Mitochondrial transformation by biolistic bombardment with DNA-coated particles failed so far in animal and in higher plant cells. For manipulation of mitochondria in animal and plant cells different strategies have thus been explored. In the first strategy proposed to manipulate the mtDNA in human cells, rho^0 HeLa cells (*i.e.* cells with mitochondria deprived of mtDNA) were fused with cytoplasts (*i.e.* enucleated cells) (King and Attardi 1989; Kagawa and Hayashi 1997). Afterwards, Hela cells were fused with cytoplasts from patients with mutant mtDNA (King, Koga et al. 1992). Through cytoplast fusion *in vitro*, correction of a mitochondrial deficiency was shown in a cell model by transfer of healthy mitochondria (Kagawa, Inoki et al. 2001). In human somatic cells, transfer of foreign mitochondria with a selectable phenotype was achieved by microinjection. The resulting cell lines were maintained *in vitro* (King and Attardi 1988). Still using microinjection, foreign mitochondria isolated from somatic cells could be effectively transferred into recipient mouse germ line cells. The occurrence of the foreign mitochondria could be identified subsequently up to the blastocyst stage (Pinkert, Irwin et al. 1997). In a further study, rodent single cell embryos were first microinjected with isolated mitochondria. From the microinjected cells, a "mitocytoplast" fraction was used for fusion with recipient cells, resulting in transfer of the organelles and restoration of mitochondrial function (Yang and Koob 2012).

The above cell biology methodologies built on the transfer of entire mitochondria may prove suitable for studying human mitochondrial gene expression, but are not directly applicable to gene therapy for diseases due to mtDNA alteration. Further approaches thus aimed to transfer exogenous DNA into the mitochondria in mammalian cells. A whole series of strategies has been documented, but no consensus methodology reproducible in different laboratories has been established until now. A number of these strategies aimed to develop "vehicles" that can take

DNA into the mammalian cell across the plasma membrane and drive it towards mitochondria (Mileshina, Niazi et al. 2015). In the Protofection approach (Khan and Bennett 2004; Keeney, Quigley et al. 2009), a protein transduction domain (PTD) (Rapoport, Salman et al. 2011) was added to the N-terminus of the mitochondrial DNA-binding protein TFAM, upstream of the regular mitochondrial targeting sequence (MTS). The PTD ensures transduction into the cells, the MTS promotes mitochondrial targeting and import, whereas TFAM carries the DNAdomain. Although some success was claimed (Iyer, Xiao et al. 2012) no direct molecular evidence was brought for mitochondrial import of the DNA through the three membranes.

Manipulation of mitochondria has also been explored by using nanocarriers, *e.g*. DQAsomes or MITO-Porters. DQAsomes are cationic, self-assembling vesicles made of dequalinium (Weissig, D'Souza et al. 2001). They can condense with DNA and localize to mitochondria, where the complexed DNA is released. Whether the DNA can subsequently be transferred across the mitochondrial membranes remains to be confirmed. MITO-Porters are liposome-based nanocarriers modified with octaarginine as a cell penetrating peptide. They can enter into cells by macropinocytosis and subsequently fuse with the mitochondrial membranes, delivering their cargo into the organelles (Yasuzaki, Yamada et al. 2010). It has been reported that MITO-Porters could deliver the nucleic acid stain propidium iodide, DNase1, green fluorescent protein or DNA into mitochondria (Yasuzaki, Yamada et al. 2010; Yamada, Furukawa et al. 2011; Yamada and Harashima 2012) and it seems to be promising.

IV.2 Import of tRNAs

Import of tRNA into mitochondria was discovered in the 1970s during the study of tRNAs in the ciliated protozoan *Tetrahymena pyriformis* (Chiu, Chiu et al. 1974; Suyama 1986). Mitochondria in most of the organisms do not encode a number of tRNAs essential for protein synthesis inside the organelles. The missing tRNAs are imported form the cytosol. The number and identity of the imported isoacceptors, as well as the transport machinery for the import of missing tRNAs are variable from species to species. For instance, the mtDNAs of *Trypanosoma* and *Leishmania* do not encode any tRNA and import all the tRNAs from the cytosol (Tan, Pach et al. 2002; Esseiva, Naguleswaran et al. 2004). There are two $tRNAs^{Met}$ needed for protein synthesis, one used for initiation and the second for chain elongation. In *Trypanosoma*, part of the elongator $tRNA^{Met}$ becomes formylated after import into mitochondria to serve for translation initiation

(Tan, Pach et al. 2002; Schneider 2011). In mammals, the mtDNA encodes all 22 tRNAs that are needed for translation of the modified genetic code used in the mitochondria (Paabo, Thomas et al. 1991; Sieber, Duchene et al. 2011). But in marsupials, the cytosolic $tRNA^{Lys}$ is imported into mitochondria (Dorner, Altmann et al. 2001), while human and rat mitochondria were claimed to import the cytosolic $tRNAs^{Gln}$ (CUG) and (UUG) (Rubio, Rinehart et al. 2008). In the yeast *S. cerevisiae*, tRNAs for all 20 amino acids are encoded by the mtDNA, but one $tRNA^{Lys}$ (Tarassov and Entelis 1992) and possibly two $tRNAs^{Gln}$ (Rinehart, Krett et al. 2005) are imported from the cytosol as well (Duchene, Pujol et al. 2009). In bean (*Phaseolus vulgaris*) mitochondria, the presence of a cytosolic $tRNA^{Leu}$ was first reported (Green, Marechal et al. 1987). After this, a number of other tRNAs (*e.g.* $tRNA^{Arg}$, $tRNAs^{Ala}$, $tRNA^{Thr}$, $tRNA^{Gly}$ and $tRNAs^{Val}$) have been reported to be encoded by the nucleus and imported into plant mitochondria (Marechal-Drouard, Guillemaut et al. 1990; Dietrich, Weil et al. 1992; Small, Marechal-Drouard et al. 1992; Kumar, Marechal-Drouard et al. 1996). As a whole, in plants one third to one half of the mitochondrial tRNAs are imported from the cytosol (Kumar, Marechal-Drouard et al. 1996; Glover, Spencer et al. 2001; Delage, Dietrich et al. 2003; Mileshina, Niazi et al. 2015). But the number and specificity for amino acids of the imported tRNAs differ from one plant system to another, even between closely related species (Kumar, Marechal-Drouard et al. 1996; Glover, Spencer et al. 2001; Schneider 2011). In *A. thaliana* 22 tRNA genes have been identified in the mitochondrial genome and about 13 tRNAs are imported from the cytosol to decode the whole set of codons. The sugar beet mitochondrial genome encodes 21 tRNAs. It has been suggested that during the evolution, the tRNA import system undergoes rapid changes (Schneider 2011).

A number of mitochondrially-encoded tRNAs of animals are unique in their features, as compared to the tRNAs found in other systems. They are structurally weaker and shorter as compared to the prokaryotic and eukaryotic cytosolic counterparts. The tRNAs of animals can be folded into an L-shaped tertiary structure, but the D-loop/T-arm interactions in the elbow part are not present. On the basis of structural characteristics, animal mitochondrial tRNAs can be classified into five groups (Watanabe 2010). Conversely, mitochondrial tRNAs of higher plants are canonical in structure (Zhang, Thieme et al. 2016). Because many plant species import a number of cytosolic tRNAs for use in mitochondria, this structure conservation is predictable. Fungal and Trypanosomatid mitochondrial tRNAs have a classical structure.

For import of tRNAs into mitochondria, several import signals have been detected. On the basis of observations in the import process, it seems that these signals vary from species to species (Salinas, Duchene et al. 2008; Schneider 2011). So, in this way the recognition signals are not common in all the imported tRNAs. For instance in protists there are two types of imported tRNAs known so far (Clayton 2016). In Type I tRNAs, import determinants are found in the D-loop (Rusconi and Cech 1996), while the tRNA import signals for type II tRNAs are in the T-arm (Suyama, Wong et al. 1998). There are also anti-determinants that can inhibit the import of some cytosolic tRNAs (Salinas, Duchene et al. 2008).

As mentioned, in the mitochondria of *T. brucei* all tRNAs have to be imported from the cytosol. Different pathways, mechanisms and proteins have been suggested for this process (Schneider 2011). The same aminoacyl-tRNA synthetases are used in the cytosol and in mitochondria of *Trypanosoma* and *Leishmania*. It is thus conceivable that they play a role in mitochondrial import of tRNAs (Duchene, Pujol et al. 2009). However, in these organisms, efficient import of tRNAs has also been observed in the absence of mitochondrial membrane potential, whereas the membrane potential is a prerequisite for protein translocation across the mitochondrial membranes. Furthermore, there might be different receptor proteins for RNAs on the mitochondrial surface.

The mechanism of tRNA import into plant mitochondria is still under investigation. There is no identified sequence or secondary structure motif that would differentiate the set of nuclear-encoded mitochondrial tRNAs from the non-imported cytosolic tRNAs. The variation in the pattern of tRNA import into plant mitochondria suggests that each tRNA is identified by specific import factors. In particular, it is commonly known that tRNAs are not free in the cytosol, but they are associated with the different proteins that are essential for cytosolic translation. Import factors are thus needed to help some tRNAs to escape from the cytosolic protein synthesis pathway and to be directed to the organelles. Aminoacyl-tRNA synthetases might be involved in such processes, as it is the case for $tRNA^{Lys}$ import into mitochondria in yeast (Kamenski, Smirnova et al. 2010). The organization of aminoacyl-tRNA synthetases is very complex in plants, due to the presence of an extra translation system in the chloroplast (Duchene, Pujol et al. 2009). The mitochondrial or chloroplastic DNA of plants does not code for aminoacyl-tRNA synthetases, which are thus nuclear-encoded and imported. Although the specificity can vary in different plant species, aminoacyl-tRNA synthetases can be divided into different groups: (A)

enzymes that are used in the cytosol, (B) enzymes that are imported into both mitochondria and chloroplasts, (C) enzymes that function in the cytosol and in mitochondria, (D) specific enzymes for chloroplasts.

In bacteria, plants and animals, the G3:U70 wobble base-pair in the acceptor stem is the major determinant in $tRNA^{Ala}$ that is recognized by alanyl-tRNA synthetase (Carneiro, Dietrich et al. 1994; Francis 2011). A U_{70} to C_{70} change, which can bring the normal structure of Watson-Crick base-pairing, abolishes binding of $tRNA^{Ala}$ to alanyl-tRNA synthetase and aminoacylation (Maréchal-Drouard, Dietrich et al. 1999). In all higher plants, mitochondrial $tRNA^{Ala}$ is encoded by the nucleus. To test a possible role of alanyl-tRNA synthetase in targeting the nucleus-encoded $tRNA^{Ala}$ into mitochondria, constructs were designed to express wild-type U_{70} or mutant C_{70} forms of the *A. thaliana* $tRNA^{Ala}$ in tobacco plants (Dietrich, Marechal-Drouard et al. 1996) . In the transgenic plants, both forms of $tRNA^{Ala}$ were found in the cytosolic fractions, but only the wild-type U_{70} $tRNA^{Ala}$ was found in mitochondria. In this way, the single U_{70}-to-C_{70} mutation was enough to stop both aminoacylation and import of $tRNA^{Ala}$, strongly supporting the hypothesis that recognition of the tRNA by the corresponding aminoacyl-tRNA synthetase is required for mitochondrial import in plants (Dietrich, Marechal-Drouard et al. 1996; Dietrich, Small et al. 1996; Delage, Dietrich et al. 2003). To find whether an aminoacyl-tRNA synthetase alone can drive a tRNA into mitochondria, potato plants have been transformed so as to express a yeast aminoacyl-tRNA synthetase able to be imported into mitochondria and to recognize a non-imported cytosolic tRNA species. Cytosolic aspartyl-tRNA synthetase was selected, because the $tRNA^{Asp}$ present in potato mitochondria is encoded by the mtDNA and potato cytosolic $tRNA^{Asp}$ is a good substrate for the yeast enzyme *in vitro* (Dietrich, Small et al. 1996). The mitochondrial targeting sequence of the β subunit of the *Nicotiana plumbaginifolia* mitochondrial ATP synthase was added to the yeast cytosolic AspRS for mitochondrial import of the enzyme. In the transgenic plants, expression and mitochondrial import of the yeast AspRS was detected by probing western blots, but the endogenous plant cytosolic $tRNA^{Asp}$ was not present in the mitochondrial fractions, illustrating that the import of aspartyl-tRNA synthetase did not lead to the import of its tRNA substrate. These results support the idea that recognition by a mitochondrially imported aminoacyl-tRNA synthetase is not enough to promote efficient import of a tRNA, implying that there must be other factors required (Dietrich, Marechal-Drouard et al. 1996). On the other hand, there are also some import determinants in tRNAs that

are distinct from the determinants for aminoacyl-tRNA synthetase recognition (Delage, Dietrich et al. 2003; Laforest, Delage et al. 2005).

Although in most organisms, the results propose that components of the mitochondrial protein translocation pathway are needed for mitochondrial uptake of tRNA *in vivo* (Schneider 2011; Tschopp, Charriere et al. 2011), an RNA import complex (RIC) has been put forward in *Leishmania tropica* (Mukherjee, Basu et al. 2007). It is composed of 11 stoichiometric proteins, is assembled at the mitochondrial inner membrane and adds up to a total of ~580 kDa. In this case, the import complex needs ATP and a membrane potential for tRNA import. There are 3 mitochondrion-encoded and 8 nucleus-encoded subunits within the RIC complex. Knockdown analyses specified that at least 6 nucleus-encoded subunits, RIC1, 4A, 6, 8A, 8B and 9 are required for import (Mukherjee, Basu et al. 2007). By using affinity the RIC complex has been isolated, and has been resolved from other mitochondrial complexes by native gel electrophoresis (Goswami, Dhar et al. 2006). Functional RIC complexes could be reconstituted from overexpressed components. During these experiments, two non-essential subunits, RIC3 and M16 metalloproteinase, have been identified. For initial tRNA binding, it is proposed that RIC1 and RIC8A are involved. RIC6 and RIC9 would constitute the translocation pore, and RIC4A and RIC8B would anchor the complex to the membrane. It was initially reported that the Rieske protein is an essential component for tRNA import but subsequent downregulation of this protein by RNA interference had no effect on import (Paris, Rubio et al. 2009). So, the mechanism for tRNA import in the kinetoplastid system is still a matter of debate.

IV.3 A tRNA-derived mitochondrial shuttling system in plants

The $tRNA^{Leu}$ isoacceptors in plants belong to those tRNAs that are encoded by the nucleus and imported into the mitochondria (Marechal-Drouard, Guillemaut et al. 1990; Kumar, Marechal-Drouard et al. 1996) . Biochemical analysis also showed that the same leucyl-tRNA synthetase is present in the mitochondria and in the cytosol (Marechal-Drouard, Weil et al. 1988; Dahan and Mireau 2013). A $tRNA^{Leu}$ from *Phaseolus vulgaris* was tentatively used as an *in vivo* import shuttle in potato (*Solanum tuberosum*) upon addition of 4 extra nucleotides into the anticodon loop. The extended tRNA was expressed from a nuclear transgene and recovered in mitochondria, suggesting at the same time that the tRNA does not need to be involved in translation to be imported. But this strategy failed when further additional sequences were added

within the $tRNA^{Leu}$ because there was no stable expression *in vivo* with extended sequences (Small, Marechal-Drouard et al. 1992). Another strategy developed by the host laboratory was to attach short extra sequences at the 5'-end of $tRNA^{Leu}$. But the additional sequences were removed by RNase P, which recognizes only the structure of the tRNA and cleaves any upstream sequence (Schon 1995; Wilusz, Freier et al. 2008). It is also not possible to rely on the natural nuclear tRNA gene transcription pathway to express a tRNA with a 5' extension because transcription by RNA polymerase III is directed by the internal promoters and the transcription start site is found only a few nucleotides upstream of the mature 5'-end of the tRNA (Geiduschek and Tocchini-Valentini 1988). Following these studies, our laboratory developed an alternative strategy of mitochondrial import based on RNA polymerase II expression of the transgene and on the use of a tRNA mimic that is not efficiently recognized by RNase P *in vivo.*

The 3'-end of the genomic RNAs of plant viruses generally folds as a tRNA-like structure (TLS) that can be aminoacylated but is not a common substrate for RNase P (Rietveld, Van Poelgeest et al. 1982; Florentz, Briand et al. 1984; Dreher 2009; Dreher 2010). This is the case in particular of the *Turnip yellow mosaic virus* (TYMV) genomic RNA (Matsuda and Dreher 2004). The TYMV genome is a single-strand RNA of 6318 nucleotide long (Kuznetsov, Daijogo et al. 2005), with a cap at the 5'-end and an 82 nucleotide long TLS at the 3'-end. The latter functionally mimics a $tRNA^{val}$, sharing many properties with this tRNA (Matsuda and Dreher 2004; Dreher 2010). The TYMV TLS can be folded into an acceptor stem, T-arm, anticodon arm and D-arm cloverleaf structure resembling that found for tRNAs. However, the acceptor stem includes a pseudoknot (Pleij, Rietveld et al. 1985), a feature that is currently considered to be present in further plant viral tRNA-like structures. Despite the additional pseudoknot, the tertiairy structure model of the TYMV TLS shows a strong similarity with tRNAs (Dreher 2004). In particular, there is a direct interaction between the T and D-loops, as found in tRNAs (de Smit, Gultyaev et al. 2002). The sequence between nucleotides 83 and 109 upstream of the TLS in the TYMV genomic RNA adopts a pseudoknot structure (UPSK) that contributes to optimal recognition by the valyl-tRNA synthetase (Dreher 2004; McCormack III 2007). The TLS and the UPSK seem to form a very stable structure that is not disturbed by the presence of the rest of the long genomic RNA of the TYMV. The TLS can be recognized and cleaved in the acceptor region by *E. coli* RNase P under certain *in vitro* conditions (Guerrier-Takada, van Belkum et al. 1988), but it does not seem to be efficiently recognized by plant RNase P *in vivo*. On the other

hand, the overall structure of the TYMV TLS mimics the aminoacylation properties of $tRNA^{Val}$ and $tRNA^{Val}$ is naturally imported into the mitochondria of all higher plants studied to date (Sieber, Duchene et al. 2011). Based on all these properties, the host laboratory developed the use of the TYMV TLS as a shuttle to import 5' cargo RNAs into mitochondria (Val, Wyszko et al. 2011; Niazi 2013).

The import shuttle was made of the last 120 nucleotides (positions 6199-6318) of the 3'-end of the TYMV genomic RNA. The UPSK sequence was included, as it optimizes the recognition by valyl-tRNA synthetase and recognition by the cognate aminoacyl-tRNA synthetase is essential for *in vivo* tRNA import into mitochondria in plant cells (Dietrich, Small et al. 1996; Salinas-Giege, Giege et al. 2015). The whole shuttle was thus called PKTLS(Val, Wyszko et al. 2011). The cargo sequences of interest were attached to the TYMV PKTLS as 5'-trailor sequences, usually through a linker. The linker sequences were adapted from loose random sequences of 40 nucleotides, so that there was no interaction between the cargo and the PKTLS. For expression of the chimeric RNAs (cargo sequences fused to the 5'-end of the PKTLS through a linker), the constructs were placed under the control of an RNA polymerase II promoter (constitutive or inducible) and terminator. The PKTLS sequence was followed at the 3'-end by the sequence encoding the *cis*-cleaving ribozyme of the *Hepatitis delta virus* (HDV). All these were transferred as a transgene into the nuclear genome of plant cells and whole plants. Expression of such constructs by RNA polymerase II produces a primary transcript with the cargo sequence, the linker, the PKTLS, the HDV *cis*-ribozyme and the termination sequence. The RNA is capped at the 5'-end and polyadenylated at the 3'-end. Self-cleavage of the HDV *cis*-ribozyme then removes the termination sequences and poly (A) tail, unmasking the 3'-CCA-end of the PKTLS. Subsequently, the resulting RNA (cargo sequence-linker-PKTLS) is exported out of the nucleus and imported into mitochondria (Val, Wyszko et al. 2011). A mutated form of the TYMV PKTLS that is aminoacylatable with methionine is also available (Dreher, Tsai et al. 1996; Albers and Czech 2016). However, $tRNA^{Met}$ is not imported into the mitochondria of higher plants (Delage, Dietrich et al. 2003). In this way, the methionine-specific PKTLS served as a negative control and was indeed unable to drive cargo sequences into mitochondria, showing that the process follows the natural pathway of tRNA uptake (Val, Wyszko et al. 2011). The host laboratory has transferred different types of cargo sequences into mitochondria, including short

viral sequences, hammerhead ribozymes designed to *trans*-cleave target RNAs in the organelles, or substrates for the mitochondrial RNA editing system.

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 the *atp9* mitochondrial mRNA. The approach was efficient and the chimeric catalytic RNA caused a strong knockdown of the target mRNA in mitochondria (Val, Wyszko 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. The host laboratory used the same strategy and stably transformed cell suspension of *N. tabacum* and whole plants of *A. thaliana* to knock down the *nad9* mitochondrial RNA through the import of a specific *trans*-cleaving hammerhead ribozyme. Again, the approach was effective and the chimeric catalytic RNA caused a strong knockdown of the target mRNA in mitochondria (Niazi 2013).

V. Recombination in mtDNA maintenance

Genome stability is a fundamental necessity for cell growth and production. Failure in the maintenance of genome stability causes mutations and gene rearrangements that can result in harmful effects. For instance, human cancer is due to genomic instability (Halazonetis, Gorgoulis et al. 2008; Smith, Guyton et al. 2016). So, it appears better to maintain the genome completely intact at the single organism level. But genetic diversity causes the reshaping of the genome. For this, a specific level of genome instability is critical. If the process of diversity is destroyed, it would also affect the resilience and adaptability of the species to a changing environment. To attain this adaptability, all organisms have specific systems that can maintain the stability of their genome but do not interfere with the process of evolution. Different proteins are involved in the process of DNA replication, recombination and repair (Gualberto, Mileshina et al. 2014; Zhang, Ruhlman et al. 2016). If a mutation happens in these proteins, it results in increased genome instability (Aguilera and Gomez-Gonzalez 2008; Mladenov, Magin et al. 2016). Different approaches have been conducted so far to understand the mechanisms involved in the stability of bacterial and nuclear genomes, but until now, little is known about how organellar genomes are preserved. Moreover, plant cells have two types of endosymbiotic organelles and for this they have to keep the stability of the three separate genomes found in the nucleus, mitochondria and

plastids. For this reason, investigating DNA maintenance in plants is also of particular interest to understand the mechanisms involved in the stability of the genomes. In flowering plants, no DNA replication, recombination and repair proteins needed for the maintenance of the organellar genome are encoded by the mtDNA (Bock 2007; O'Brien, Zhang et al. 2009). Mitochondria of flowering plants have thus to import these proteins from the cytosol so that they can keep the genetic material functional. In this context, different research groups have identified a series of families of nuclear-encoded proteins involved in the maintenance of plant organellar genome stability (Gualberto, Mileshina et al. 2014; Oldenburg and Bendich 2015; Smith and Keeling 2015; Zhang, Ruhlman et al. 2016). A major function of these protein families is the control of recombination between repeated sequences in the mtDNA. Suppression or mutation of these proteins causes large scale rearrangements of the organellar genome that ultimately disrupt the function of mitochondria. Such mutations cause different phenotypes in plants, *e.g.* leaf varigation and cytoplasmic male sterility (CMS), but also help in understating the functions of these proteins in the stability of organellar genomes in plant development (Gualberto, Mileshina et al. 2014).To maintain genome stability, different repair pathways exist in the cells and are described below, together with the involved proteins.

V.1 Mismatch repair (MMR)

Different factors can cause damage to the DNA in the cells over time, such as exogenous chemicals and physical agents (*e.g.* polychlorinated biphenyls, cigarette smoke, ultraviolet light, asbestos, dioxin, radon, etc.), or endogenous reactive metabolites (*e.g.* reactive oxygen and nitrogen species, ROS and NOS). DNA damage is also possible during DNA metabolism, in DNA processing reactions such as DNA replication, recombination, and repair (Polo and Jackson 2011). Errors during DNA synthesis cause nucleotide mis-incorporation and generate DNA base-base mismatches. If the mismatch in the DNA remains unrepaired, it can cause a mutation in somatic or germline cells that can change the cellular phenotype and lead to dysfunction and disease. Mismatch repair (MMR) allows recognition and excision of mismatches generated during DNA replication, thereby preventing mutations becoming permanent in dividing cells (Modrich and Lahue 1996; Kolodner and Marsischky 1999; Li, Pearlman et al. 2016).

In *E.coli*, three proteins, MutS, MutL, and MutH called as the MutHLS system, are involved in MMR (Iyer, Pluciennik et al. 2006; Hermans, Laffeber et al. 2016). First MutS (homodimer)

identifies and binds the mismatched base in the dsDNA (Seidel and Szczelkun 2013). After this, MutL (homodimer) interacts with the MutS-DNA mismatch complex, stabilizes the complex and activates the MutH endonuclease (Putnam 2016). In this process, MMR requires to distinguish the newly synthesized strand from the correct native strand of the DNA, so that it can remove the mismatched base. But the mismatch does not have a signal for such recognition. The MMR system in *E.coli* thus looks for the nearest d(GATC) methylation site to the mismatch and uses the lack of methylation of the newly synthesized strand to direct the repair (Putnam 2016). The MutH endonuclease nicks the unmethylated strand at the hemimethylation site. The UvrD DNA helicase unwinds the DNA and an ssDNA-specific exonuclease then digests the single-stranded tail until it passes the mismatch. Re-synthesis of the digested strand is then done by DNA polymerase and the final nick is sealed by DNA ligase. In *E.coli*, as methylation gives the signal for MMR, the process is called as methyl-directed MMR (Goellner, Putnam et al. 2015; Putnam 2016). Homologues of MutS and MutL of *E.coli* are found in all organisms and homologues for MutH of *E.coli* have been found in the majority of eukaryotes and in most bacteria (Modrich 2016).

In eukaryotes, strand discontinuity serves as a signal to direct MMR to a specific strand of the mismatched duplex *in vivo*, as. newly synthesized strands in living cells contain discontinuities at 3'-ends or at termini of Okazaki fragments (Lujan, Williams et al. 2012; Grin and Ishchenko 2016). Nicked plasmid DNAs having a strand discontinuity thus served as a model system for biochemical characterization of eukaryotic MMR (Fukui 2010). The excision reaction removed the shorter path from a nick to the mismatch, showing that 5'-and 3'-directed MMR are distinct. The 5'-3' exonuclease activity of exonuclease 1 (EXO1) was required for both 5'- and 3'-directed strand removal (Kadyrova and Kadyrov 2016). The need for 5'-3' exonuclease activity in 3'-directed MMR was unexplained because it was considered that strand discontinuity was also the entry point for the excision reaction. But later on this issue was resolved through the discovery that the human MutL homologue MutLα (MLH1-PMS2 heterodimer) and the yeast homologue MutLα (MLH1-PMS1 heterodimer) have a latent endonuclease activity that nicks the discontinuous strand of the mismatched duplex (Iyer, Pluciennik et al. 2015). Thus, the 3'- and 5'- sides of a mismatch are both incised by MutLα and EXO1 digests the segment spanning the mismatch (Dexheimer 2013).

V.2 Repair of double-strand breaks

Double-strand breaks (DSBs) are the most harmful lesions in the DNA because they can cause a loss of genetic information, deletions or rearrangements, chromosomal instabilities or distortion of the double helix. DSBs can be produced by ionizing radiations, nuclease dysfunction, ROS, UV and when a replication fork stops. DSBs can be repaired by two main repair pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ). Both of these repair pathways can also generate diversity, mutations, deletion or insertions. In the HR pathway, recombination involving similar sequences present in different regions of the genome can cause rearrangements. In the case of multiple breaks, the NHEJ pathway can join ends that are unrelated, causing deletions or insertions.

V.3 Repair by non-homologous end joining

The non-homologous end-joining (NHEJ) pathway participates in DSB repair and telomere maintenance in all kinds of cells from bacteria to human. The V(D)J recombination mechanism in lymphocytes also involves NHEJ factors (Gellert 2002; van Gent, IJspeert et al. 2016). In eukaryotes, the main proteins involved in the NHEJ pathway are DNA ligase IV, XRCC4, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), the Ku proteins, and Artemis (Chang and Lieber 2016). The process is initiated by the binding of the Ku70/Ku80 heterodimer to the broken DNA ends. Both strands of DNA molecules are encircled by Ku proteins, forming a ring around the DNA duplex. Ku proteins help to align the broken DNA ends and to protect them from degradation, and also prevent the attachment of non-relevant proteins. At the same time, Ku proteins leave access to the polymerases, nucleases and ligases required for end joining. Broken ends of DNA cannot necessarily be directly ligated, but need proper processing by a series of specific proteins. Ku heterodimers recruit DNA-PKcs, which stabilizes the proximity of the two ends and allows complementary microhomology regions to hybridize. DNA-PKcs also phosphorylates the Artemis protein, which subsequently cleaves remaining overhangs. After the blunt ends are created, the XRCC4/DNA ligase IV complex is recruited to join the DNA ends together (Rulten and Grundy 2017).

V.4 Repair by homologous recombination

The homologous recombination pathway (HR) is considered to be the main mechanism for the repair of DSBs in bacteria and consists of various steps: end resection, strand invasion, repair

synthesis, branch migration and Holiday junction resolution. The steps of this process are conserved, but the factors involved can differ between organisms. HR starts with DNA-end processing. Each end of the break is incised from 5’ to 3’ to generate a single-stranded 3’-end region. In bacteria, this strand resection can be carried out by different helicase-nuclease complexes: RecBCD, AddAB, or AdnAB. RecB has a 3’-5’ helicase domain associated with a nuclease domain. RecC comprises a recognition domain for the crossover hotspot instigator (Chi) sequence (sometimes designated with the Greek letter χ). Chi sequences are 8 nucleotide motifs near which HR is most likely to occur. The motif is 5'-GCTGGTGG-3' in *E. coli*, but differs between groups of closely related organisms. The Chi sequence exists in many copies in the bacterial genome and can thus stimulate recombination. RecD is a 5'-3' helicase. RecD and RecB unwind the DNA and RecB nicks the strand with the Chi motif that is recognized by RecC. Unwinding continues and produces a 3' single-stranded tail with the Chi near its terminus. The 3’ single stranded region is then loaded with RecA, which mediates the invasion of the single-stranded DNA into a homologous double-stranded region in the genome and it’s pairing with the complementary strand. In this way, a structure called as D-loop is generated. In *E.coli*, genetic studies of RecBCD mutants have also revealed an auxiliary bacterial DNA end-resection pathway that is dependent on the RecQ helicase and RecJ exonuclease. Unwinding of the duplex template is done by the RecQ helicase, while the RecJ exonuclease resects the 5’ strand. This pathway is dependent on the RecFOR recombination mediator complex and also loads RecA on the single-stranded DNA for strand invasion.

After the establishment of a D-loop, different pathways can be set up and produce end products with a different organization. In the case of “break-induced replication” (BIR), the D-loop may turn into a replication fork because there is no second single stranded end. When the replication fork is in place, the synthesis of DNA proceeds until the end of the chromosome and generates a newly formed strand. When the D-loop dissociates once the invading strand has been extended, the process switches to synthesis-dependent strand annealing (SDSA). The newly extended strand in this case re-anneals with the second single stranded region of the initial break. The latter functions as a template for repair and gap filling. In such a mechanism, there is no crossover and rearrangements are avoided, as the 3’-ends on each side of the break are extended along the initial homologous complementary strand.

There are also two mechanisms in which RecA is not involved: single strand annealing (SSA) and microhomology-mediated end joining (MMEJ), also known as alternative non-homologous end-joining (Alt-NHEJ). The SSA mechanism uses repeated regions oriented in the same direction as the identical sequences required for HR repair and proceeds without RecA. When two repeats of over 30 nucleotides are present in the flanking regions of a DSB, the corresponding single-stranded sequences can anneal after resection of the DSB ends. The 3' non-homologous sequences are cleaved, the gaps are filled by a DNA polymerase and the strands are sealed by a ligase. But during this process, the genetic information between the two repeats is lost. In yeast, the evidence for SSA was provided by the observation that the HO endonuclease can stimulate deletions between *ura3* sequences *in vivo*. Microhomology-mediated end joining (MMEJ) is a similar pathway in eukaryotes, when a repeat of 5 to 30 nucleotides is located in the 3'-overhang resulting from resection. Chromosome abnormalities such as deletions, translocations, inversions and other complex rearrangements are frequently associated with MMEJ.

In eukaryotes, the process of DSB end resection is similar to that of RecFOR in bacteria. Multiple RecQ homologues are observed that are involved in the DNA-end resection. The first step of HR in eukaryotes involves the binding of protein factors to the broken DNA-ends, which initiates the subsequent processing of such ends into the single-stranded 3'-overhangs required for stand invasion. The MRN complex (Mre11-Rad50-Nsb1) in human, and the MRX complex (Mre11-Rad50-Xrs2) in yeast are involved in the creation of these 3'-overhangs for HR (Symington 2016). Mre11 has both single-strand endonuclease and 3'→5' double-strand exonuclease activity (Gobbini, Cassani et al. 2016). The Mre11-Rad50 (MR) complex binds to the DNA at DSBs. Binding triggers conformational changes, which allows inter-complex coiled-coil interactions that in turn increase DNA binding (Moreno-Herrero, de Jager et al. 2005). The major requirement for HR is base-pairing between the brocken DNA strand and an intact homologous strand. For this reason, it is necessary that the damaged DNA strand base-pairs with an undamaged strand in a sister DNA molecule. For this purpose, in eukaryotes the Rad51 protein is first coated on the single-stranded DNA resulting from resection (Godin, Sullivan et al. 2016). The formation of the Rad51 protein nucleo-filament has an inverted polarity in eukaryotes, as compared to bacteria, and occurs in the 3'→5' direction (Murayama, Kurokawa et al. 2008). Strand invasion promoted by Rad54 and recombinases then allows base-pairing with

the complementary strand in the double-stranded sister DNA molecule and establishes a branch point (Sung, Krejci et al. 2003; Heyer, Ehmsen et al. 2010). Later on, the second resected single stranded end of the DSB base-pairs with the complementary sequence in the loop formed upon strand invasion, which results in a four-stranded cross-shaped structure known as Holliday Junction (Holliday 1964). The two branch points move along the DNA in opposite directions, while DNA polymerase copies the missing information from the undamaged template and fills the gaps. A DNA ligase seals the repaired strands and the Holliday junction is resolved by specific resolvases such as Mus11, Yen1 or Slx-Slx-4 (Svendsen and Harper 2010).

V.5 Mitochondrial repeated sequences and recombination in plants

Repeated sequences are the main sites for intramolecular recombination in plant mtDNA (Kühn and Gualberto 2012). As mentioned above, recombination mostly involves pairs of direct or inverted repeats in the kb range (Röhr, Kües et al. 1999). Recombination across such repeated sequences leads to the generation of subgenomic DNA molecules (Alverson, Rice et al. 2011; Mower, Case et al. 2012). Besides frequent large size repeat recombination, intermediate size repeats (100-500 bp) recombine infrequently, and recombination involving short repeats (4-50 bp) is very rare (Kitazaki and Kubo 2010; Kühn and Gualberto 2012; Wallet, Le Ret et al. 2015).

V.5.1.1 Recombination across large repeats

Large repeated sequences mediate reciprocal DNA exchange that subdivides the mitochondrial genome. If multiple pairs of large repeats are present in the genome, recombination produces a complex, inter-genomic population of heterogeneous molecules (Wolstenholme and Fauron 1995). Generally, recombination across large repeats produces equal amounts of parental and recombined forms. All the flowering plants analyzed so far have large repeats, but their number varies between different species. Three copies of a single recombination repeat are present in the *Petunia hybrida* genome (Folkerts and Hanson 1989). These three repeats recombine and generate nine different subgenomic configurations. The C24 *A. thaliana* accession has two pairs of large repeats (6.5 kb and 4.2 kb) in direct and in inverse orientation (Thompson and Knauf 2009). These two pairs of large repeats are conserved in other *A. thaliana* accessions but not in the same orientation. Large repeats do not necessarily have functions in mitochondrial stability or activities.

V.5.1.2 Recombination involving intermediate-size repeats

Intermediate size repeated sequences (IRs) mediate low frequency, often asymmetric DNA exchange. Plant mtDNAs have many intermediate size repeats, as compared to the few large repeats. Recombination due to IRs generates complex rearrangements in the genome that lead to novel DNA polymorphism, intraspecific genomic variation and substoichiometric shifting (SSS) (Kühn and Gualberto 2012). CMS can be associated with genomic rearrangements in the process of SSS (Tanaka, Tsuda et al. 2012). While SSS activity can take place spontaneously, but at low frequency, similar rearrangements are also observed in tissues. The best documented examples of rearrangements by IRs are found in the mitochondrial genome of *A. thaliana*, but similar processes occur in other plant species as well. It was highlighted that 72 repeats of 50 to 626 bp are present in beet, 32 repeats of 100 bp to 830 bp in maize, 26 repeats of 100 to 405 bp in tobacco (Arrieta-Montiel, Shedge et al. 2009; Arrieta-Montiel and Mackenzie 2011). In *A. thaliana*, the DNA exchange activity through IRs can be increased by disruption of nuclear genes like *MSH1, OSB1* or *RECA3* (Arrieta-Montiel, Lyznik et al. 2001; Zaegel, Guermann et al. 2006; Miller-Messmer, Kuhn et al. 2012). Most IRs become active in recombination in the case of *msh1* mutants. In the *recA3* mutants, mtDNA rearrangements due to IRs are less extensive, as compared to *msh1* mutants (Shedge, Arrieta-Montiel et al. 2007). It has been proposed that IRs are involved in the break-induced replication and single strand annealing pathways, and in DSB repair (Woloszynska and Trojanowski 2009). Recombination through smaller repeats tends to be asymmetric, resulting in just one of the two possible recombination products (Davila, Arrieta-Montiel et al. 2011). These can subsequently be amplified to a much higher frequency through SSS (Kühn and Gualberto 2012). Initial substoichiometric forms have been estimated at levels as low as one copy per 100-200 cells of the plant (Abdelnoor, Yule et al. 2003).

V.5.1.3 Small repeated sequences

Small (4-50 bp) repeated sequences are also involved in mitochondrial genome changes and mtDNA evolution in plants (Kühn and Gualberto 2012). These repeats participate in non-homologous end joining (NHEJ). Chimeric regions are generated in the genome, due to small repeated sequences at the junction of unrelated sequences (Hanson and Bentolila 2004). As mentioned, generation and expression of chimeric genes are often associated with CMS. Such a chimeric gene is expressed in T-CMS in maize (Dewey, Levings et al. 1986). *C. reinhardtii* and

C. eugametos have small repeated sequences in the intergenic spacers (Boudreau, Otis et al. 1994). AT-rich small repeated sequences are found in the mitochondrial genome of *Prototheca wickerhamii*. Various foreign DNA introgressions of plastid, nuclear and viral sequences into the mitochondrial genome of plants are also observed (Kubo and Newton 2008). These too are considered as a consequence of NHEJ activity. Small repeats and even stretches of sequence homology are found at the junctions of sequence chimeras, suggesting that they have supported DNA exchange (Kubo and Newton 2008).

VI. Genes involved in mtDNA replication, repair, and recombination

A number of genes that are involved in plant mtDNA replication, repair and recombination have been characterized. Some of these genes are specific to plants. Conversely, others appear to have a prokaryotic origin (Ku 2016). The latter do not seem to be conserved in yeast and mammalian mitochondrial mtDNA maintenance mechanisms. Nuclear-encoded factors required in mammals for mtDNA replication include DNA Polγ (DNA polymerase gamma) and the Twinkle helicase (McKinney and Oliveira 2013). When DNA Polγ or Twinkle genes become nonfunctional, age-dependent mutations accumulate in the mtDNA (Wanrooij, Luoma et al. 2004). Different factors are involved in mtDNA and plastidial genome maintenance in plants, encoded by small gene families. Some of these factors are specific for the chloroplast or the mitochondrion, whereas a number of them are dual-targeted to both types of organelles.

VI.1 DNA Polymerases

In plants two nuclear-encoded DNA polymerases, DNA polymerase 1A (Pol-1A) and DNA polymerase 1B (Pol-1B) have been characterized. They are both targeted into mitochondria and plastids, and are redundant in their functions (Elo, Lyznik et al. 2003; Ono, Sakai et al. 2007; Arrieta-Montiel, Shedge et al. 2009; Gualberto, Mileshina et al. 2014). Structural analyses indicate that these DNA polymerases are different from animal mitochondrial DNA polymerase gamma and other animal nuclear DNA polymerases, but they have a similar phylogenetic relationship with bacterial DNA polymerase Pol1 and other members of the family of A-type DNA polymerases. The organellar DNA polymerase of tobacco has been expressed in bacteria, which showed similar characteristics to bacterial DNA polymerase I activity (Ono, Sakai et al. 2007). The data suggest that Pol-1A and Pol-1B DNA polymerases have specific functions in plant organelles and have temporal and spatial differences in their expression. In *A. thaliana,*

Pol-1B functions as a DNA repair enzyme in plastids and allelic mutations in Pol-1B raise the sensitivity to DNA damaging agents, whereas mutants in DNA Pol-1A displayed no such susceptibility phenotype (Parent, Lepage et al. 2011). But mutants of Pol-1A have a reduced growth rate and seed production, as compared to control plants (Cupp and Nielsen 2014). Mutants of Pol1-B in *A. thaliana* also showed a slight difference in the growth rate during germination, and had 30% less copies of mtDNA, as well as a lower respiration rate. Interestingly, the expression level of Pol1-A transcripts in mutants of Pol1-B was enhanced up to 70% (Cupp and Nielsen 2014). The double mutant of Pol-1A and Pol-1B is not viable, suggesting that these DNA polymerases are redundant for organelle genome replication. There are also two dual-localized organellar DNA polymerases in maize, but the *w2* DNA polymerase gene seems to be responsible almost completely for plastid DNA replication. Mutation of this gene causes a 100-fold reduction in plastid DNA and in plastid gene expression. These mutants have only a slight decrease in mtDNA copy number, suggesting that may be in maize other organellar DNA polymerases may function in mtDNA replication (Udy, Belcher et al. 2012).

VI.2 DNA primase/helicase

Replication of the mtDNA in plants is expected to need both primase and helicase enzymes. An ortholog of the bacteriophage T7 gp4 protein that has both DNA primase and helicase activity has been identified in *A. thaliana* (Diray-Arce, Liu et al. 2013). Orthologs of the T7 gp4 protein are found in all eukaryotes, with the exception of fungi (Shutt and Gray 2006). In animal mitochondria, the ortholog is called Twinkle and it is the replicative helicase required for unwinding of the mtDNA during replication fork progression (Patel and Picha 2000; Korhonen, Gaspari et al. 2003).While in animal Twinkle essential residues of the primase domain have been lost, in plants these resides are conserved, so that the *A. thaliana* Twinkle has both DNA primase and helicase activities (Diray-Arce, Liu et al. 2013; Gualberto, Mileshina et al. 2014). The DNA primase activity has been associated with the N-terminal domain of this protein and the C-terminal domain has helicase activity (Shutt and Gray 2006). Twinkle is dual-targeted to both mitochondria and chloroplasts in *A. thaliana* (Carrie, Kuhn et al. 2009). The function of the plant Twinkle protein in mtDNA replication, recombination and repair is still unknown.

VI.3 DNA Ligase

DNA ligases are enzymes that join the adjacent polynucleotides and have a vital role in DNA metabolism. Plants have three genes encoding DNA ligase, possibly four in *A. thaliana* (Bryant JA 2008). DNA ligase 1 is found in all eukaryotes and joins the fragments generated during DNA synthesis. It is also involved in DNA repair (Wu, Hohn et al. 2001). The plant DNA ligase 1 gene expresses one major and two minor mRNA transcripts, differing in the length of the 5' untranslated leader, so as to produce an isoform targeted to the nucleus and another one to the mitochondria (Sunderland, West et al. 2006). It has been suggested that the extra reading frame encodes an isoform for the chloroplast (Bryant JA 2008). Still there is no experimental evidence for that. The mitochondrial isoform contains both a mitochondrial targeting sequence (MTS) and a nuclear localization signal (NLS). Yet, it localizes only to mitochondria, implying a dominance of the MTS over the NLS (Sunderland, West et al. 2006). DNA ligase 1 in *A. thaliana* has a vital role in DNA replication and excision repair pathways (Dyrkheeva, Lebedeva et al. 2016).

VI.4 Topoisomerase

Topoisomerases are required for releasing the tension in the double-stranded DNA molecules ahead of and behind DNA replication forks. The first topoisomerase, Topoisomerase I, was discovered in *E. coli* (Pommier, Leo et al. 2010). Topoisomerases generate transient cuts in the DNA to allow the passage of one double-stranded DNA segment across another, and subsequently reseal the strands. Topoisomerases are classified into two types, depending on the number of strands cut in one round of action. Type I can cleave only one DNA strand, while Type II, also called as gyrases in the prokaryotic system, cut both strands of one DNA double helix. In prokaryotes, Type II topoisomerase, also called gyrase, has a vital role in DNA replication and transcription. Gyrase is the only enzyme known in prokaryotes that can catalyze DNA supercoiling (Nitiss 1994). Both types of topoisomerases I and II have been purified from a range of plant species (Singh, Sopory et al. 2004) such as pea (Chiatante, Claut et al. 1993), carrot (Carbonera, Cella et al. 1988), tobacco (Heath-Pagliuso, Cole et al. 1990), cauliflower (Fukata, Ohgami et al. 1986) and *A. thaliana* (Carrie, Kuhn et al. 2009). Dual-targeted topoisomerases called as topo-I and topo-II have been characterized in *A. thaliana* (Carrie, Kuhn et al. 2009). Mutation in topo-II showed an embryo lethal phenotype (Wall, Mitchenall et al. 2004). From several species, cDNAs encoding topo-II have been cloned (Singh, Sopory et al.

2004) and tobacco topo-II rescued successfully a temperature sensitive topoisomerase II mutant in budding yeast.

VI.5 The MutS homologue MSH1

MSH1, a homologue of the bacterial MutS protein appears to be absent in mammals (Reenan and Kolodner 1992), while it is present in fungi and well conserved in higher plants (Abdelnoor, Christensen et al. 2006). In yeast it is an essential component for mtDNA recombination and it has a very important role in the surveillance of mtDNA recombination in plants (Gualberto, Mileshina et al. 2014). When there is little sequence homology, MSH1 might recognize heteroduplexes in strand exchange complexes and then would promote their rejection (Abdelnoor, Christensen et al. 2006). The protein encoded by the *MSH1* gene is dual-targeted to both mitochondria and plastids in plants (Gualberto, Mileshina et al. 2014; Virdi, Wamboldt et al. 2016). The MSH1 protein sequence comprises six conserved domains, three of which might be involved in DNA-binding and recombination (Virdi, Wamboldt et al. 2016). Mutations in *MSH1* cause alterations in the organellar genome stability (Davila, Arrieta-Montiel et al. 2011) and impact numerous pathways such as growth rate, flowering timing, reproductively incompetent juvenile vegetative phase of seed plants and abiotic stress response (Xu, Santamaria Rde et al. 2012). Mutants of this gene have a mixture of two types of phenotype resulting from mtDNA rearrangement by recombination and chloroplast dysfunction: i) variegated leaves with rarely leaf distortion and ii) delay in growth (Sakamoto, Kondo et al. 1996; Shedge, Arrieta-Montiel et al. 2007; Xu, Arrieta-Montiel et al. 2011). When *MSH1*was knocked-down by RNA silencing, mtDNA recombination was increased with a male sterility phenotype (Sandhu, Abdelnoor et al. 2007). Double mutants of MSH1 and RECA3 in *A. thaliana* by knock-out showed significant variations in the mtDNA, changes in nuclear transcripts and sensitivity to temperature (Van Aken and Whelan 2012). As described above, in bacteria, MutS is a protein that recognizes mismatches and recruits enzymes of the MMR pathway (Schofield and Hsieh 2003). In plants there are no counterparts for the MutH MMR factor, but MSH1 has a GIY-YIG domain. It has been proposed that this domain plays a role of detection but also of endonuclease.

VI.6 RecA-like recombinases

Recombination of the mtDNA needs one or more DNA recombinases. Bacterial RecA catalyzes DNA strand exchange during HR (Seitz, Brockman et al. 1998; Borgogno, Monti et al. 2016). In

mitochondria and chloroplasts of higher plants RecA-like proteins are present. Mitochondrial recombinases have been characterized in *A. thaliana* (Khazi, Edmondson et al. 2003), soybean and turnip (Manchekar, Scissum-Gunn et al. 2006). Two RecA-like proteins, RECA2 and RECA3 are targeted to mitochondria in *A. thaliana* and in all flowering plants (Gualberto, Mileshina et al. 2014). RECA3 is only targeted to mitochondria, while RECA2 was described to be dual-targeted to mitochondria and chloroplasts (Shedge, Arrieta-Montiel et al. 2007; Miller-Messmer, Kuhn et al. 2012). *A. thaliana* mutants of *RECA2* and *RECA3* have different phenotypes (Shedge, Arrieta-Montiel et al. 2007; Miller-Messmer, Kuhn et al. 2012). Both *recA2* and *recA3* mutants show an increase in recombination involving IRs. But *recA2* and *recA3* mutants have normal mtDNA copy numbers, showing that RecA functions are not essential for replication (Gualberto, Mileshina et al. 2014). *recA2* mutants are unable to develop past the seedling stage, suggesting that in the absence of RECA2 the mtDNA structure is very unstable, while *recA3* mutants have a normal phenotype. The phenotype of *recA2* mutants might be due to the shuffling of the mitochondrial genome by recombination (Gualberto, Mileshina et al. 2014). The precise activity of these two RECA proteins remains to be characterized. Eukaryotes, including plants, also possess Rad51 recombinases. It has been reported that Rad51 is targeted to mitochondria in human cells and promotes mtDNA synthesis under conditions of increased replication stress (Sage and Knight 2013).

VI.7 Single-stranded DNA-Binding Proteins

Single-stranded DNA (ssDNA) generated during replication, recombination or repair is sensitive to degradation by nucleases, and to environmental damage (Errol, Graham et al. 2006; Gualberto, Mileshina et al. 2014). It can also fold into secondary structures that will impair the action of other factors involved in DNA metabolism. In this situation, ssDNA-binding proteins (SSBs) are needed for protection against nuclease degradation and environmental damage, but also to promote recombination and replication (Shereda, Kozlov et al. 2008).

VI.7.1.1 Bacterial type SSBs

In plant mitochondria, two types of bacterial-like SSBs were identified that are involved in mtDNA replication, recombination and repair (Edmondson, Song et al. 2005; Zaegel, Guermann et al. 2006; Rendekova, Ward et al. 2016). SSB1 and SSB2 proteins are directed to mitochondria and plastids. SSB1 was shown to have properties analogous to those of *E. coli* SSB: It binds to

unwound DNA at the replication fork to prevent reannealing. The activity of mitochondrial recombinase RECA is also stimulated by SSB1 (Edmondson, Song et al. 2005). SSB2 has been shown to have essential functions in *A. thaliana* because the *ssb2* mutants are unable to develop past the seedling stage (Gualberto, Mileshina et al. 2014). In bacteria, SSB is separated from the ssDNA by further factors such as translocases, or different recombination mediators such as RecO or Rad52. These functions might be ensured by ODB, OSB and WHY proteins in plant mitochondria (Janicka, Kuhn et al. 2012).

VI.7.1.2 SSBs specific to organelles (OSBs)

The second type of great affinity SSB proteins in plant mitochondria is the organellar single-stranded DNA-binding (OSB) proteins. There are four types of OSB proteins in *A. thaliana,* OSB1 and OSB4 are localized to mitochondria, OSB2 to chloroplasts and OSB3 to both types of organelles (Zaegel, Guermann et al. 2006; Odahara, Masuda et al. 2015). Only plant organelles have these proteins (Odahara, Masuda et al. 2015). They have one, two or three C-terminal PDF domains, specific to plants, that are involved in the interaction with the DNA (Kühn and Gualberto 2012). In bacteria, SSB can prevent the binding of RecA on the ssDNA (Suksombat, Khafizov et al. 2015). Depending on the DNA metabolic process, an ssDNA-binding helper protein might contribute to displace SSB. May be OSB fulfills such a role in plant mitochondria, but there is no data yet to support this idea.

OSB1 and OSB4 mutants showed an increase of aberrant mtDNA sequences due to ectopic recombination (Zaegel, Guermann et al. 2006; Gualberto, Mileshina et al. 2014)). Mutants of OSB1 and OSB4 had a phenotype of variegated and distorted leaves, and were partially sterile. It is estimated that this phenotype resulted from recombination across IRs (Zaegel, Guermann et al. 2006). In the following generation, the phenotype became worse and was associated with an SSS process through which some of the recombined forms of the mtDNA became predominant. This indicates that OSB1/OSB4 have a role in controlling the stoichiometry of alternative forms of the mtDNA (Zaegel, Guermann et al. 2006; Kühn and Gualberto 2012). It has been suggested that OSB1 is involved in recombination surveillance, to stop the transmission of aberrant mtDNAs to new mitochondria (Zaegel, Guermann et al. 2006).

VI.7.1.3 Whirly ssDNA-binding proteins

Solanum tuberosum Whirly1 (StWhy1) is a typical Whirly protein that was foremost characterized as a nuclear transcriptional regulator of the pathogenesis-related gene PR-10a in potato (Desveaux, Marechal et al. 2005; Boyle and Despres 2010). But Whirly proteins have active transit peptides at their N-terminus that can target them to mitochondria or plastids (Krause, Kilbienski et al. 2005). All angiosperms for which the complete genome is known have at least one mitochondrion-targeted and one plastid-targeted Whirly protein (mtwhirlies and ptwhirlies) (Ruhlman and Jansen 2014), indicating that these play vital roles in both types of organelles (Krause, Kilbienski et al. 2005). The dual nuclear and plastid localization of barley (*Hordeum vulgare*) Whirly1 (HvWhy1) was demonstrated by immunolocalization and cell sub-fractionation experiments (Grabowski, Miao et al. 2008). In *A. thaliana*, the WHY2 protein is localized to mitochondria, while WHY1 and WHY3 are targeted to chloroplasts (Krause, Kilbienski et al. 2005). The plastid-targeted whirly in maize, ZmWhy1, localizes to the stroma of chloroplasts and is bound to the thylakoid membrane by its contact with plastid DNA (Prikryl, Watkins et al. 2008). Whirly proteins specially bind to ssDNA. Furthermore, there is multiple evidence that they take part in the DNA metabolism of plant organelles, and in the regulation of transcription in the nucleus. Whirlies have also been involved in the maintenance of telomere length in nuclear chromosomes (Yoo, Kwon et al. 2007). Their participation in repressing non-homologous recombination has been revealed in plastids and mitochondria. Transcription of several mitochondrial genes is affected by the over-expression of WHY2. However, this effect may be non-specific and due to masking of the mtDNA by WHY2. Mutants of the *WHY2* gene show an increase in recombination between microhomologies in mitochondria when plants are treated with ciprofloxacin. Ciprofloxacin blocks gyrase, which is targeted to the organelles, and causes DSBs in the mtDNA. Thus, it was suggested that WHY2 is involved in the surveillance of illegitimate recombination, avoiding error-prone pathways of DSB repair through sequence microhomologies (Marechal and Brisson 2010).

VI.7.1.4 Homologues of Rad52 (ODBs)

RAD52 is a nuclear factor involved in DNA DSB repair mediated by HR in human and *S. cerevisiae*. It binds ssDNA ends, contributes to annealing of complementary DNA strands and recruitment of the DNA recombinase RAD51 (Hanamshet, Mazina et al. 2016; Rendekova,

Ward et al. 2016). Rad52 is involved in SSA or MMEJ in yeast (Ceccaldi, Rondinelli et al. 2016). RAD52 homologues are also found in plants. In *A. thaliana* two RAD52-like genes have been identified that can generate four different *orfs* through alternative splicing (Samach, Melamed-Bessudo et al. 2011). Two isoforms of RAD52-like proteins have been detected and called ODB (organellar DNA-binding). ODB1 is targeted to mitochondria and ODB2 to chloroplasts (Kühn and Gualberto 2012). The plant RAD52-like mitochondrial protein ODB1 might be an extra recombination mediator. It is a short protein essentially homologuous to the single strand annealing (SSA) domain of RAD52. ODB1 binds ssDNA with no apparent sequence specificity, but has less affinity for double-stranded DNA (Rendekova, Ward et al. 2016). Mutants of ODB1 when treated with ciprofloxacin show a lower homologous recombination rate as compared to wild-type plants and the absence of ODB1 favors error-prone repair like microhomology-mediated repair (MHMR) or single-strand annealing (SSA) (Janicka, Kuhn et al. 2012). ODB1 has similarity also with Mgm101, involved in the recombination of yeast mtDNA. The comparisons of ODB1, Rad52 and Mgm101 suggest that ODB1 has analogous functions in the mitochondria of plants (Rendekova, Ward et al. 2016). WHY2 and ODB1 were co-immunoprecipitated and seem to have functional similarities, which suggests that they might be redundant. Indeed, the *why2* and *odb1* single mutants did not show a phenotype. Double mutants *why2*/*odb1* were not obtained in *A. thaliana* because the two genes are only 8 kb apart on the same chromosome. Notably, ODB1 is a bi-functional protein involved in both HR and splicing of two introns in plant mitochondria (Gualberto, Le Ret et al. 2015).

VII. Framework and objectives of the thesis

As it appears from the above data, the mitochondrial genome of the different organisms varies from each other in terms of size, shape, structure and gene content. The complex expression of the mitochondrial genome in the various species makes it difficult to understand the mitochondrial machineries. In human beings, neurodegenerative diseases caused by mtDNA mutations are still waiting for the development of gene therapy methodologies. In plant breeding, mitochondrial biology has a very important role to generate valuable hybrids to increase the production of crop yield and to understand the different types of CMS in plants. For these reasons, there is a strong need to manipulate mitochondrial genetics in mammals and in plants. From the plant mitochondrial manipulation, we can in particular understand the cross-talk

between the organelles and the nucleus. In such a context, different strategies have been developed to import DNA or RNA into the mitochondria.

As described above, the RNA shuttling strategy developed in our laboratory opened the unprecedented possibility to import passenger RNAs into mitochondria in plant cells. This strategy is based on the natural mechanism of nuclear-encoded tRNA import from the cytosol into mitochondria in plants. Our laboratory has shown that passenger RNAs associated with a tRNA mimic and expressed from a nuclear transgene are imported into the mitochondria in the transformed plants. In my PhD project, I used this strategy to introduce into mitochondria in *A. thaliana* the *orf77* RNA characteristic for S-CMS in maize. That the *orf77* RNA is the main cause of the S-type CMS in maize is currently the most popular working hypothesis (Gabay-Laughnan, Kuzmin et al. 2009; Chen, Zhang et al. 2016). By importing this CMS RNA into the mitochondria of otherwise normal *A. thaliana* plants, we wanted to investigate whether it can indeed affect the mitochondrial transcriptome and trigger further signaling. The objective was both to contribute to the deciphering of mitochondrial regulation and to progress in the understanding of CMS in plants. A longer term aim was to determine a possible effect on fertility, with the prospect of generating a new tool to promote CMS.

Recombination is still considered to be rare in mammalian mitochondria. But in plant mitochondria various recombination processes are active. They generate the subgenomic organization of the mtDNA. The recombination activities are also involved in repair, replication and formation of alternative configurations of plant mtDNA. Segregation and substoichiometric shifting of the latter contribute to genome evolution. Ectopic recombination leads to heteroplasmy of the mtDNA and for this reasons it is precisely controlled by nuclear-encoded factors. Mutation of the nuclear genes encoding these proteins releases the surveillance and allows a high frequency of ectopic recombination. As a consequence, new mtDNA configurations are formed and can subsequently segregate, creating mitochondrial genetic variability. This constitutes the second approach used in our laboratory to manipulate mitochondrial genetics, with the prospect of producing new phenotypes of interest. A number of proteins involved in mtDNA recombination and repair processes, such as recombinases, DNA polymerases or ssDNA binding proteins, have been characterised previously in the laboratory and mutants of the corresponding nuclear genes have been analyzed in terms of developmental and molecular phenotype. But still more factors are required in these mechanisms, in particular

5'→3' exonucleases that would ensure resection of DNA ends. In plant mitochondria, no 5'→3' exonuclease had been identified so far. The second aspect of my PhD project was thus to characterize plant mitochondrial 5'→3' exonucleases and to analyze the variability of the mitochondrial genome in the corresponding mutants. The objective was to contribute to the understanding of the recombination mechanisms involved in plant mitochondria and to be able to manipulate the mitochondrial genome through ectopic recombination.

RESULTS CHAPTER I

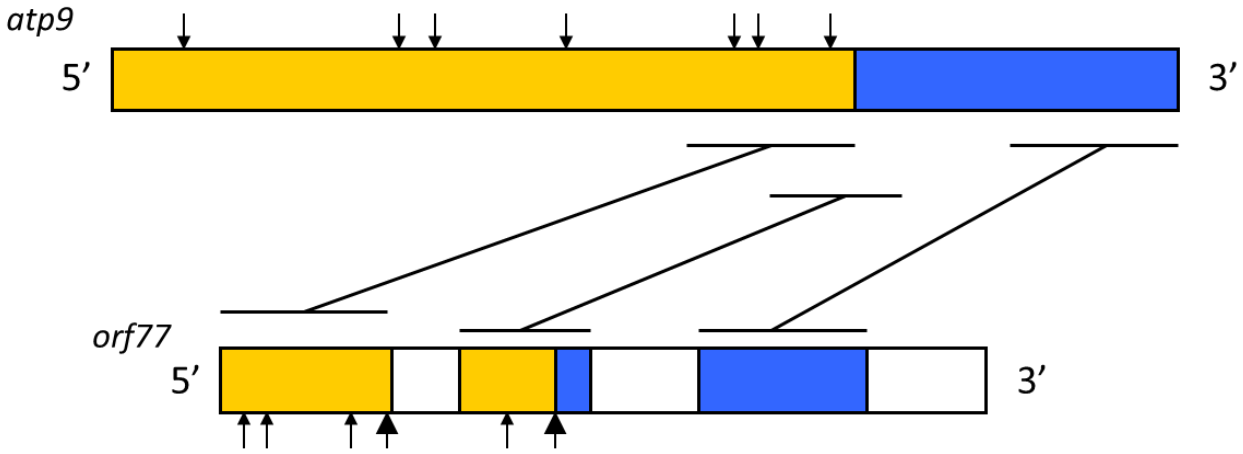


Figure 8: Common sequences between *atp9* and *orf77*. The arrows indicate observed RNA editing sites

The *orf77* sequence is a chimera combining atypical sequences with three regions that are almost identical to parts of the coding region or 3'-flanking region of the *atp9* mitochondrial gene (Figure 8). Due to this partial identity with *atp9*, it is currently hypothesized that *orf77* is the main cause of S-CMS in maize, potentially through competition with *atp9* at the RNA or protein level. To explore these mechanisms and determine whether it is possible to trigger directed CMS, we have coupled the *orf77* sequence, supplemented with a 6 histidine tag, to the PKTLS tRNA mimic. We have expressed the corresponding *orf77*-PKTLS RNA in *A. thaliana* plants from a nuclear transgene and analyzed the effect of the import of this RNA into mitochondria on the steady-state level of the mitochondrial RNAs, especially *atp9*.

I. Preparation of gene constructs for *in vivo* expression

A PCR product comprising the complete *orf77* with 4 nucleotides 5'-UTR and 92 nucleotides 3'-UTR were amplified using total DNA from S-CMS maize as a template. The forward primer comprised a *Hind*III site and a sequence encoding a 6xHis tag. The reverse primer had a *Bam*HI site. The resulting product was cloned into the *Hind*III and *Bam*HI sites of the pUCAP-PKTLS-HDV plasmid upstream of the sequences encoding the TYMV PKTLS and the HDV *cis*-ribozyme. On my arrival in the host laboratory, the pUCAP-PKTLS-HDV was already prepared. After confirmation by sequencing, the assembled *orf77*-PKTLS-HDV construct was re-extracted upon digestion with *Asc*I and *Pac*I and cloned into the *Asc*I and *Pac*I restriction sites of the estradiol-inducible transcription unit (O_{LexA}-46 estradiol-inducible promoter) of the pER8 vector. The resulting plasmid called pER8-*orf77*-PKTLS-cHDV was used for transformation of *A. thaliana* plants. For control, plants were transformed with empty pER8 plasmid.

I.1 Nuclear transformants of *A. thaliana* plants

A. thaliana plants (ecotype Col-0) were transformed *via A. tumefaciens* through floral dip. Seeds from transformed plants were subsequently recovered and were germinated on hygromycin for transformant selection. Plants were cultivated on agar plates under long day conditions (16h light/8h dark). The presence of the construct was confirmed by PCR. Four different homozygous lines of the transformant were selected and named as At-*orf77*-4.1 to At-*orf77*-4.4. For the control plants, three lines transformed with the empty pER8 vector were selected and named as At-*EMT*-5.1 to At-*EMT*-5.3. Transgenic *A. thaliana* plants were subsequently grown on solid agar medium that was overlaid with β-estradiol-supplemented liquid medium for transgene

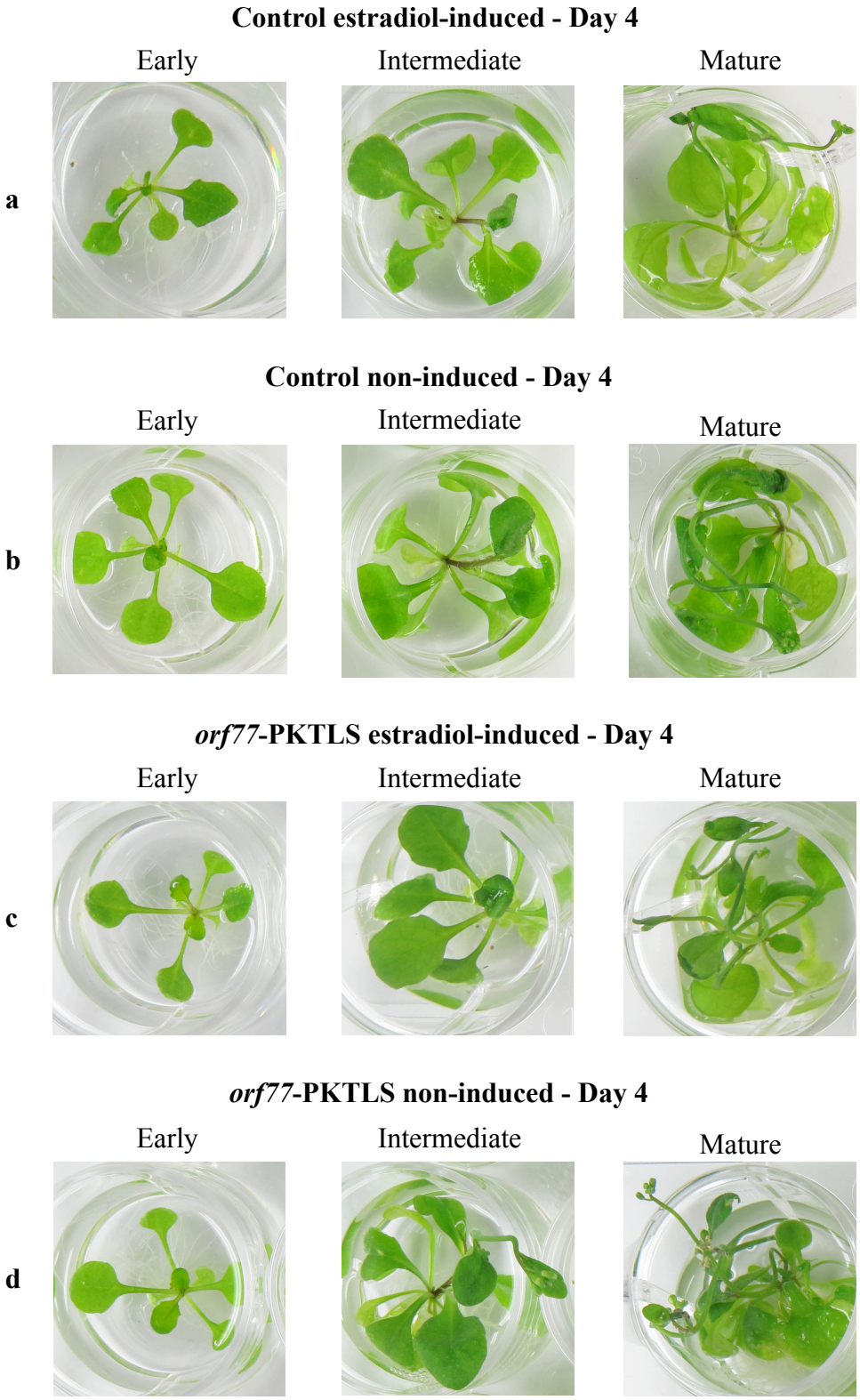


Figure 9: Developmental stages of light-grown *A. thaliana* transformed lines used for the induced expression of the *orf77*-PKTLS RNA. Seeds were sown on Petri dishes containing solid MS-agar medium and transferred at the appropriate developmental stage (early, intermediate or mature) to wells in culture plates containing either liquid medium supplemented with estradiol (estradiol-induced) or regular culture medium (non-induced). The pictures were taken at day 4 after induction and show that neither the expression of the *orf77*-PKTLS RNA (panel **a** versus **c**), nor the estradiol treatment by itself (panel **a** versus **b**, panel **c** versus **d**) had a phenotypic effect within the 4-day time frame considered.

induction. Alternatively, plants grown on solid agar were transferred to culture plates containing liquid medium supplemented with β-estradiol and the roots were dipped into the medium.

I.2 Expression of the construct

We evaluated transgene expression in the homozygous transformed plants. We decided to see the expression level in light-grown plants at different developmental stages (early, intermediate and mature stage) and in dark-grown seedlings. In all growth conditions, the plants were treated with β-estradiol for transgene induction and harvested each day till day 4 after induction. The control plants for result normalization, *i.e.* plants transformed with the empty vector, were grown and treated in the same way, in particular to take into account side effects of estradiol treatment. β-estradiol can promote a slight increase in the expression level of some mRNAs (Niazi 2013). In all growth conditions, the transformants did not have a particular phenotype (Figure 9 and 10). To evaluate *orf77*-PKTLS expression, total RNAs were extracted from the induced plants in all growth conditions and analyzed by reverse transcription and real time PCR (RT-qPCR). For the preparation of cDNAs, random hexamer primers were used, whereas qPCR was run with different couples of specific primers (Table 7). In addition, the absence of amplification in the absence of reverse transcriptase (RT^-) showed that there was no contamination by DNA after effective treatment with RNase-free DNase. The mRNAs from the *actin2* (At3g18780) and *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) (At1g13440) nuclear genes were chosen as reference for normalization of the expression of all transcripts studied. The pairs of primers used for the amplification of *actin* and *GAPDH* by qPCR were 31-actine2araFw/31-actine2araRv and GAPDH-Fw/GAPDH-Rv, respectively (Table 7). No relationship between these RNAs and mitochondria has been reported to date, these served as extra-mitochondrial reference during this study. On the basis of the first results of transgene expression, we selected the At-*orf77*-4.4 transformant for most of the further experiments. The At-*EMT-5*.1 was essentially used as a control.

I.3 Import of the orf77-PKTLS RNA into mitochondria

To confirm that the *orf77*-PKTLS RNA expressed in the transformants was targeted to the organelles, mitochondria were isolated from light-grown plants at day 2 after induction with β-estradiol. Intact isolated organelles were treated with RNase and mitochondrial RNA was extracted. Total RNA was prepared in parallel from the same plants. Mitochondrial and total

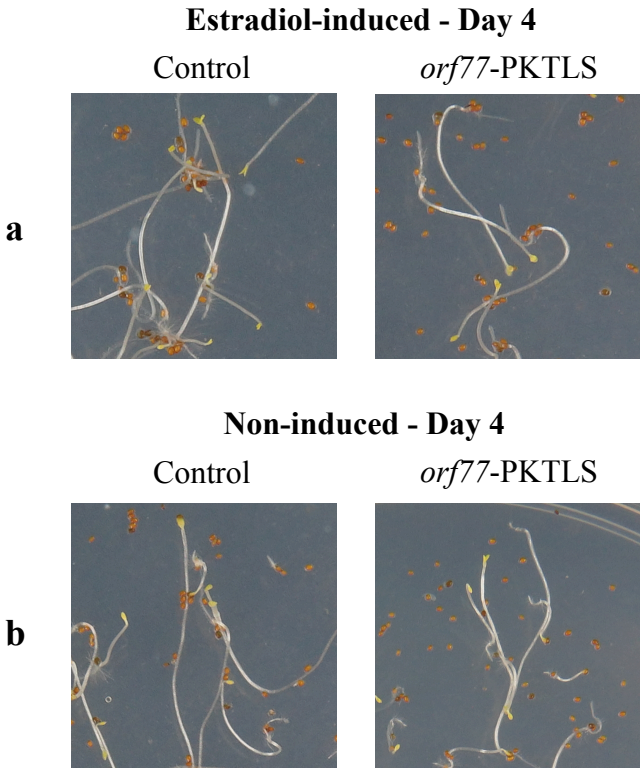


Figure 10: Dark-grown *A. thaliana* transformed lines used for the induced expression of the *orf77*-PKTLS RNA. Seeds were sown on Petri dishes containing solid MS-agar medium that was overlayed after appropriate seedling growth with either liquid medium supplemented with estradiol (estradiol-induced) or regular culture medium (non-induced). The pictures were taken at day 4 after induction and show that neither the expression of the *orf77*-PKTLS RNA (panel **a**, left versus right), nor the estradiol treatment by itself (panel **a** versus **b**) had a phenotypic effect within the 4-day time frame considered.

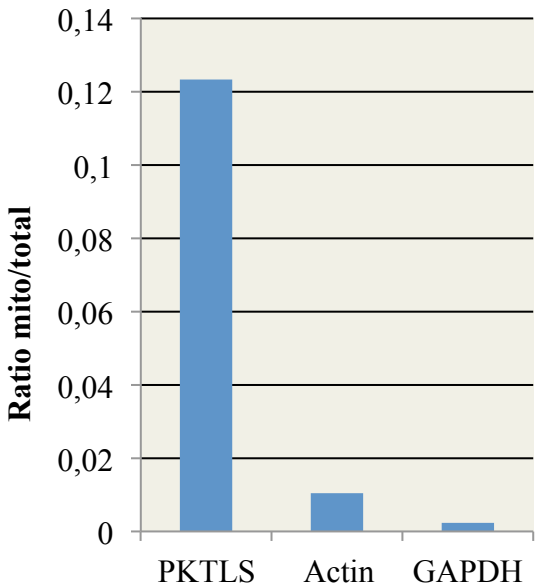


Figure 11: Import of the *orf77*-PKTLS RNA into mitochondria in *A. thaliana transformants*. Total and mitochondrial RNAs from plants expressing the *orf77*-PKTLS RNA were probed by RT-qPCR for PKTLS, actin and GAPDH RNA. The results are given as the mitochondrial-N0/total-N0 ratio.

RNAs were probed by RT-qPCR for *orf77*-PKTLS, for specific nuclear transcripts and for selected mitochondrial transcripts, so as to characterize mitochondrial enrichment and confirm the absence of significant contamination. RT-qPCR analysis showed about 10 fold enrichment of the *orf77*-PKTLS RNA in the mitochondrial fraction *versus* the actin and GAPDH nuclear transcripts (Figure 11), confirming the import into the organelles.

II. Collection of RNAs probed by RT-qPCR

We wanted to characterize the effects of the *orf77*-PKTLS expression on the levels of essentially all mitochondrial RNAs in the transformed *A. thaliana* plants at different growth stages and under different growth conditions. From the NADH dehydrogenase complex (Complex-I of the oxidative phosphorylation (OXPHOS) chain), we followed the variations in the level of the *nad1b, nad2b, nad4, nad5a, nad5b, nad6*, *nad7* and *nad9* mRNAs. From the cytochrome c reductase complex (Complex III), we checked the changes in the level of the cytochrome b (*cob*) mRNA. For the Cytochrome oxidase complex (Complex IV), we determined the variations in the expression of the *cox1, cox2 and cox3* mRNAs. From the ATP synthase complex (Complex V), we followed the variations in the expression of the *atp4, atp6-1* and *atp9* mRNAs. The ATP4 and ATP9 proteins are part of the F_0 ATP synthase sub-complex. No subunit of the succinate dehydrogenase complex (Complex II) is encoded in the mitochondrial genome of *A. thaliana*. We included the mRNAs coding for four cytochrome c maturation (*ccm*) proteins: *ccmFN2, ccmB, ccmFN1* and *ccmFC*. We also selected RNAs related to mitochondrial protein synthesis, to see how much the translation machinery is affected in response to *orf77*-PKTLS expression. For this, in addition the two rRNAs, *rrn18* and *rrn26*, we followed the changes in three mRNAs encoding proteins of the small ribosomal subunit, *rps3, rps4* and *rps7*, and two mRNAs encoding proteins of the large ribosomal subunit, *rpl2* and *rpl5*. Finally, we also selected two mRNAs deriving from nuclear genes, *i.e. aox1a* and *aox1d*. Both of these mRNAs code for the alternative oxidase (AOX), a protein that has an important role for mitochondria in conditions such as pathogen attack and heat or drought stress. AOX uncouples respiration from mitochondrial ATP production and may improve plant performance under stressful environmental conditions by preventing excess accumulation of reactive oxygen species

The following results correspond to RT-qPCR analysis performed on total RNAs from three biological replicates of *orf77*-PKTLS plants at different growth stages and under different

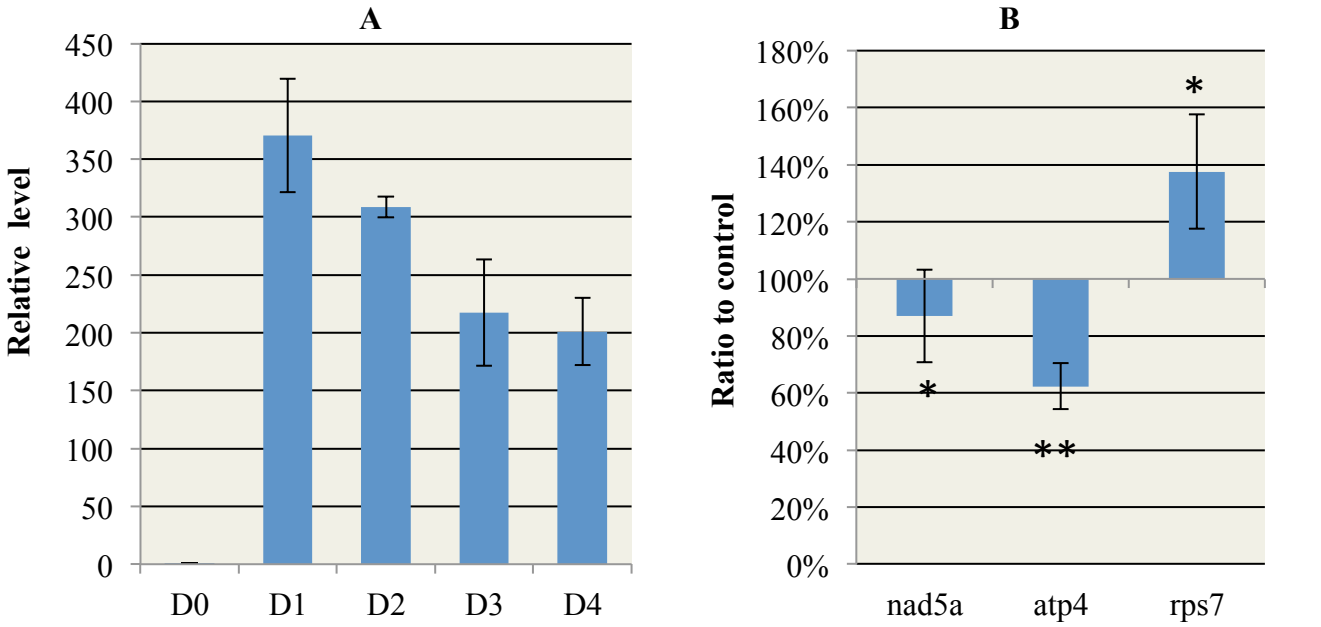


Figure 12: A) Kinetics of the *orf77*-PKTLS RNA expression at the early stage of development in light-grown transformed *A. thaliana* plants. Analysis by RT-qPCR on total RNA. For transgene induction, β-estradiol was added on D0, and samples were harvested till D4. D0: before treatment with β-estradiol; D1 to D4: days after onset of the induction. Relative expression levels of *orf77*-PKTLS RNA as compared to the expression at D0. **B) Effect of *orf77*-PKTLS RNA expression at the early stage of development on the level of selected mitochondrial RNAs.** Analysis by RT-qPCR on total RNA. Results are given as the percentage of the selected mitochondrial RNAs at D2 as compared to control. *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001.

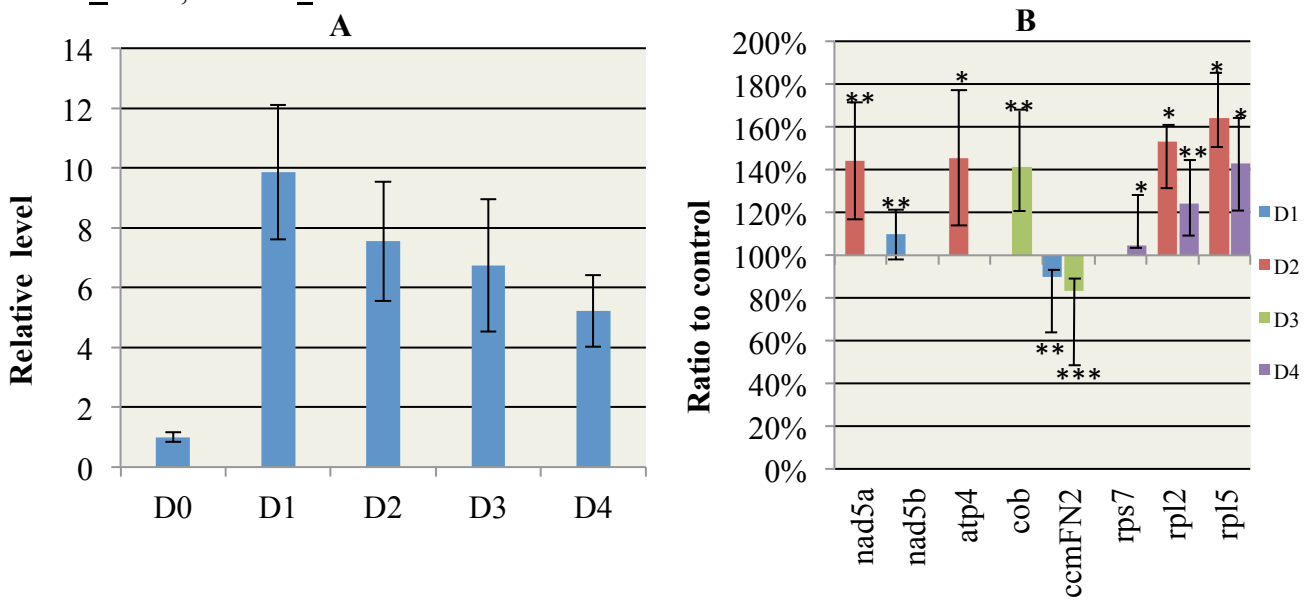


Figure 13: A) Kinetics of the *orf77*-PKTLS RNA expression at the intermediate stage of development in light-grown transformed *A. thaliana* plants. Analysis by RT-qPCR on total RNA. D0: before treatment with β-estradiol; D1 to D4: days after onset of the induction. Relative expression levels of *orf77*-PKTLS RNA as compared to the expression at D0. **B) Effect of *orf77*-PKTLS RNA expression at the intermediate stage of development on the level of selected mitochondrial RNAs.** Analysis by RT-qPCR on total RNA. Results are given as the percentage of the selected mitochondrial RNAs at D1, D2, D3 and D4 as compared to control. *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001.

growth conditions. For the determination of statistically significant and non-significant effects on the steady-state level of RNAs, we analyzed the results of the three biological replicates with the Graph Pad Prism version 7.01 software.

III. Expression of the *orf77*-PKTLS RNA at the early stage of plant development and impact on the mitochondrial transcriptome

We studied the expression level of the *orf77*-PKTLS RNA and its impact on the mitochondrial transcriptome in young light-grown plants at about 12 days after germination. As mentioned above, plants grown up to that stage were induced with β-estradiol and from that day taken as day 0 (D0) we harvested samples every subsequent day till day 4 (D4). At that stage of development, the expression of the transgene was maximum at D1. After this, the expression was going down until D4 (Figure 12A). At that early stage, there was almost no impact of the *orf77*-PKTLS expression on the mitochondrial transcriptome. We only observed that the *nad5a* mRNA from Complex-I and the *atp4* mRNA from ATP synthase decreased significantly at D2, while the *rps7* mRNA from the small ribosomal subunit increased (Figure 12B). All other mitochondrial RNAs showed no statistically significant variation.

III.1 Expression of the *orf77*-PKTLS RNA at the intermediate stage of plant development and impact on the mitochondrial transcriptome

We then studied the expression level of *orf77*-PKTLS and its impact on the mitochondrial transcriptome in light-grown plants at an intermediate developmental stage, *i.e.* at about 21 days after germination. As before, we induced the plants at the chosen stage with β-estradiol and harvested samples every day for 4 days. The maximum level of *orf77*-PKTLS expression was again at D1 (Figure 13A), followed by a decrease till D4. At that developmental stage, the mitochondrial transcriptome started to become more reactive to the expression of the *orf77*-PKTLS RNA. We recorded changes in the steady-state level of two mRNAs coding for Complex I subunits: *nad5b* significantly increased at D1, while *nad5a* increased significantly at D2. From Complex III, *cob* significantly increased at D3, whereas *atp4* from complex V increased at D2. The *ccmFN2* mRNA decreased at D1 and D3. The *rpl2* and *rpl5* mRNAs increased at D2 and D4, while *rps7* increased only at D4 (Figure 13B).

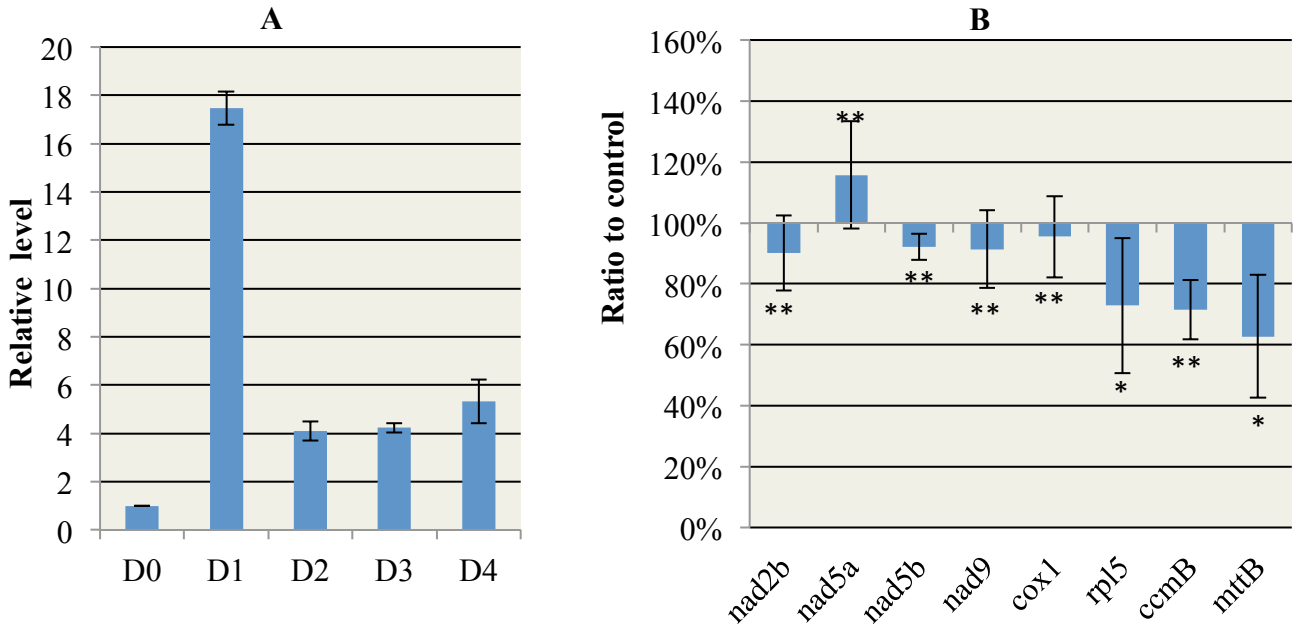


Figure 14: A) Kinetics of the *orf77*-PKTLS RNA expression at the mature stage of development in light-grown transformed *A. thaliana* plants. Analysis by RT-qPCR on total RNA. D0: before treatment with β-estradiol; D1 to D4: days after onset of the induction. Relative expression levels of *orf77*-PKTLS RNA as compared to the expression at D0. **B) Effect of *orf77*-PKTLS RNA expression at the mature stage of development on the level of selected mitochondrial RNAs.** Analysis by RT-qPCR on total RNA. Results are given as the percentage of the selected mitochondrial RNAs at D1 as compared to control. *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001.

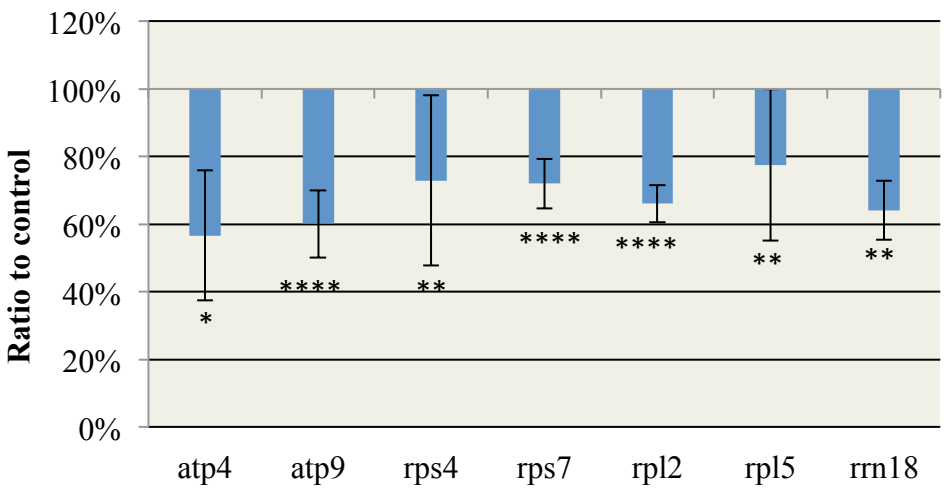


Figure 15: Effect of *orf77*-PKTLS RNA expression at the mature stage of development on the level of selected mitochondrial RNAs. Analysis by RT-qPCR on total RNA. Results are given as the percentage of the selected mitochondrial RNAs at D2 as compared to control. *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001.

III.2 Expression of the *orf77*-PKTLS RNA at the mature stage of plant development and impact on the mitochondrial transcriptome

The expression level of *orf77*-PKTLS and its impact on the mitochondrial transcriptome was further investigated in light-grown mature plants that were induced with estradiol at about 32 days after germination. The maximum expression of the *orf77*-PKTLS RNA was subsequently on D1, the level dropped to a large extent on D2 and then remained almost constant till D4 (Figure 14A). At that developmental stage, we observed a large response of the mitochondrial transcriptome, with mostly a negative impact. On D1, when the *orf77*-PKTLS expression was maximum, only *nad5a* increased, while the steady-state level of *nad2b, nad5b, nad9, cox1, rpl5, ccmB* and *mttB* decreased substantially (Figure 14B. On D2, the levels of *atp4, atp9, rpl2, rpl5, rps4, rps7* and *rrn18* were significantly decreased (Figure 15). On D3, *nad1b* and *nad5a* levels were substantially increased, while the other Complex-I mRNAs *nad2b, nad4, nad5b, nad6* and *nad9* decreased. Further RNAs that were decreased included *cob* (Complex-III), *cox1, cox2* and *cox3* (Complex-IV), *ccmB, ccmFC, ccmFN1* and *ccmFN2* (cytochrome c biogenesis), the two rRNAs *rrn18* and *rrn26*, and the RNA of unknown function *mttB* (Figure 16A). On D4, *cox1*, *atp9, ccmFC* and *rrn26* were decreased, while *rpl5* and *mttB* were increased substantially (Figure 16B). Strikingly, regarding the above-mentioned hypothesis on the role of the *orf77* in maize S-CMS, only at that developmental stage we observed an effect on the *atp9* mRNA, with a decrease at D2 and D4 and an increase at D3.

III.3 Expression of the *orf77*-PKTLS RNA in dark-grown seedlings and impact on the mitochondrial transcriptome

We finally checked the expression level of *orf77*-PKTLS and its impact on the mitochondrial transcriptome in dark-grown seedlings. In such growth conditions, plants develop their hypocotyls terminated with the two cotyledons and do not make leaves. In this case, the *orf77*-PKTLS expression was low at D1 and about maximal from D2 to D4 (Figure 17A). The response of the mitochondrial transcriptome was in between those of the intermediate and mature developmental stages of the light-grown plants, in terms of the number of RNAs affected, and this response was somehow balanced between increased and decreased RNAs. On D1, *nad2b, nad5b, nad9* and *rsp7* increased significantly and *nad5a*, *cox2*, *rpl5*, *rrn26* and *matR* decreased (Figure 17 B). At D2, *nad4, nad5b* and *cox1* increased, while *ccmFN2* decreased (Figure 18A).

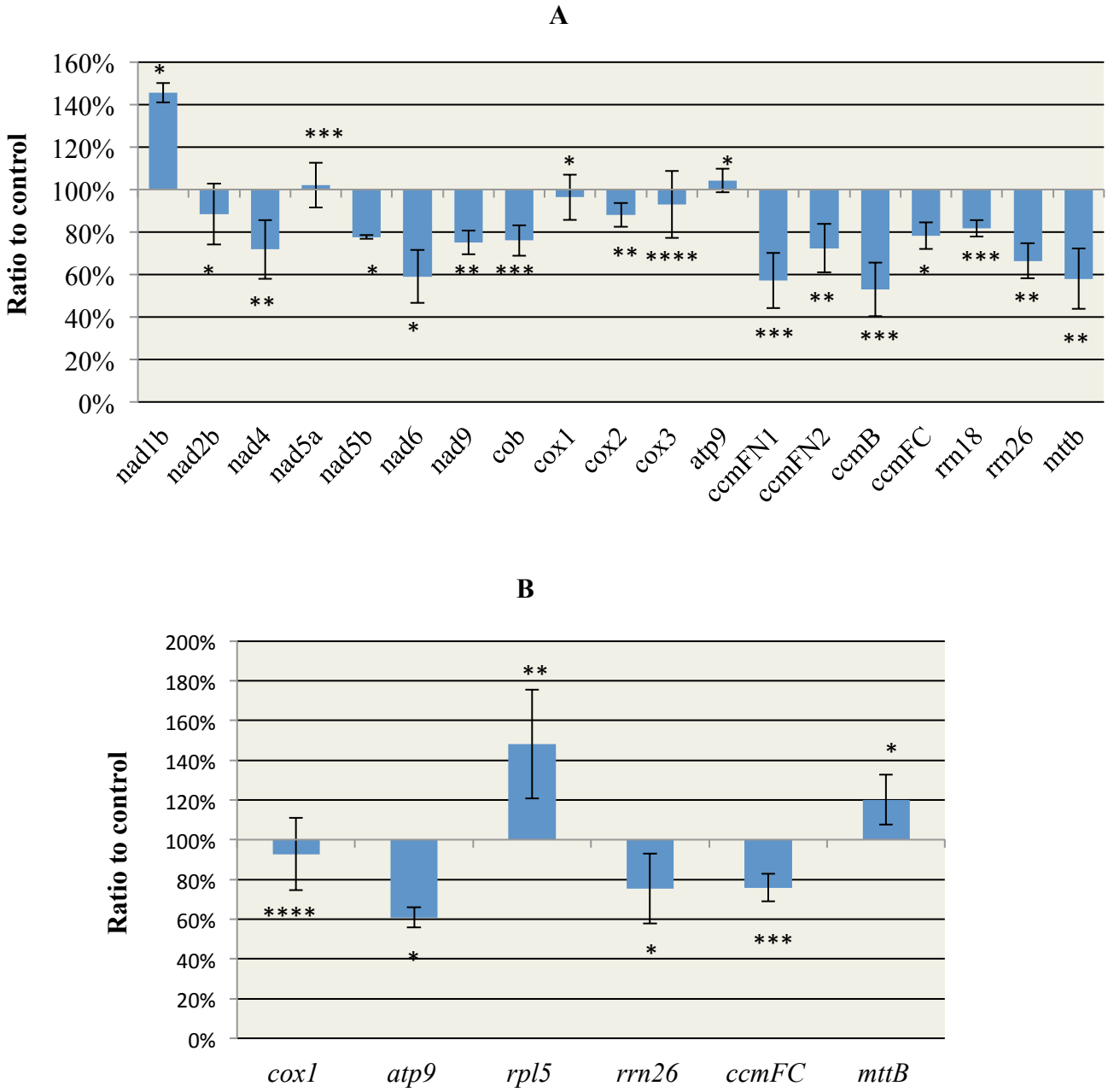


Figure 16: Effect of *orf77*-PKTLS RNA expression at the mature stage of development on the level of selected mitochondrial RNAs. Analysis by RT-qPCR on total RNA. **A)** Results are given as the percentage of the selected mitochondrial RNAs at D3 as compared to control. **B)** Results are given as the percentage of the selected mitochondrial RNAs at D4 as compared to control. *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001.

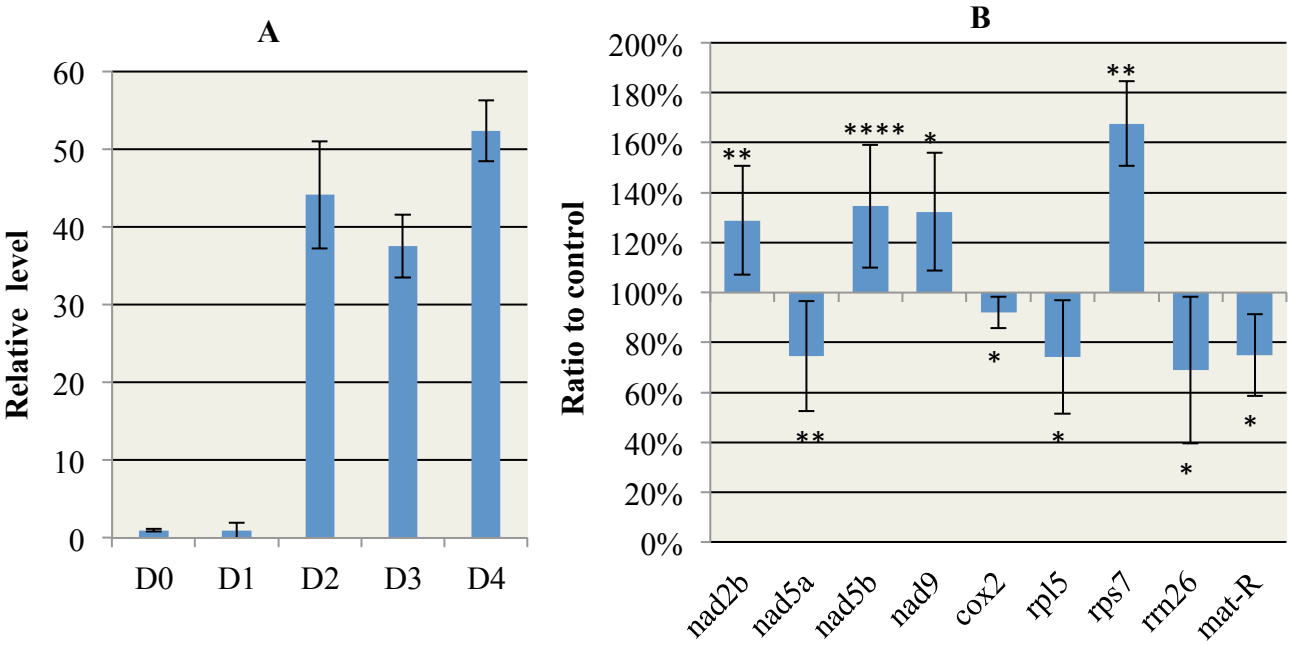


Figure 17: A) Kinetics of the *orf77*-PKTLS RNA expression in dark-grown transformed *A. thaliana* seedlings. Analysis by RT-qPCR on total RNA. D0: before treatment with β-estradiol; D1 to D4: days after onset of the induction. Relative expression levels of *orf77*-PKTLS RNA as compared to the expression at D0. **B) Effect of *orf77*-PKTLS RNA in dark-grown seedlings on the level of selected mitochondrial RNAs.** Analysis by RT-qPCR on total RNA. Results are given as the percentage of the selected mitochondrial RNAs at D1 as compared to control. *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001.

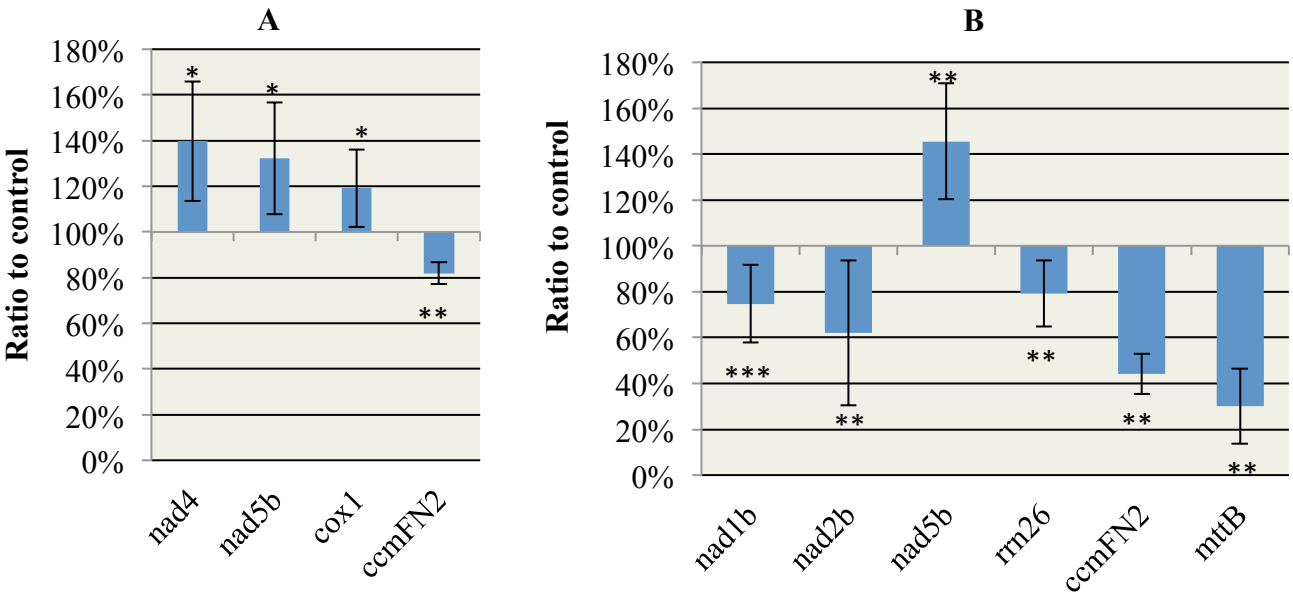


Figure 18: A**) Effect of *orf77*-PKTLS RNA in dark-grown seedlings on the level of selected mitochondrial RNAs.** Analysis by RT-qPCR on total RNA. Results are given as the percentage of the selected mitochondrial RNAs at D2 as compared to control. **B)** Results are given as the percentage of the selected mitochondrial RNAs at D3 as compared to control. *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001.

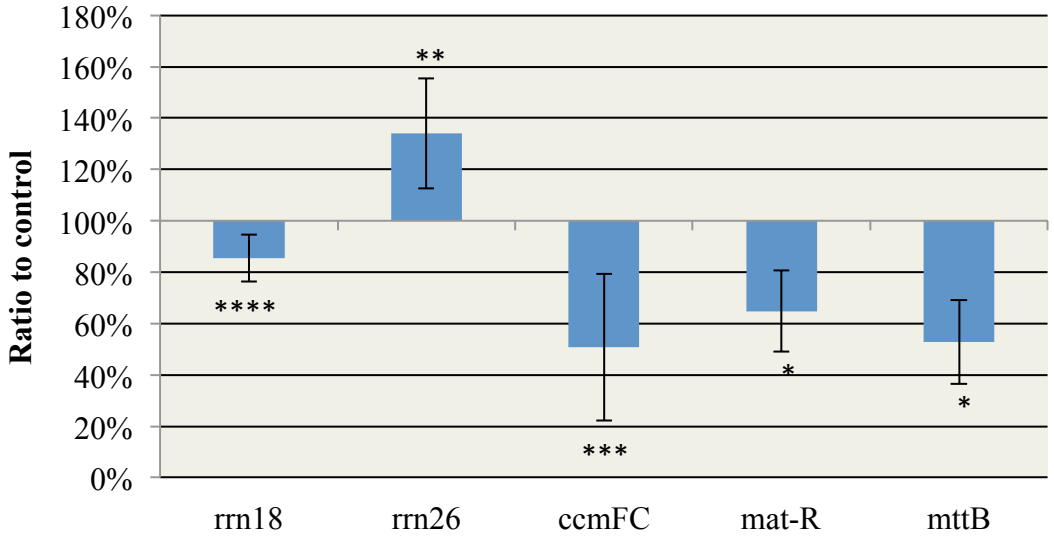


Figure 19: Effect of *orf77*-PKTLS RNA in dark-grown seedlings on the level of selected mitochondrial RNAs. Analysis by RT-qPCR on total RNA. Results are given as the percentage of the selected mitochondrial RNAs at D4 as compared to control. *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001.

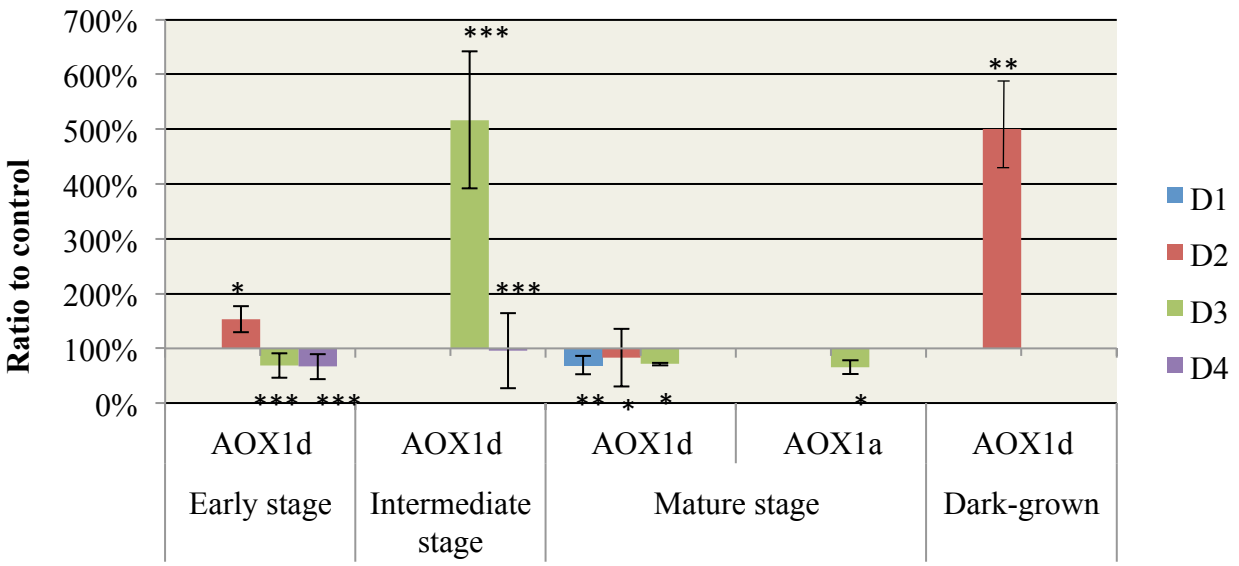


Figure 20: Effect of *orf77*-PKTLS RNA on the level of selected nuclear RNAs in the different growth conditions of transformed *A. thaliana* plants. Analysis by RT-qPCR on total RNA. Results are given as the percentage of the selected nuclear RNAs at D1, D2, D3 and D4 as compared to control. *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001.

At D3, *nad1b*, *nad2b*, *rrn26*, *ccmFN2* and *mttB* decreased, while only *nad5b* increased (Figure 18B). Finally at D4, *rrn18*, *matR*, *mttB* and *ccmFC* decreased and only *rrn26* increased (Figure 19).

III.4 Expression of the *orf77*-PKTLS RNA and impact on the level of selected nuclear mRNAs

Transformed *A. thaliana* plants expressing the *orf77*-PKTLS RNA upon estradiol induction of the transgene also presented significant variations in the expression of the RNAs encoding the alternative oxidase, *aox1a* and *aox1d*. Such results are striking, since the AOX protein is encoded by the nuclear genome. In light-grown plants at the early stage of development, *aox1d* was increased at D2, but was decreased versus control plants at D3 and D4, as the expression of the transgene was slowly going down. Conversely, at the intermediate stage of development, *aox1d* increased at D3 and D4. In the mature plants, *aox1d* was decreased at D1, D2 and D3, while *aox1a* was decreased only at D3. Finally, in dark-grown seedlings, *aox1d* was highly increased at D2, when the expression of the *orf77*-PKTLS RNA was maximum (Figure 20).

Conclusion

As summarized in Table 1, our results show that the presence of the *orf77* CMS RNA in the mitochondria is indeed capable of disturbing the organellar transcriptome, but mainly in light-grown mature plants. The response is thus strongly dependent on the developmental stage and the culture conditions, which implies appropriate regulation mechanisms. The level of the *atp9* mRNA, which shares sequences with the *orf77*, was significantly affected only in the frame of the maximal response in mature plants. Interestingly, in all conditions tested, plants expressing the *orf77*-PKTLS RNA showed positive or negative variations in the level of nuclear RNAs coding for the mitochondrial alternative oxidase. Introducing the *orf77* into mitochondria thus seems to trigger also a mitochondrial retrograde signaling followed by a nuclear anterograde response.

Conditions	Day 1	Day 2	Day 3	Day 4
Light, early stage		nad5a*		
		atp4**		
		rps7*		
		AOX1d*	**AOX1d*****	**AOX1d*****
Light, intermediate stage	nad5b**	nad5a**		
			cob**	
		atp4*		
		rpl2*, rpl5*		rpl2**, rpl5*, rps7*
	cmFN2**		ccmFN2***	
			AOX1d***	**AOX1d*****
Light, mature stage	nad2b**, nad5a**, nad5b**, nad9**		nad1b*, nad2b*, nad4**, nad5a***, nad5b*, nad6*, nad9**	
			cob***	
	cox1**		cox1*, cox2**, cox3****	cox1****
		atp4,* atp9****	atp9*	atp9*
	rpl5*	rps4**, rps7****, rpl2****, rpl5**, rrn18**	rrn18***, rrn26**	rpl5**, rrn26*
	ccmB**		ccmFN2**,ccmB***, ccmFN1***, ccmFC*	ccmFC***
	mttB*		mttB**	mttB*
	AOX1d**	**AOX1d***	**AOX1a*, AOX1d***	
Dark-grown	nad2b**, nad5a**, nad5b****, nad9*	nad4*, nad5b*	nad1b***, nad2b**, nad5b**	
	cox2*	cox1*		
	rpl5*, rrn26*, rps7**		rrn26**	rrn18****, rrn26**
		ccmFN2**	ccmFN2**	ccmFC***
			mttB**	mttB*
	mat-R*			mat-R*
		AOX1d**		

Table 1. Mitochondrial RNAs whose level increases (in black) or decreases (in red) significantly in relation with the expression and mitochondrial import of the *orf77*-PKTLS RNA. The significant variations of the nuclear RNAs coding for the alternative oxidase (AOX1, underlined) are also included. *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001.

RESULTS CHAPTER II

I. Mitochondrial exonucleases

In higher plants, the mitochondrial DNA (mtDNA) is rich in small repeated sequences. These repeats recombine with each other at low frequency and cause rearrangements of the mtDNA that result in increased heteroplasmy of the mtDNA and genetic diversity that drive the rapid evolution of the plant mtDNA. Such rearrangements affecting the mitochondrial genome can lead to cytoplasmic male sterility (CMS), a trait that is widely used in crops for breeding and hybrid maintenance. Several genes that are involved in plant mtDNA replication, repair and recombination have been identified. Several of these genes are specific to plants, but many others are related to α-proteobacterial orthologs (Ku 2016) (see part-V of introduction for details). The mitochondrial recombination machinery is also likely to require 5'-3'exonucleases, to generate single-stranded DNA ends through resection, which is a first step in the repair of DNA double strand breaks by homologous recombination (Figure 21). But up to now no such enzyme has been identified in the mitochondria of plants. In *Arabidopsis thaliana* we identified two genes encoded by the nuclear genome that code for two exonucleases that we called as Organellar Exonuclease 1 and 2 (OEX1 and OEX2, genes At3g52050 and At1g34380 respectively). Interestingly, we found that the OEX1 and OEX2 sequences are related to the 5'-3' exonucleases domain of bacterial DNA polymerase I (DNA pol I) (Figures 22 and 23). The two sequences were predicted to be targeted to mitochondria or chloroplasts. The protein sequences databases were searched by BLAST using the Arabidopsis OEX1 and OEX2 sequences as queries, to identify plant orthologs and do a phylogenetic analysis (Figure 24). OEX1 and OEX2 are apparently conserved in all plant species, including mosses and algae. The alignments showed that both OEX1 and OEX2 are highly conversed in plants (Figures 25).

II. Intracellular localization of OEX:GFP fusions

All plant OEX sequences found have N-terminal extensions that are poorly conserved between species that could correspond to organellar targeting peptides. The *Arabidopsis thaliana* OEX1 and OEX2 are predicted to be targeted to organelles. To confirm this assumption, the OEX1 and OEX2 coding sequences were cloned in the pUCAP-GFP vector, fused in frame at the C-terminal extremity to eGFP. Subcellular localization of the fusion proteins was analyzed in *Nicotiana benthamiana* leaf epidermal cells, after transfection by particle bombardment and observation at the confocal microscope. The OEX1:eGFP fusion protein was found in

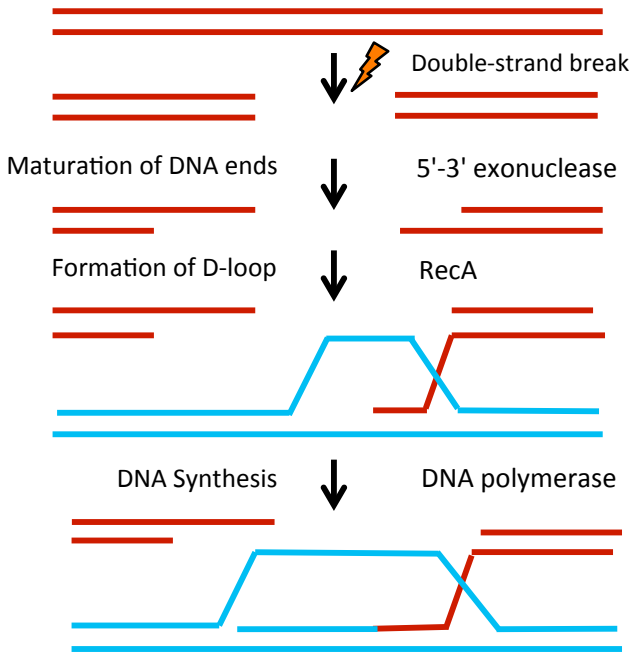


Fig 21: During DSB repair by homologous recombination 5'-3'exonucleases are needed to generate single-stranded DNA ends

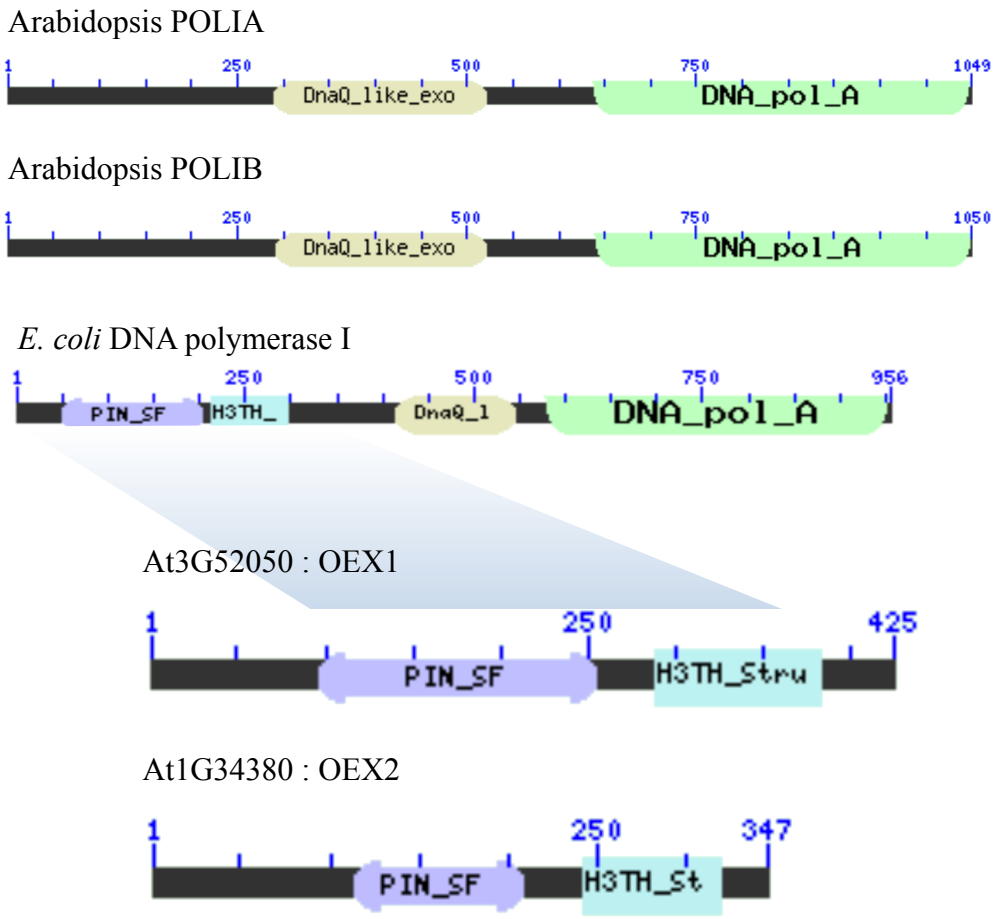


Figure 22: The 5'-3 exonuclease domain of bacterial DNA polymerase I is absent from the Arabidopsis organellar DNA polymerases POLIA and POLIB. The OEX1 and OEX2 proteins are similar to that domain.

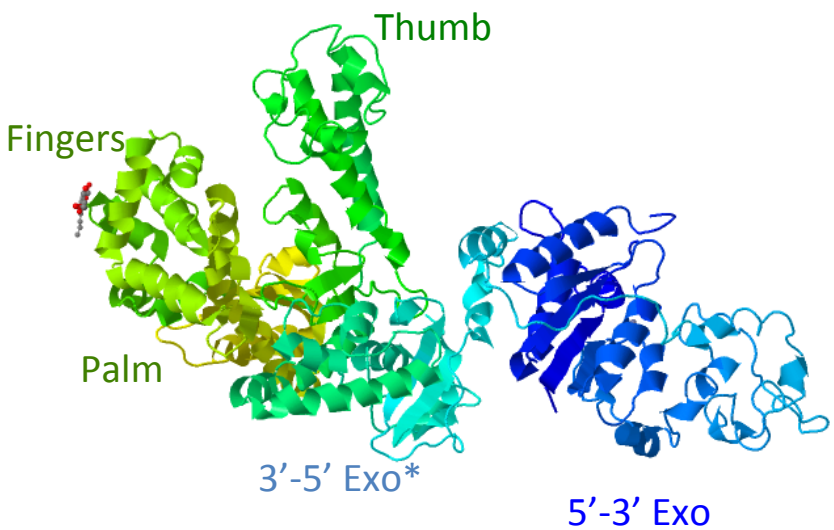


Figure 23: Structure of bacterial DNA polymerase I. The different domains are indicated.

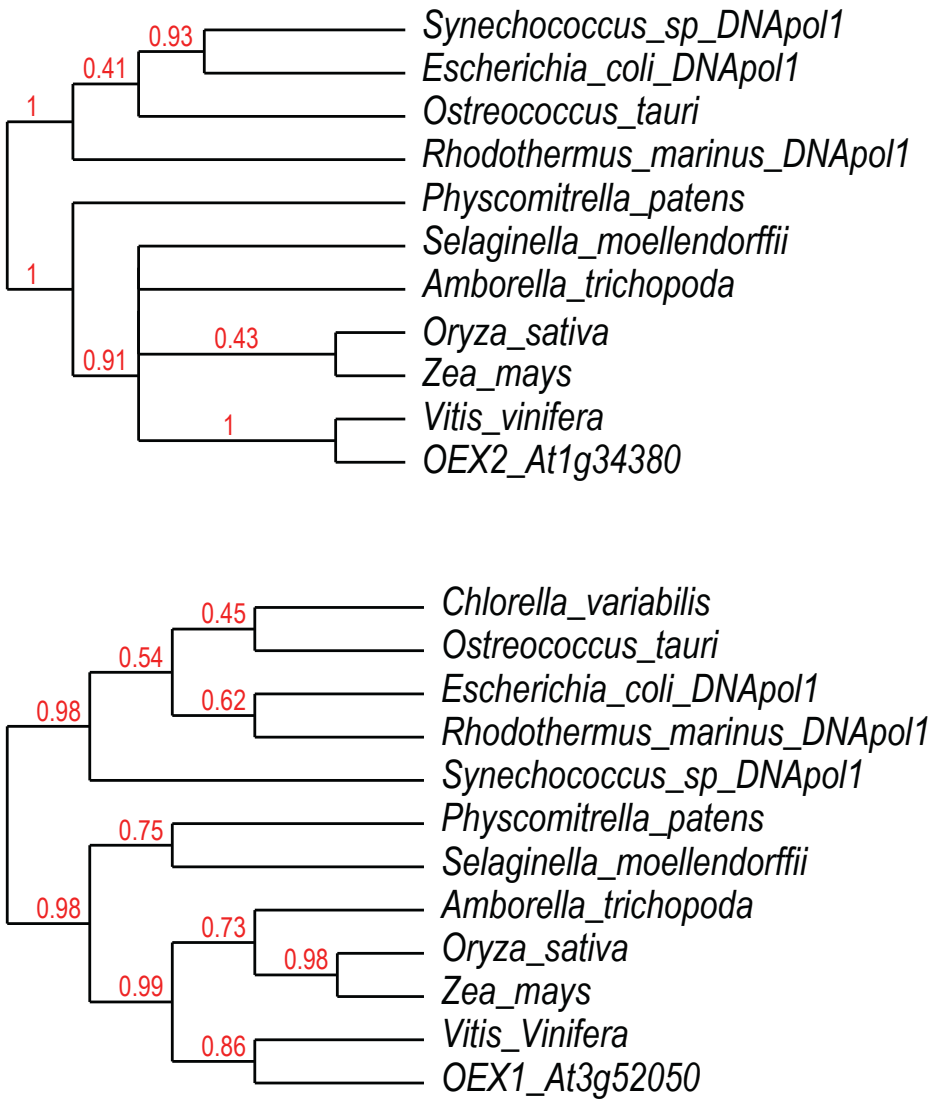


Figure 24: Unrooted phylogenetic tree of plant OEX1 and OEX2 proteins and the 5'-3' exonuclease domain of bacterial DNA polymerase I. The closer relationship to the DNA polymerase I of cyanobacteria suggest that plant *OEX* genes were inherited from the symbiotic ancestor of chloroplasts. Bootstrap values are indicated.

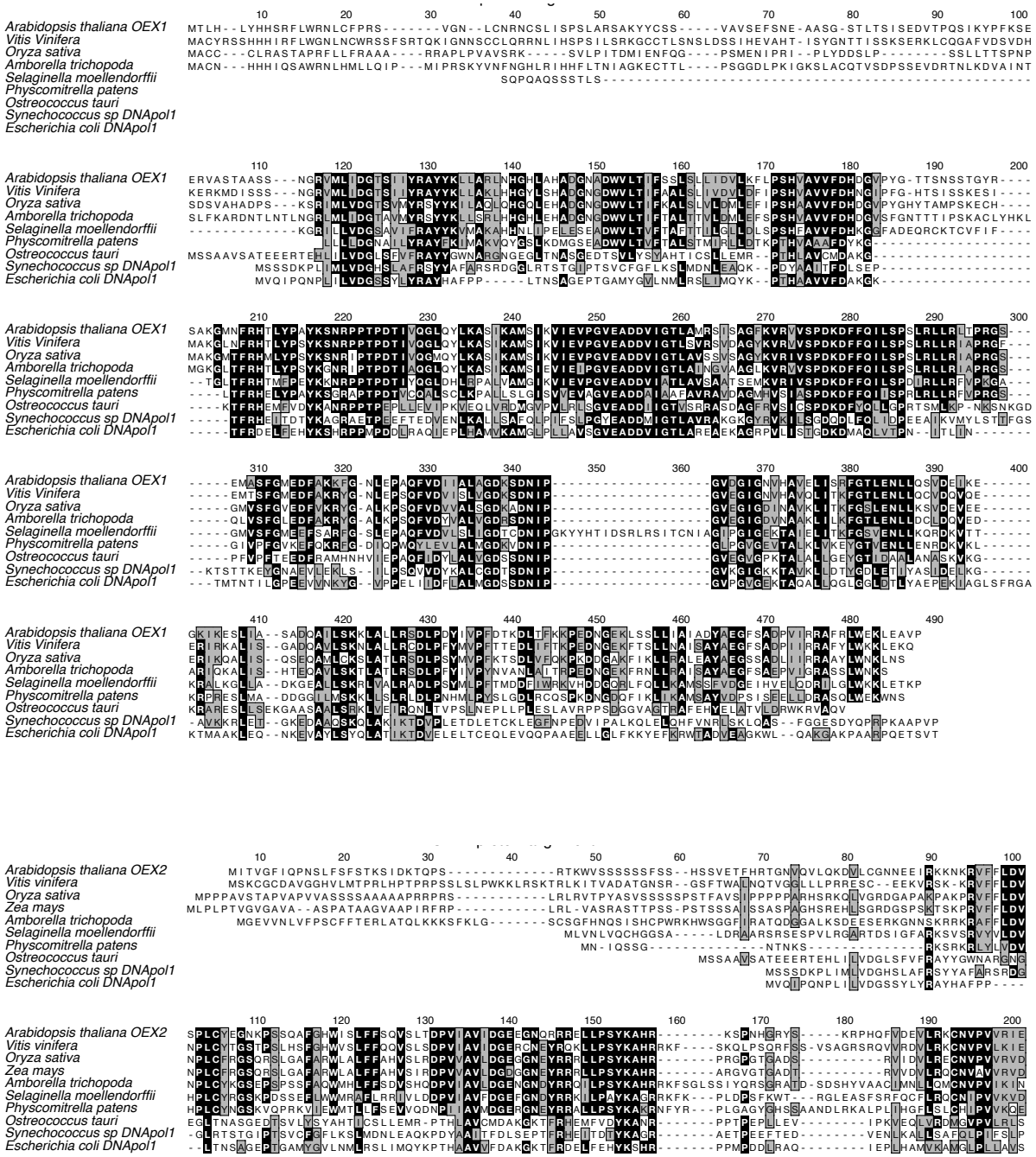
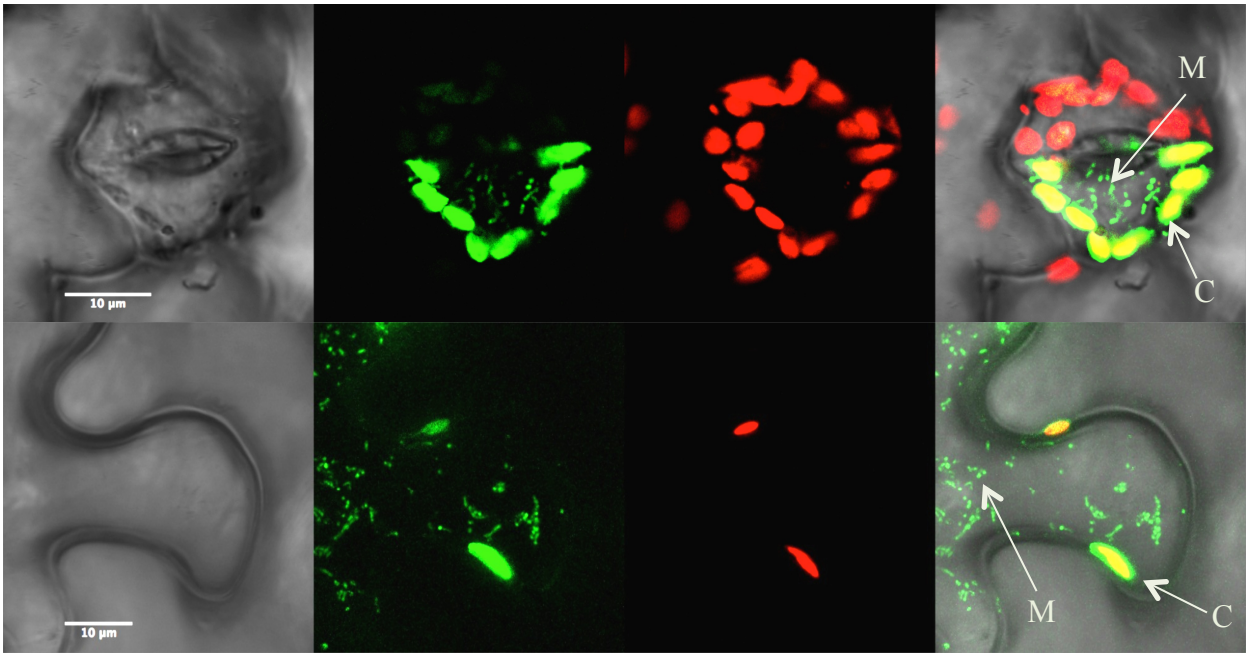


Figure 25: Alignment of the OEX1 and OEX2 protein sequences and the 5'-3' exonuclease domain of bacterial DNA polymerase I. Generated using the ClustalW multiple sequence alignment program.



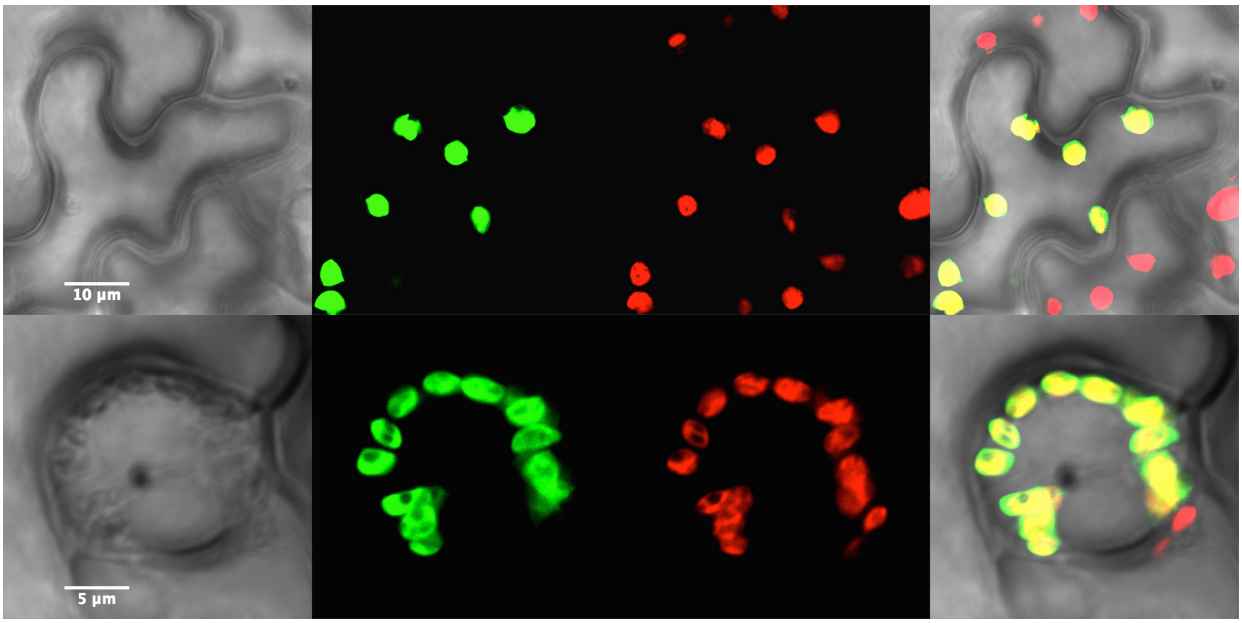


Figure 26: Transient expression of the OEX1-GFP and OEX2-GFP constructions in *Nicotiana benthamiana* leaf epidermal cells showing dual targeting of OEX1 to mitochondria (M) and chloroplasts (C) and targeting of OEX2 to chloroplasts. Chloroplasts are identified by the auto fluorescence of the chlorophyll. Mitochondria are recognizable as mobile small round dots.

chloroplasts, as determined by the co-localization with the fluorescence of the chlorophyll. But small spots that looked like mitochondria were also observed. So, apparently, OEX1 is targeted into both chloroplasts and mitochondria. On the contrary, observation of the OEX2:eGFP fusion protein showed that OEX2 is only localized in chloroplasts (Figure 26).

III. Biochemical activity test

The alternative splicing of *OEX1* transcripts produces two alternative forms of *OEX1 that* we called as *OEX1a* and *OEX1b* (Figure 27). Both alternative cDNA sequences of *OEX1* were cloned into the pET28a expression vector and then the recombinant proteins fused to a N-terminal His-tag were expressed in the Rosetta (D3) host strain under induction by IPTG. The expression and sizes of the proteins were confirmed by SDS-PAGE analysis (Figure 28). Immobilized metal ion affinity chromatography (IMAC) and gel filtration were used for the purification of the proteins.

The biochemical activities of the purified proteins were tested on different potential DNA substrates prepared by hybridization of complementary oligonucleotides: single-stranded DNA (ssDNA), double stranded DNA (dsDNA), Y-shaped branched DNA, 3'-overhanged ssDNA and 5'-overhanged ssDNA. The 5'-ends of the substrates were labeled with radioactive ^{32}P, by phosphorylation of one of the oligonucleotides. The substrates were purified in 8% polyacrylamide gels, and incubated with the OEX1a and OEX1b recombinant proteins. The biochemical activity was revealed by analysis on 8% polyacrylamide gels and autoradiography. The biochemical activity tests (Figure 29) showed that both alternative forms of OEX1 do not recognize ssDNA as substrate for degradation. But all the DNA structures containing double stranded DNA regions were recognized as substrates and cleaved by both alternative forms of OEX1.

IV. Characterization of plant mutants

IV.1 Localization of T-DNA insertions

From a search of the available Arabidopsis T-DNA insertion mutant collections, only one confirmed line was obtained, line GK_911E05 from the GABI-Kat collection that was named *oex1-1*. It is derived from ecotype Col-0 and contains a T-DNA insertion in intron 9. The map of the *OEX1* gene showing the location of the insertion is shown in (Figure 30). Genotyping of the

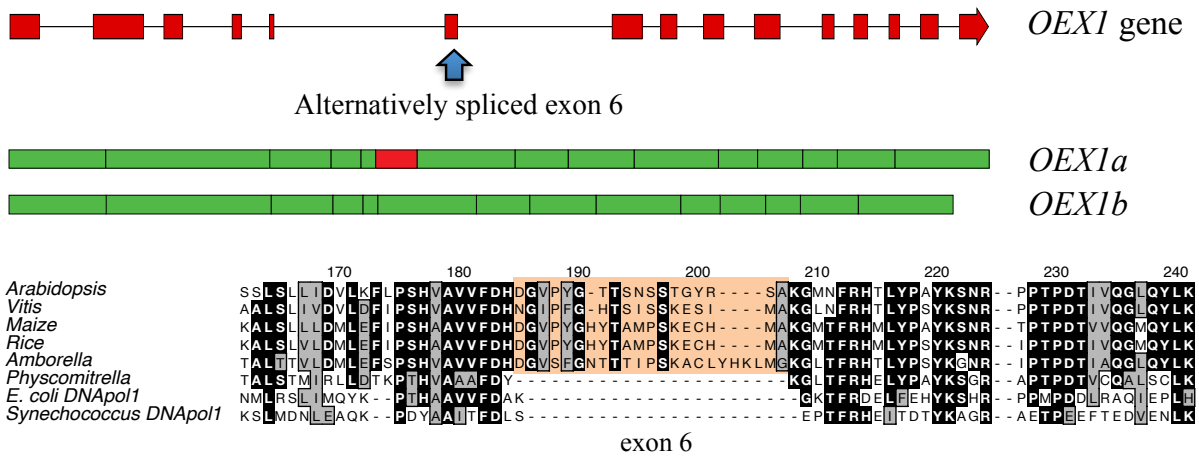


Figure 27: Alternative splicing of *OEX1* exon 6 generates two proteins.

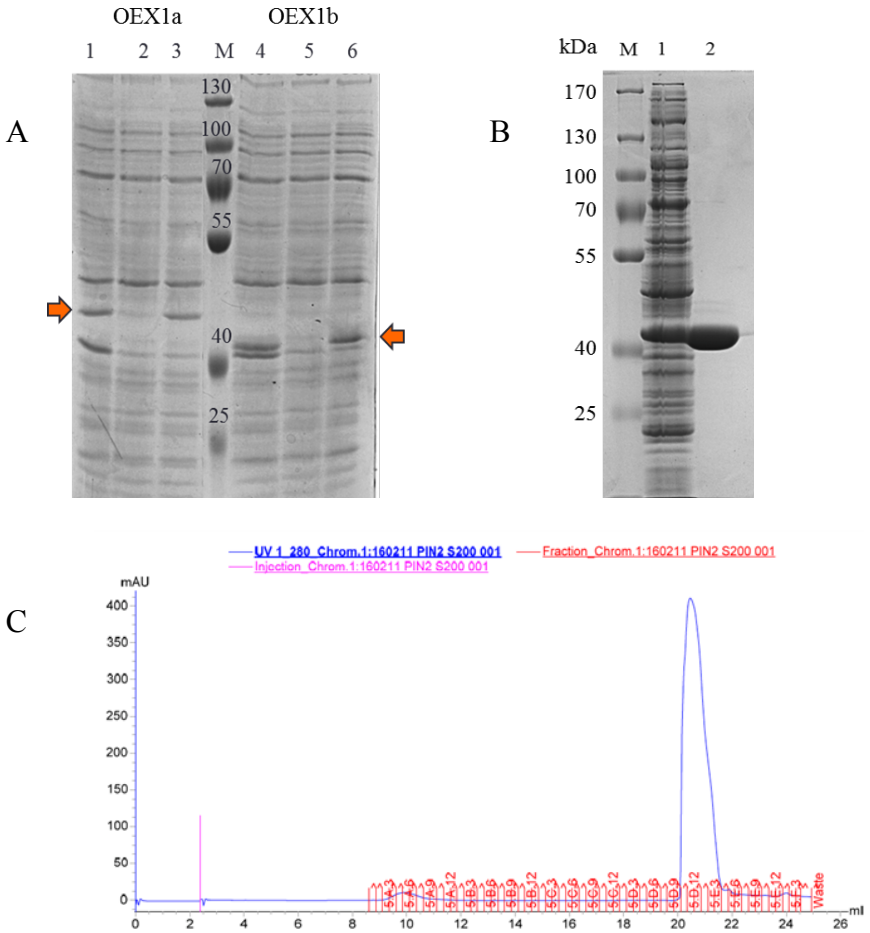


Figure 28: Expression and purification of OEX1 proteins by IMAC and gel filtration.

(A) OEX1a and OEX1b were purified from *E. coli* by a combination of IMAC and gel filtration chromatography. Lanes 1 and 4, total bacterial protein extract after IPTG induction; lanes 2 and 5, total soluble protein before IPTG induction; lanes 3 and 6, total soluble protein after IPTG induction; M, molecular weight marker. The recombinant proteins are indicated by arrowheads.

(B) Purity of OEX1b, evaluated by Coomassie staining. M, molecular weight marker; Lane 1, total soluble fraction; lane 2, OEX1b protein purified after IMAC and gel filtration.

(C) Gel filtration chromatogram of OEX1b, showing the monodispersion of the protein.

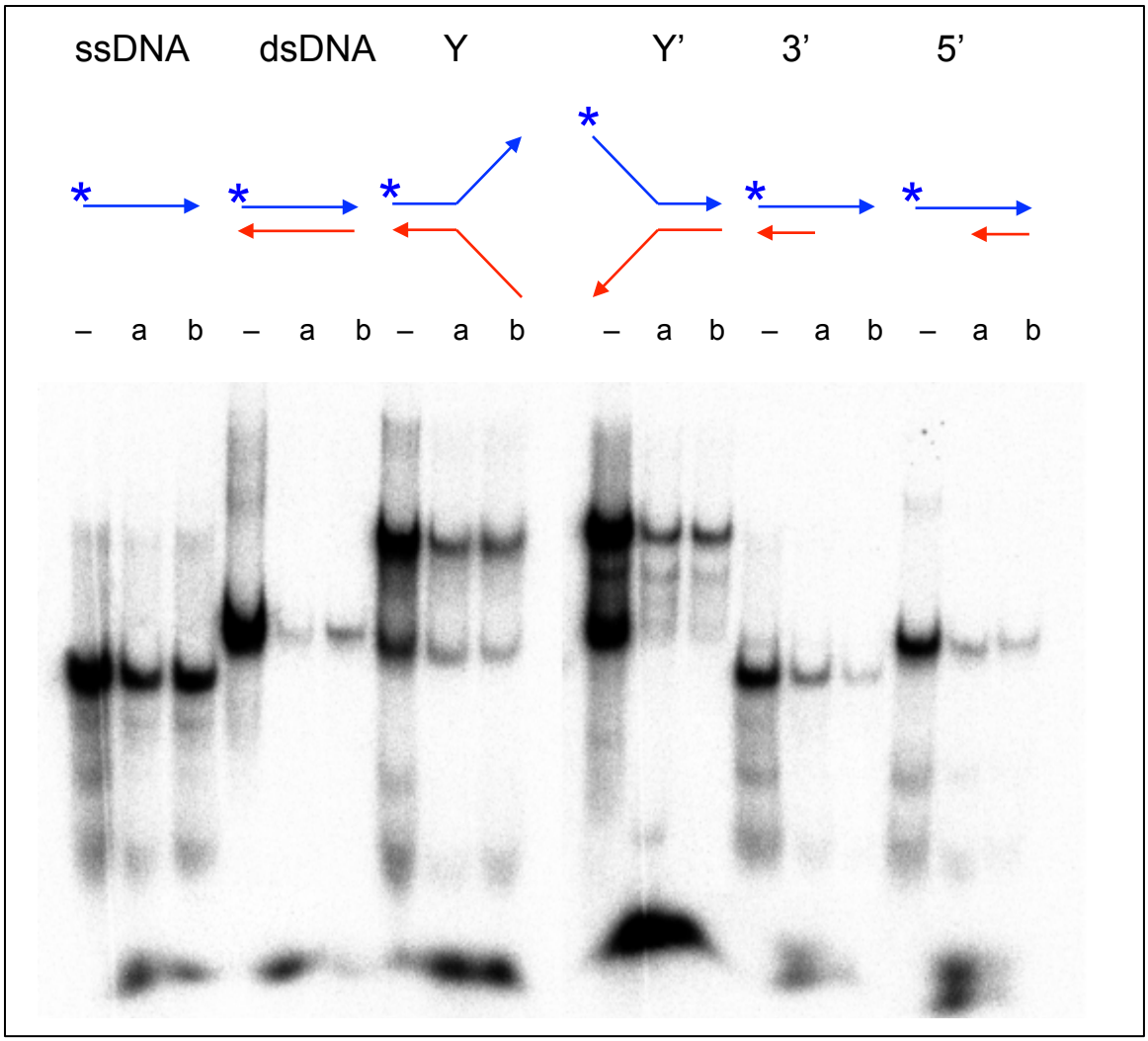


Figure 29: Biochemical activity assays of the nuclease activity of both alternative forms of OEX1. The substrates consisted of ssDNA, dsDNA, y-shaped branched DNA, 3′-overhang and 5′-overhang (star denotes 5' radiolabelling site), (-) is substrate control, no enzyme added, (a) OEX1a protein added, (b) OEX1b protein added. The reaction products were analyzed on 8% polyacrylamide gels and revealed by Phosphorimager.

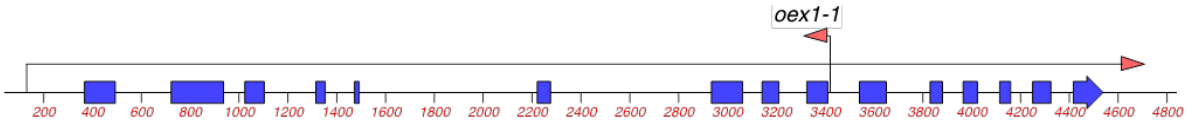


Figure 30: Schematic representation of the Arabidopsis *OEX1* gene. Exons are shown as blue boxes. The position of the T-DNA insertion in the mutant line is shown, with the arrow indicating the orientation of the left border of the T-DNA.

F1 F2 F3

Figure 31: Phenotypes of *oex1-1* plants grown on soil. The plants were more severally affected in their development in the F2 and F3 generations.

oex1-1 mutant was done by PCR using primers which frame the T-DNA insertion region. Sequencing of the amplification products allowed us to determine the exact position of the T-DNA insertion in the mutant. The different mutant plants of *oex1-1* were studied from F1 to F3. It has been shown that mutants of different genes involved in the surveillance of organellar genome recombination or involved in repair by recombination can show visible phenotypes of reduced growth, distorted leaves, leaves variegation, and reduced fertility (Liberatore, Dukowic-Schulze et al. 2016; Odahara, Kobayashi et al. 2016). Similar and quite severe phenotypes were also observed in the first generation of the homozygote *oex1-1* mutants (probably F2 plants). Under normal growing conditions, all homozygote plants displayed phenotypes of reduced growth, mottled and distorted leaves and very reduced fertility (Figure 31). In the next generations (F3) the phenotype of the plants became severer, and plants were completely unable to produce seeds (Figure 32).

V. Study of mutants at the molecular level

In the *oex1* mutant there is increased accumulation of mtDNA recombination involving small interspersed repeats (SIRs)

Changes in the mt-genome in *oex1-1* mutants were first studied by analysis of the recombination involving SIRs. The ones selected were the small interspersed repeats L (256 bp) and EE (126 bp) which are present in two copies in the mtDNA of Col-0 and can be involved in infrequent recombination leading to characteristic crossover products. Recombination involving these repeats is often increased in mutants of genes involved in recombination surveillance, such as the mutants of the *OSB1, RECA3, MSH1* and *RECG1* genes (Zaegel, Guermann et al. 2006; Shedge, Arrieta-Montiel et al. 2007; Miller-Messmer, Kühn et al. 2012; Wallet, Le Ret et al. 2015). Their levels were evaluated by qPCR in two individual plants, *oex1-1#1* and *oex1-1#2* and compared to the levels in wild type Col-0 plants. Relative copy numbers of each repeat sequence (L-1/1, L-2/2, EE-1/1, EE-2/2) and reciprocal recombination products (L-1/2, L-2/1, EE-1/2, EE-2/1) were standardized against three nuclear reference genes: the actin gene *ACT1* (At2G37620), the polyubiquitin gene *UBQ10* (At4G05320) and the 18S rRNA gene (18S RRNA). As shown, (Figure 33) the crossover products of repeats L and EE were amplified up to 32 and 256 fold respectively in the mtDNA of *oex1-1#1* and *oex1-1#2* plants as compared to

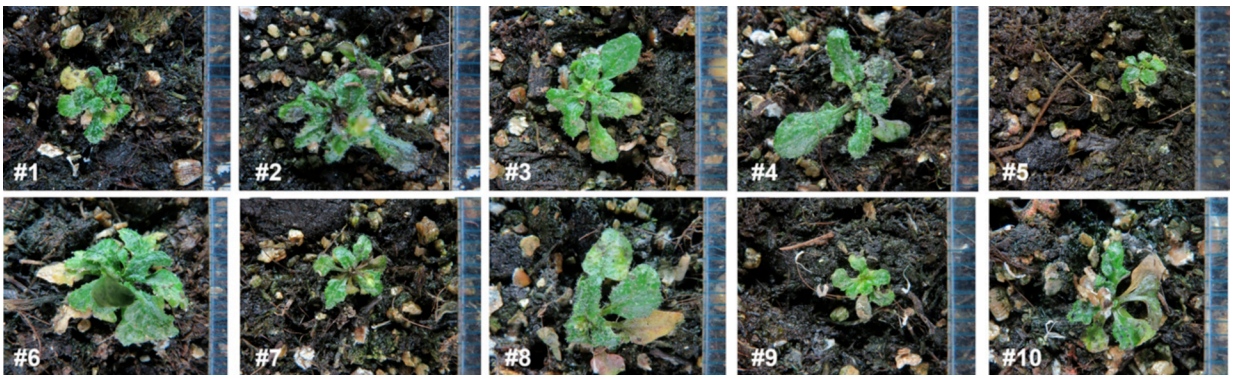


Figure 32: Phenotypes of individual *oex1-1* plants selected for analysis. The plants show severe phenotypes of dwarfism, distortion and sterility.

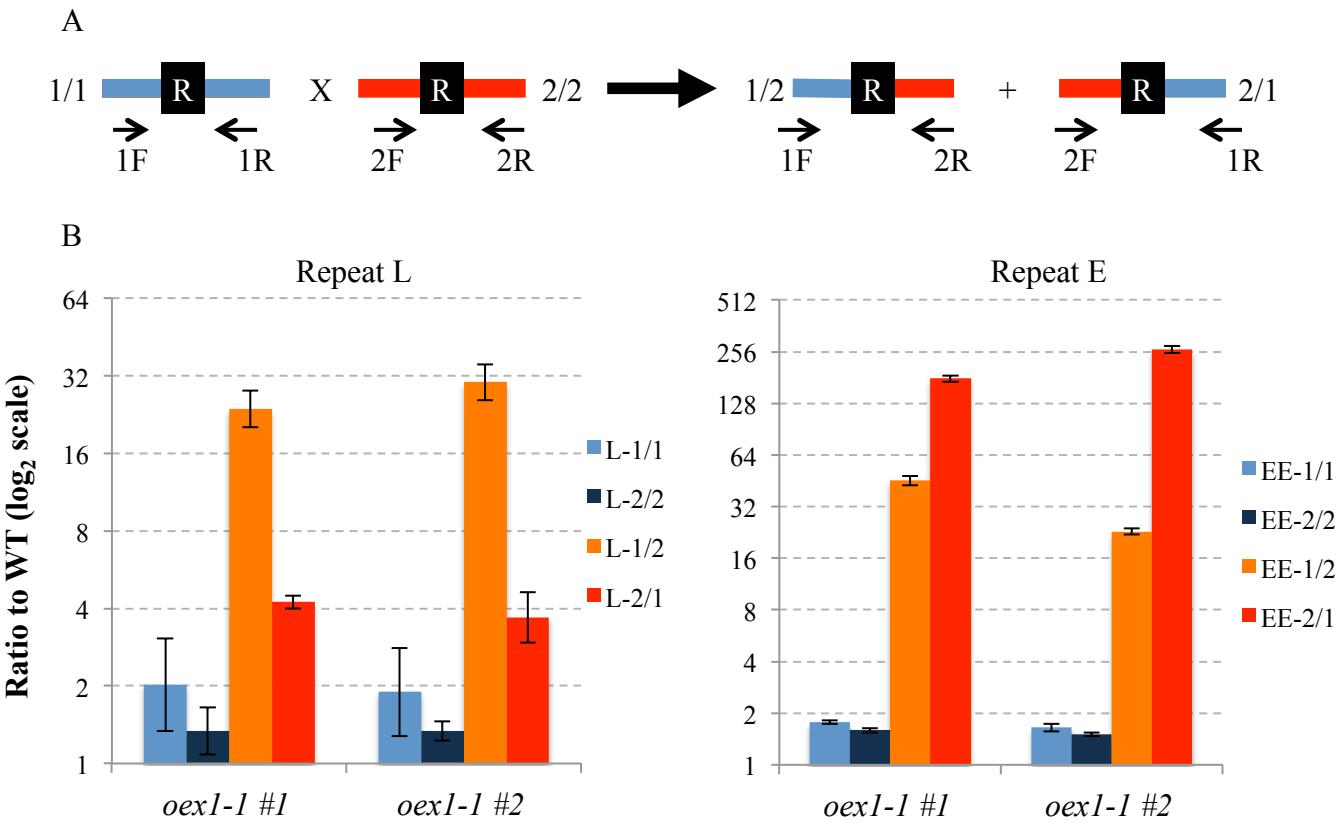


Figure 33: Analysis by qPCR of the recombination involving repeats L and EE.

(A). Scheme of the qPCR relative quantification of parental sequences 1/1 and 2/2 that comprise a same repeated sequences (R detonates repeat) and of the corresponding crossover products 1/2 and 2/1, using combinations of forward (F) and reverse (R) primers.

(B). Analysis of the accumulation of crossover products in two individual *oex1-1* plants (*oex1-1 #1* and #2 as compared to Wild-type plants. qPCR results for repeat L and EE were normalized against actin gene *ACT1* (At2G37620), the polyubiquitin gene *UBQ10* (At4G05320) and the 18S rRNA gene (18SRRNA). Results are presented in a $\log_2$ scale.

wild-type plants, suggesting that OEX1 is involved in recombination processes that suppress recombination involving small interspersed repeats (SIRs).

V.1 Changes in the stoichiometry of the different mtDNA regions

To study recombination events in the mitochondrial genome of the *oex1-1* mutants, the relative copy numbers of the different mtDNA regions were analyzed. In different individual plants from the F3 generation (picture of plants in Figure 32), the relative copy number of the different mtDNA regions was determined by qPCR, using a set of primer pairs spaced from 5 to 10 kb apart along the genome (Wallet, Le Ret et al. 2015). The published Arabidopsis Col-0 mtDNA sequence (accession JF729201) was taken as a reference. Values were standardized against three nuclear reference genes: the actin gene *ACT1* (At2G37620), the polyubiquitin gene *UBQ10* (At4G05320) and the 18S rRNA gene (18S rRNA). In all *oex1-1* plants, significant variations in the relative copy number of the different mtDNA regions were detected in comparison to the WT Col-0 plant mtDNA. In some regions, we could detect an increase between 1.5 up to 4.5 fold, but in others a decrease from 0.5 to near zero. These variations were different in each plant, considering the genomic regions and the levels. The results for 4 individual plants are shown in (Figure 34). A possible explanation for the sharp increase of certain mtDNA regions as compared to neighboring ones could be the recombination between directly oriented SIRs generating circular DNA sequences that replicate autonomously. Such example was observed and described in an Arabidosis *recG1* mutant, involving recombination between repeats EE of 126 bp and generating a circular episome of 8 kb (Wallet, Le Ret et al. 2015). An analysis of candidate examples in *oex1-1* plants was performed using both direct and inverse PCR, followed by sequencing. The sequencing results revealed that indeed different regions that are amplified in the *oex1-1* plant apparently are circular subgenomic molecules generated by recombination involving direct repeats (Figure 35). It was not possible to further characterize these molecules by other methods because of the very little amount of DNA that was available from the very small and severely affected plants. The circular molecules that were analyzed resulted from the recombination between directly oriented repeats A, EE and L (Figure 35), but also from recombination involving a small imperfect repeat and from microhomologies. All these circular molecules contain highly expressed genes. It is tempting to speculate that the transcription of these genes is related to the ability of the subgenomes to replicate autonomously. For instance, it is possible that high transcription activity generates RNA primers required for replication

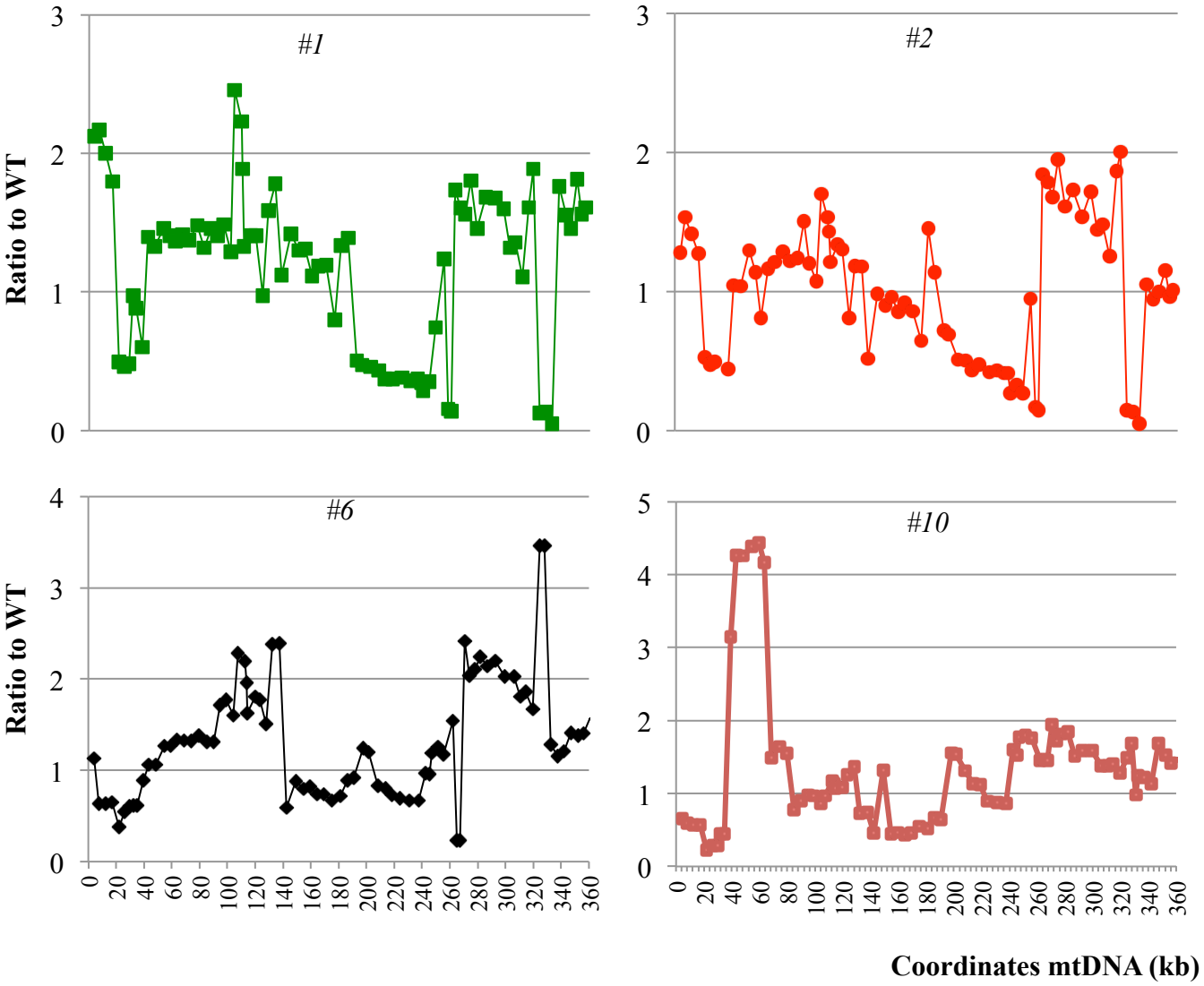


Figure 34: Scanning of the mtDNA for changes in the relative copy numbers of the different mtDNA regions. Sequences spaced 5 to 10 kb apart on the mtDNA were quantified by qPCR on individual *oex1-1* plants (and corresponding wild-type Col-0). Coordinates are those of the published Col-0 mtDNA sequence.

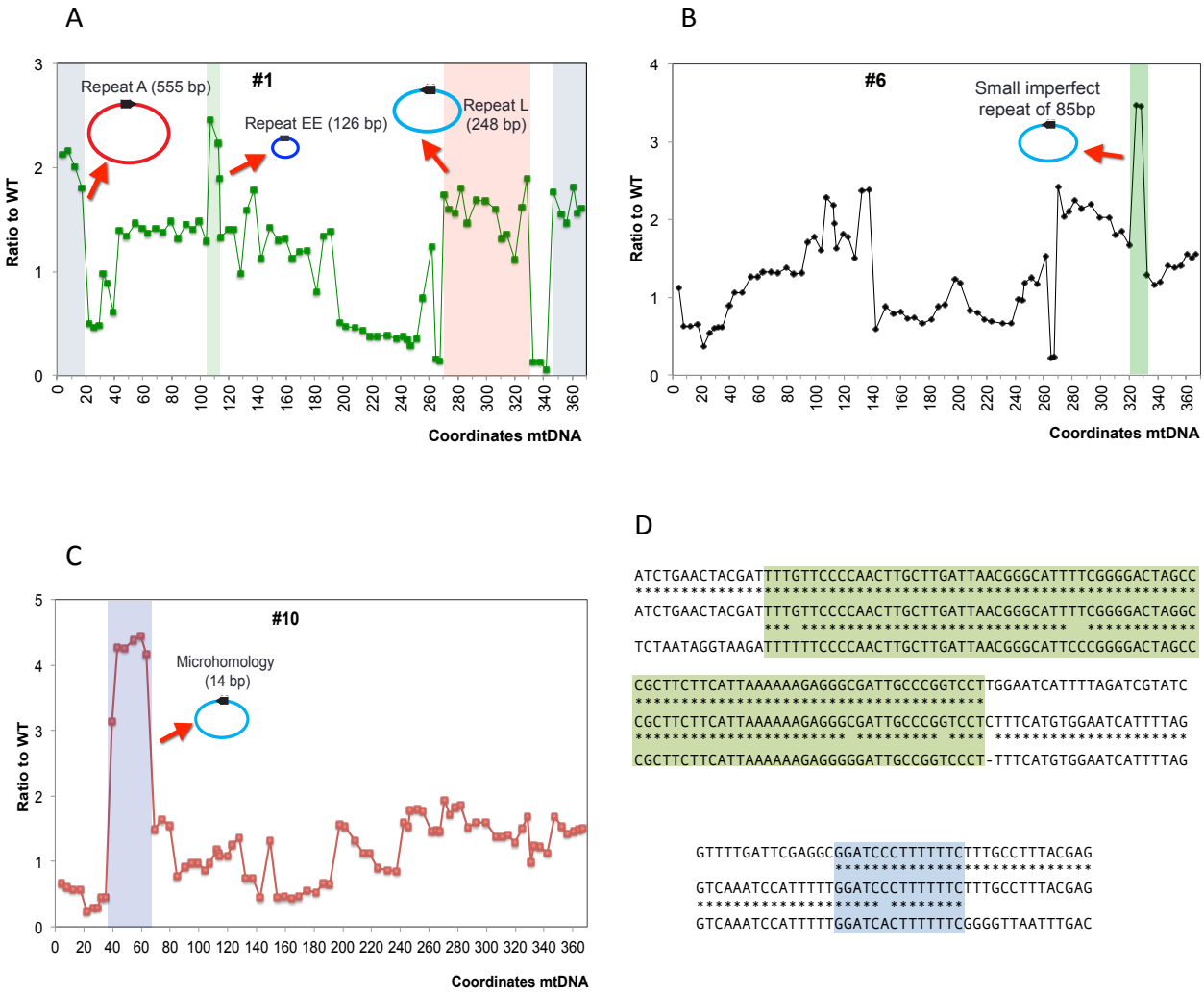


Figure 35: Changes in the copy number and stoichiometry of mtDNA sequences partially results from recombination involving directly oriented repeats that generate subgenomes that replicate autonomously. Regions of the mtDNA that were augmented in copy number in mutant plants #1, #6 and #10 (see **Figure 32**) were analyzed by inverted PCR and sequenced, to determine their borders. They contained the signatures of recombination involving direct repeats. These could be small interspersed repeats of more than 100 bp - repeats A, EE and L - in several regions of plant #*1* (A), a small imperfect repeat in a specific locus of the mtDNA in plant #6 (B) and a sequence microhomology in plant #10 (C). The imperfect repeats involved in the recombination events of B and C are shown in panel D.

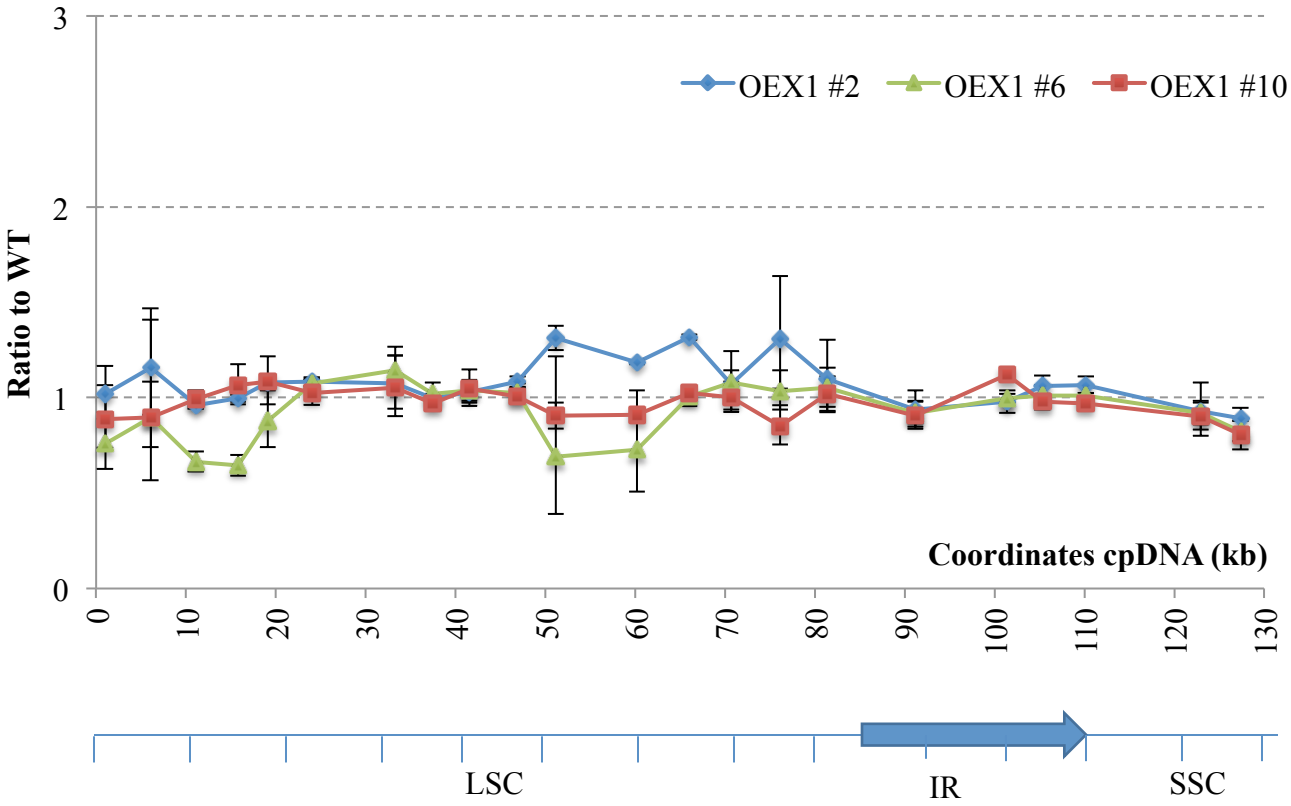


Figure 36: Scanning of the cpDNA of *oex1-1* mutants for changes in the relative copy numbers of the different cpDNA regions. Sequences spaced 5-10 kb apart on the cpDNA were quantified by qPCR as explained in Figure 33. Coordinates correspond to the ones of the published Col-0 cpDNA sequence. The three major regions of the cpDNA are indicated: LSC, large single copy region; SSC, small single copy region; IR, inverted repeat.

initiation. The table 2 gives the details of the repeats that are involved in these recombination events.

Repeat	Repeat size (bp)	Coordinates	Expressed genes in the circular molecule
A	555	19679:20234 / 345151:345706	*COX2*
EE	126	106114:106240 / 114177:114303	*ATP1*
L	248	269745:269993 / 330834:331082	*ATP9* and *COX3*
Small imperfect repeat	56	320428:3204833 / 32022:332077	*COX3*
Microhomology	14	36633:36646 / 64755:64768	*18S* and *26S*

Table No 2. Detail of the repeats, repeat sizes, coordinates in the published mtDNA sequence of Col-0, names according to genome annotation and highly expressed genes contained in the circular subgenomes.

V.2 Chloroplast DNA (cpDNA) copy numbers in *oex1-1* plants

The OEX1 protein is apparently dually targeted to mitochondria and chloroplasts. We therefore considered the hypothesis that loss of OEX1 also affects the stability of the plastidial genome (cpDNA). The complete cpDNA of several individual plants was also scanned by qPCR using a set of primer pairs spaced 5 to 10 kb apart along the genome. The published *Arabidopsis* Col-0 cpDNA sequence was taken as a reference. No changes were detected between the cpDNA of mutant plants *oex1-1#2, oex1-1#6* and *oex1-1#10* and the one of wild-type, apart from a minor increase in the copy number of some regions, but at the level of technical errors (Figure 36). Thus, loss of OEX1 does not seem to significantly affect the stoichiometric replication and segregation of the cpDNA in *Arabidopsis*.

VI. Characterization of double mutants

The two organellar DNA polymerases (DNAPs) from Arabidopsis, POLIA and POLIB, are involved in the replication and in the repair of the organellar genomes. In Arabidopsis, the two proteins are dually targeted to both mitochondria and plastids, and (Parent, Lepage et al. 2011)

Figure 37: Synergetic effect of the *polIA-1* mutation on *oex1-1*.
Variable severity of the phenotypes developed by *polIA-1 oex1-1* double mutants.

Figure 38: Bushy phenotype of several heterozygous *polIA-1 oex1-1* plants. Plants developed many floral amps showing loss of apical dominance.

reported that it is not possible to obtain double mutants *pol1A pol1B,* confirming essential and redundant functions for the organellar DNAPs. However, the POLIB polymerase seemed to be preferentially involved in DNA repair, and double mutants of *polIB* with *why1 why3* (mutants of plastidial Whirly proteins WHY1 and WHY3) or with *recA1* (mutant of plastidial RecA-like protein RECA1) were synergistically more affected in repair of the Arabidopsis cpDNA (Zampini, Lepage et al. 2015). The preferential involvement of POLIB but not of POLIA in mtDNA repair was also observed in our laboratory (unpublished results).

Because OEX1 is similar to the 5-3' exonucleases domain of bacterial DNA pol I, it is possible that OEX1 works *in trans* with POLIA and/or POLIB in organellar genomes replication and/or repair. We therefore tried to create double mutants with organellar DNA polymerases (*pol1A oex1, pol1B oex1*). The mutant lines that were used were SALK_020119 (*polIA-2*) and GK-291F05 (*poI1B-2*). Because *polIA* and *pol1B* mutants are viable and present no visible developmental phenotypes while *oex1-1* plant are severely affected in growth and fertility, for the crosses we used *pol1A* and *pol1B* mutants as female plants and *oex1-1* as pollen donor.

VI.1 *polIA-1 oex1-1* plants

We studied the developmental phenotypes of mutants *pol1A-1 oex1-1* from F1 to F2. The total DNA of the segregating plants was extracted using a rapid method (Edwards, Johnstone et al. 1991) the genotypes were determined and compared to the visible development phenotypes. As expected, in the F1 generation plants were heterozygous for both mutations. In the F2 generation, homozygous double mutants could be selected. These plants were all affected, to different extents, by phenotypes of dwarfism, deformed and mottled leaves, and sterility (Figure 37). Phenotypes were similar to the ones of homozygote *oex1-1* single mutant plants, but apparently severer, suggesting a synergistic effect of the double mutation. Surprisingly, several F2 plants displayed a "bushy" phenotype, with many floral amps growing at the same time, indicative of a loss of apical dominance (Figure 38). Genotyping results showed that these plants were heterozygote both for *polIA-2* and for *oex1-1*.

The different molecular genotypes and visible phenotypes of plants are summarized in Table 3. The expected theoretical segregation following the Law of Independent Assortment (Mendel Second Law) and the actual results are compared in Table 4.

No.	*oex1-1*	*polIA-1*	Phenotype	No.	*oex1-1*	*pol1A-1*	Phenotype
A1	+/-	+/-	WTP	E1	+/-	+/-	WTP
A2	+/-	+/-	WTP	E2	+/+	+/-	WTP
A3	+/+	+/-	WTP	E3	+/+	-/-	WTP
A4	+/-	+/-	WTP	E4	+/+	-/-	WTP
A5	+/-	+/-	BP	E5	+/-	-/-	WTP
A6	+/+	+/-	BP	E6	+/-	Nd	WTP
A7	+/+	-/-?	BP	E7	+/+	+/-	WTP
A8	+/+	+/-	BP	E8	+/-	+/-	WTP
A9	+/-	+/-	BP	E9	+/-	Nd	WTP
A10	+/-	-/-?	WTP	E10	+/-	-/-	WTP
A11	+/+	+/+	WTP	E11	+/+	+/-	WTP
A12	+/-	+/+	BP	E12	+/+	+/-	WTP
B1	+/+	+/+	WTP	F1	+/+	+/-	WTP
B2	+/+	+/-	WTP	F2	+/-	-/-	WTP
B3	+/+	+/-	BP	F3	+/-	+/-	WTP
B4	+/-	+/-	WTP	F4	+/-	+/-	WTP
B5	+/-	+/-	WTP	F5	+/-	-/-	WTP
B6	+/-	+/-	WTP	F6	+/-	+/-	WTP
B7	+/-	+/+	WTP	F7	+/-	nd	WTP
B8	+/-	+/+	WTP	F8	+/-	+/+	WTP
B9	+/+	+/-	BP	F9	+/-	+/-	WTP
B10	+/-	+/+	BP	F10	+/-	+/-	WTP
B11	+/-	+/-	WTP	F11	+/+	+/-	WTP
B12	+/+	-/-?	WTP	F12	+/-	+/-	WTP
C1	+/-	+/-	WTP	G1	+/-	-/-	WTP
C2	+/-	+/-	WTP	G2	+/+	-/-	WTP
C3	+/-	+/+	WTP	G3	+/-	-/-	WTP
C4	+/-	+/+	WTP	G4	+/-	+/+	WTP
C5	+/+	+/-	WTP	G5	+/+	-/-	WTP

Table 3: Genotypes and phenotypes of *polIA-1 oex1-1* plants, generation F2. (--), homozygous; (+ +), Wild-type; (+ -), heterozygous; P, *oex1* phenotype; nd, no PCR band; WTP, Wild-type phenotype; sm, small size

No	Genotype (*OEX1/POLI1A)*	Obtained	Expected
1	EE (+-/+-)	22	22
2	EW (+-/++)	9	11
3	WE (++/+-)	13	11
4	EO (+-/--)	7	11
5	OE (--/+-)	9	11
6	WW (++/++)	2	5.5
7	WO (+-/--)	7	5.5
8	OW (--/++)	4	5.5
9	OO (--/--)	9	5.5

Table 4: Segregation of *pol1A oex1-1* plants at the F2 generation. Comparison between the expected segregation results and the experimental results. (--), homozygous; (++), Wild-type; (+–), heterozygous

VI.2 *polIB-2 oex1-1* plants

Mutants of *pol1B-2 oex1-1* were studied from F1 to F3. F1 plants did not display any visible developmental phenotype. In the F2 generation, from 96 plants tested we could not obtain any double homozygote mutant. We have therefore segregated F3 plants from plants that had been genotyped as being homozygous for *polIB-2* and heterozygous for *oex1-1.* However, in the progeny of these plants we still observed segregation of *polIB-2,* indicating either an erroneous genotyping or mis-identification of the plants. The molecular phenotypes and visible phenotypes of the segregating mutants are shown in Table 5. The expected segregation of the genotypes and the obtained results are compared in Table 6. Still, from 48 F3 plants that were genotypes we could not identify any double homozygous mutant *pol1B-2 oex1-1,* or any plant homozygous for one mutation and heterozygous for the other. Thus, it might not be possible to get homozygous double mutants plants. But because the *POLIB* genes and *OEX1* genes are in the same chromosome, although far apart (12 Mb) and in different chromosome arms, we cannot exclude that the difficulty in obtaining double mutants results from the difficulty in segregating the two gene locus. Further work involving the genotyping of many more plants will be necessary before drawing conclusions.

VII. Study of molecular phenotypes of *po1IA-1 oex1-1* double mutants

Individual F2 *po1IA-2 oex1-1* plants were scanned for changes in the stoichiometry of the different mtDNA regions, as described above for (Figure 34). All *polIA-2 oex1-1* plants tested showed substantial changes in the stoichiometry of mtDNA sequences as compared to the mtDNA of wild-type plants (Figure 39). An increase in the copy number of large regions was mainly observed, that could be as high as 4-fold in some cases. The mtDNA sequences affected and the level of the fluctuations varied from plant to plant, with an involvement of common specific regions. As described above for the individual *oex1-1* plants*,* such changes may be explained by processes of recombination involving certain small interspersed repeats (ex. repeats EE, A and L) leading to the formation of circular subgenomes that replicate autonomously.

VIII. Copy numbers of the mtDNA and cpDNA in plants with bushy phenotype

F2-generation plants selected for displaying a bushy phenotype (Figure 40) were tested for changes in the copy number of the mtDNA and cpDNA. Values were standardized against two nuclear reference genes: the actin gene *ACT1* (At2G37620) and the polyubiquitin gene *UBQ10*

No.	*polIb*	*oex1-1*	Phenotype	No.	*polIb*	*oex1-1*	Phenotype
A1	+/-	nd	WTP	E1	nd	+/+	WTP
A2	-/-	+/+	WTP	E2	+/+	+/+	WTP
A3	+/-	+/-	WTP	E3	+/+	+/+	WTP
A4	+/-	+/-	WTP	E4	+/+	+/+	WTP
A5	+/-	+/-	WTP	E5	+/+	+/+	WTP
A6	+/-	+/-	WTP	E6	+/+	+/+	WTP
A7	+/-	+/-	WTP	E7	+/+	+/+	WTP
A8	+/-	+/-	WTP	E8	+/+	+/+	WTP
A9	+/-	+/-	WTP	E9	+/+	+/+	WTP
A10	+/-	+/-	WTP	E10	+/+	+/+	WTP
A11	+/+?	+/-	WTP	E11	+/+	+/+	WTP
A12	+/+?	-/-?	P	E12	+/+	+/+	WTP
B1	wb	+/+	WTP	F1	wb	+/+	WTP
B2	+/-	+/-	WTP	F2	+/+	+/+	WTP
B3	-/-	+/+	WTP	F3	nd	+/+	WTP
B4	+/-	+/-	WTP	F4	+/+	+/+	WTP
B5	+/-	+/-	WTP	F5	+/+	+/+	WTP
B6	+/-	+/-	WTP	F6	+/+	+/+	WTP
B7	wb?	+/-	WTP	F7	+/+	+/+	WTP
B8	-/-?	+/+	WTP	F8	+/+	+/+	WTP
B9	-/-	+/+	WTP	F9	+/+	+/+	WTP
B10	+/-	+/-	WTP	F10	+/+	+/+	WTP
B11	+/-	+/-	WTP	F11	+/+	+/+	WTP
B12	-/-	+/+	WTP	F12	+/+	+/+	WTP
C1	wb?	+/-	WTP	G1	+/+	+/+	WTP
C2	+/+	-/-	P	G2	+/+	+/+	WTP
C3	-/-	+/+	WTP	G3	+/+	+/+	WTP
C4	-/-	+/+	WTP	G4	+/+	+/+	WTP
C5	+/-	+/-	WTP	G5	+/+	+/+	WTP
C6	+/-	+/-	WTP	G6	+/+	+/+	WTP
C7	+/-	+/-	WTP	G7	+/+	+/+	WTP
C8	+/-	+/-	WTP	G8	+/+	+/+	WTP
C9	wb	-/-	P	G9	+/+	+/+	WTP
C10	+/-	+/-	YL	G10	+/+	+/+	WTP
C11	+/-	+/-	WTP	G11	+/+	+/+	WTP
C12	+/-	+/-	WTP	G12	+/+	+/+	WTP
D1	+/-	+/-	WTP	H1	nd	+/+	WTP
D2	-/-	+/+	WTP	H2	+/+	+/+	WTP
D3	+/+	-/-	P	H3	+/+	+/+	WTP
D4	+/-	+/-	WTP	H4	+/+	+/+	WTP
D5	+/-	+/-	WTP	H5	+/+	+/+	WTP
D6	-/-	+/+	WTP	H6	+/+	+/+	WTP
D7	+/-	+/-?	P	H7	+/+	+/+	WTP
D8	+/-	+/-	WTP	H8	+/+	+/+	WTP
D9	+/-	+/+	WTP	H9	+/+	+/+	WTP
D10	+/-	+/+	WTP	H10	+/+	+/+	WTP
D11	+/-	+/+	WTP	H11	+/+	+/+	WTP
D12	nd	+/+	WTP	H12	+/+	+/+	WTP

Table 5: Genotypes and phenotypes of *polIB-2 oex1-1* plants, generation F2. (--), homozygous; (++), wild-type; (+-), heterozygous; P, *oex1* phenotype; nd, no PCR band; WTP, Wild-type phenotype; sm, small size; YL, yellow leaves: ?, not sure for PCR band.

No	Genotype (*OEX1/POLI1B)*	Obtained	Expected
1	EE (+-/+-)	26	24
2	EW (+-/++)	4	12
3	WE (++/+-)	3	12
4	EO (+-/--)	0	12
5	OE (--/+-)	0	12
6	WW (++/++)	49	6
7	WO (+-/--)	4	6
8	OW (--/++)	9	6
9	OO (--/--)	0	6

Table 6: Segregation of *pol1B-2 oex1-1* plants at the F2 generation. Comparison between the expected segregation results and the experimental results. (--), homozygous; (++), Wild-type; (+–), heterozygous

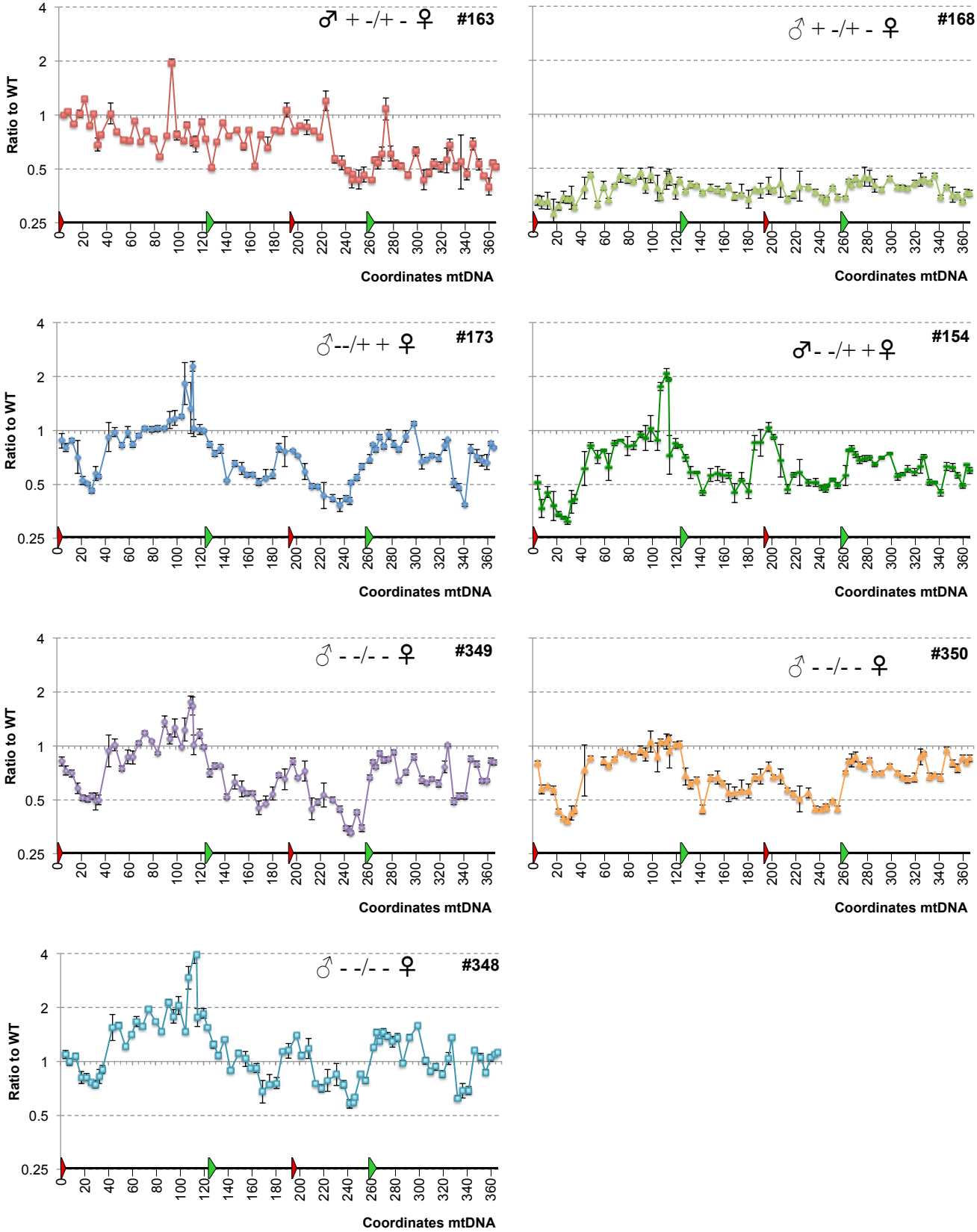


Figure 39: Changes in the copy number and stoichiometry of mtDNA sequences in *pol1A-1 oex1-1* mutant plants. The mtDNA was scanned for differences in copy number as described in **Figure 34**. The linear representation of the Col-0 mtDNA is displayed below the graphics, with the coordinates in kilobases and the two pairs of large repeated sequences LR1 and LR2 (green and red harrowheads).(+ +), Wild-type; (+ -), heterozygous; (--), homozygous; In this study the pollen from *oex1-1* plants was used as male ♂ and *pol1A* plants used as a female ♀. The results show significant overall decrease of the mtDNA copy number in bushy plant #168, but no significant differences between single homozygous *oex1-1* plants (#173 and #154) and double homozygous *pol1-2 oex1-1* mutant plants (#348, #349 and #350)

Figure 40: Phenotypes of plant analyzed by qPCR for mtDNA and cpDNA copy number. *pol1A-1* used as female while pollen of o*ex1-1* used as male

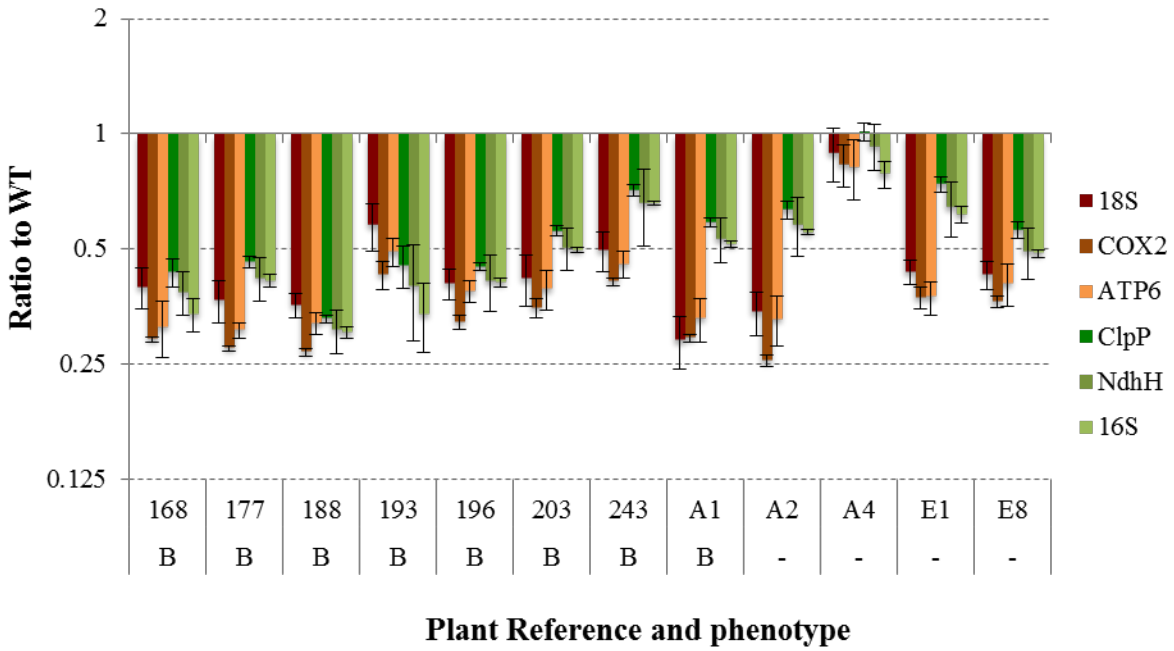


Figure 41: Copy numbers of mtDNA and cpDNA in plants with bushy phenotype. Copy numbers were standardized against two nuclear reference genes: the actin gene *ACTIN* and the polyubiquitin gene *UBQ10*. (E), heterozygous; (O), homozygous; (W), Wild-type.

(At4G05320). The results revealed that the copy number of the *18S rRNA*, *COX2*, and *ATP6* gene sequences from the mtDNA and *ClpP*, *NdhH* and 16S rRNA gene sequences from the cpDNA decreased up to 0.5 fold (Figure 41). It is possible that the decrease in copy number of the organellar genomes is linked to the bushy phenotype of those plants. But these are still preliminary results, and further studies have to be done to follow the segregation of the molecular and visible phenotypes in the progeny of these plants.

Conclusion

The mitochondrial genome of plants is large and more complex as compared to mammalian or fungi mitochondrial genomes, due to recombination activities that can create complex structures. OEX1 is targeted to mitochondria and chloroplasts, while OEX2 seems to be solely targeted to plastids. The *OEX1* gene codes for two alternative proteins, OEX1a and OEX1b, by alternative splicing. Both alternative protein forms cannot recognize ssDNA as substrate, but can degrade dsDNA. OEX1 is involved in recombination processes that if unchecked, as in the *oex1* mutant, can generate circular subgenomic molecules from the main mtDNA that apparently replicate independently from the rest of mtDNA. These sub-genomes contain highly expressed mitochondrial genes, whose transcription might be linked to the replication of the circular sub-genomes. Apparently because of these mtDNA rearrangements the *oex1-1* mutants are severely affected in their development and have very reduced fertility. However the chloroplast genome remains stable in all *oex1-1* mutants. Double homozygous mutants of *pol1A-1 oex1-1* have severe visible phenotypes but at the molecular level are not significantly more affected than the single *oex1-1* homozygote plants.

DISCUSSION

The molecular strategy established in the host laboratory (Val, Wyszko et al. 2011) brings a key advance in the study of mitochondrial regulation and in the manipulation of mitochondrial genetics. Until now, the lack of transformation methodologies restricted the study of RNA expression and metabolism to *in vitro* or *in organello* analyses. Through the PKTLS-driven import of specific cargo RNAs into the organelles, it is now possible to perform direct *in vivo* analyses. It has been reported that about half of the sequences in plant mitochondrial genomes are not known in origin and functions (Kubo and Newton 2008). Surprisingly, a maximum of these sequences are transcribed, including also aberrant new sequences associated with CMS and coding for chimeric polypeptides. This is the case for maize S-CMS, which is associated with the presence of specific mitochondrial transcripts carrying two open reading frames, *orf355* and *orf77* (Gabay-Laughnan, Kuzmin et al. 2009; Matera, Monroe et al. 2011; Vančetović, Ignjatović-Micić et al. 2013). The expression of these transcripts is complex and occurs in both sense and antisense orientation. Moreover, *orf77* (234 nucleotides) is a chimera combining regions of the mitochondrial *atp9* gene with atypical sequences (Su, Song et al. 2016). It is suspected to be the main cause of S-CMS, potentially through competition with *atp9* at the RNA or protein level. Because ATP9 is essential in energy metabolism of the mitochondria, it has been suggested that the *orf77* chimeric sequence causes malfunction of ATP9 (Hanson and Bentolila 2004). CMS is commonly exploited in maize hybrid breeding to avoid extra labor for artificial emasculation and possible hybrid contaminations. For these reasons, different laboratories are trying to understand the molecular mechanism of S-CMS in maize.

Using the PKTLS shuttle, we imported the maize S-CMS *orf77* RNA with its 5'- and 3'-UTR into mitochondria in *A. thaliana* plants. We then studied the effect of the *orf77* at the level of the organellar transcriptome at different developmental stages or in different growth conditions, with the long term prospect to better understand the mechanism of S-CMS and to generate CMS in non-CMS plants. Oxidative phosphorylation chain complexes consist of subunits encoded by the mitochondrial genome and subunits expressed by the nuclear genes. In this way, building the oxidative phosphorylation chain needs coordination between the nuclear and mitochondrial genomes, which is performed by the anterograde and retrograde regulatory pathways in plants.

For this reason, we started to extend to nuclear genes the effects of the expression and mitochondrial import of *orf77*-PKTLS.

Mitochondrial transcripts of the S-CMS-associated open reading frames *orf355* and *orf77* were detected from the early stages of microspore development in maize (Wen and Chase 1999). However, in our studies, expression and mitochondrial targeting of the *orf77*-PKTLS RNA affected the levels of very few mitochondrial RNAs at the early and intermediate stages of transgenic plant development in the light. Such observations are in agreement with the fact that generally CMS plants show no phenotype at early and intermediate stages of development. Conversely, mature plants expressing the *orf77*-PKTLS RNA showed a significant variation in the levels of many mitochondrial RNAs, with as much as 19 RNAs affected at day 3 after transgene induction. The response was sequential, as the RNAs affected mostly differed from one day to the other, but each day the concerned set included RNAs for subunits of mitochondrial complexes, RNAs for factors involved in the biogenesis of the complexes and RNAs of the mitochondrial translation system (ribosomal RNAs and RNAs encoding ribosomal proteins). This suggests a cascade effect of interdependent and coordinated functions, although the variations of the mRNAs encoding Complex I subunits were not necessarily in the same direction: *e.g.* at day 3 *nad1b* and *nad5a* increased in contrast to *nad2b, nad4, nad5b*, *nad6* and *nad9*, which decreased. Strikingly, whichever the day after induction, the vast majority of these RNAs was negatively affected, but with no observable phenotype, raising the question of the relative importance of the mitochondria at that developmental stage. On the other hand, the mature plants used in our studies were actually reaching the beginning of the flowering stage. One might thus speculate that the impairment of mitochondrial RNAs that we observed in our plant samples, which are essentially composed of leaf tissue, are somehow representative for the molecular events that would subsequently occur in the flowers of S-CMS plants and lead to male sterility. One prospect of the present studies is thus to induce the expression of the *orf77*-PKTLS RNA at different stages of the flowering process and to analyze the impact on mitochondrial RNA levels in the inflorescences.

Altogether, our results do not strengthen the idea that the *atp9* mRNA would be the main target of *orf77* in S-CMS, as this mRNA was only affected in light-grown mature plants at days 2, 3 and 4 after induction. The level of *atp9* decreased at day 2, in parallel with that of *atp4*, suggesting an effect of one mRNA on the other mRNA of the same complex. In earlier studies of

our laboratory, when the level of *atp9* mRNA was knocked down through mitochondrial import of a specific *trans*-ribozyme-PKTLS RNA, the level of *atp4* was also strongly affected (Niazi 2013). However, the level of *apt9* increased at day 3, possibly to compensate the effect of day 2, and decreased again at day 4, with no effect on the level of other mRNAs coding for ATP synthase subunits.

In dark-grown seedlings, the response to the induced expression of the *orf77*-PKTLS RNA was much more limited at day 3 than in the case of the mature light-grown plants. Significant differences also appeared in the sets of responding mitochondrial RNAs and the distribution between RNA increase and decrease was better balanced, suggesting that the pattern of regulation depends on the growth conditions. Nevertheless, the general comments made on the response of mature light-grown plants mostly also apply to dark-grown seedlings. Notably, neither *atp9*, nor the RNAs for other subunits of the ATP synthase were affected in dark conditions, which again disagrees with the idea that *atp9* would be the primary target of *orf77*.

In these studies, we also followed the variations in the level of nuclear mRNAs, and especially those encoding the mitochondrial alternative oxidase. The AOX provides a non-phosphorylating by-pass in the mitochondrial respiratory chain and it is known in particular that the level of AOX increases under stress conditions (Clifton, Lister et al. 2005). Remarkably, expression and mitochondrial targeting of the *orf77*-PKTLS RNA affected the level of the *AOX1d* nuclear mRNA at all developmental stages of light-grown plants, as well as in dark-grown seedlings. It thus seems that in all conditions the presence of the *orf77* RNA in mitochondria might somehow trigger a retrograde signaling and that the AOX would be part of the anterograde response. Surprisingly, when the levels of *atp9* decreased at day 2 in light-grown mature plants, the *AOX1d* nuclear mRNA also decreased, while the level of *AOX1d* would rather be expected to increase, so as to compensate for a possible malfunction of the ATP-synthetase.

As a whole, our experiments show that the *A. thaliana* mitochondrial transcriptome is very little disturbed by the targeting of the *orf77* RNA into the organelles at the first stages of plant development. These results reinforce the conclusions of another work performed in our laboratory by Adnan Khan Niazi. As mentioned above, in this case *trans*-ribozymes designed to specifically knock down individual RNAs were targeted into the mitochondria of transformed *A. thaliana* plants thanks to the PKTLS shuttle. The expected knockdown turned out to be

impossible at the early developmental stage and was rapidly counteracted at the intermediate stage. On the contrary, an efficient knockdown was obtained starting from the mature plant stage and beyond. It thus appears that the plant mitochondrial transcriptome might be controlled by a "buffering" mechanism at the first stages of development, as mitochondrial functions are essential at that point.

The next task in these experiments is to determine whether the mitochondrial targeting of the *orf77* RNA is able to generate male sterility. In a preliminary step, we analyzed the effects of long term induction of *orf77*-PKTLS expression on the phenotype and fertility of the plants. For this, *orf77*-PKTLS expressing plants and control plants were grown on solid agar plates and β-estradiol in liquid growth medium was applied every four days to keep permanent expression of the transgene. In this way, the plants were kept induced until the flowering stage and silique formation. When compared to control plants, plants expressing permanently the *orf77*-PKTLS RNA had a significantly lower wet weight yield and showed a 50% reduced number of siliques. This suggests a possible effect of the *orf77* RNA on fertility in the transformed *A. thaliana* plants. To draw further conclusions, we are now preparing transgenic plants of *A. thaliana* expressing the *orf77*-PKTLS RNA under a constitutive promoter.

In relation with the problem of mitochondrial genetic diversity, the second part of the PhD project was devoted to the characterization of the plant organellar recombination mechanisms and factors, with a special interest for plant mitochondrial 5' to 3' exonucleases. DNA double-strand breaks (DSB) can be repaired by several mechanisms, including Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR). While NHEJ seems to be the predominant mechanism of repair in the nucleus of eukaryotes, repair by recombination is the main process in prokaryotes (Pitcher, Wilson et al. 2005). HR-dependent repair also seems to be predominant in plant organelles, probably because of the prokaryotic origin of mitochondria and plastids. In bacteria, the first step in HR-dependent repair of DSB is the resection of the DNA extremities (Figure 21). The RecBCD complex unwinds the double stranded DNA and generates 3'- single stranded ends through resection (Azeroglu and Leach 2017). This complex contains both 5'-3' exonuclease and helicase activities. A 5'-3' exonuclease activity is also involved in certain steps of repair of modified bases by the Base-Excision Repair (BER), Nucleotide Excision Repair (NER) or Mismatch Repair (MMR) pathways, although up to now only certain

types of BER and maybe of MMR have been reported to exist in plant organelles (Kimura, Tahira et al. 2004).

In plant mitochondria, the 5'-3' exonuclease proteins involved in this processes are unknown. Comparing bacterial DNA polymerase I and *Arabidopsis thaliana* DNA polymerases IA and IB sequences (POLIA and POLIB), we have found that the plant organellar DNA polymerases lack the 5'-3' exonuclease domain present in bacterial DNA Pol I (Figure 22). The OEX1 and OEX2 Arabidopsis proteins are homologous to such a domain and we have shown that OEX1 is targeted into both chloroplasts and mitochondria, while OEX2 is only localized into chloroplasts. Biochemical activity tests revealed that both alternative forms of OEX1 can degrade different DNA structures containing double stranded DNA regions, suggesting that indeed the protein has exonuclease activity.

It is possible that plant OEX are the nucleases required for the resection of DNA extremities during repair of DSBs in the organelles, or for transforming nicks in the DNA into gaps that can be repaired by DNA polymerase. To better understand the role(s) of OEX1 we have analyzed Arabidopsis T-DNA insertion mutants. Mutant plants for OEX1 are severally affected in their development, showing that OEX1 has important functions. Different pleiotropic phenotypes were observed: wrinkled mottled leaves, growth retardation, reduced fertility and sterility. Similar phenotypes were described in the mutants of *recA3, msh1, osb1* and *osb4,* but with low penetrance or only in late mutant generations (Gualberto and Newton 2017). Recently it has been shown that no phenotype was observed in homozygous *recG1* plants, even in the T5 generation. However, double mutants *recG1-1 recA3-2* (Wallet, Le Ret et al. 2015) had similar phenotypes as the homozygous *oex1* mutant. The wrinkled and mosaic phenotypes can affect the whole plant or sometimes only parts of it. As mitochondrial dysfunction affects chloroplasts (Woodson and Chory 2008; Van Dingenen, Blomme et al. 2016), the mutants often display chlorotic strips. It is possible that the mottle leaves, variegation and sterility phenotypes are linked to the expression of genes essential for oxidative phosphorylation. Variegated leaves phenotypes have been observed in several other *Arabidopsis* mutants lessened in mtDNA recombination process (Kühn and Gualberto 2012). Looking at molecular phenotypes, we have shown that Arabidopsis *oex1* mutants gained alternative mtDNA sequences due to recombination between short interspersed repeats, showing that OEX1 is involved in mitochondrial recombination processes. In normal growth conditions the copy number of some parts of the mtDNA in *oex1-1* mutant plants can

increase up to 4.5 fold in comparison to the WT mtDNA. Circular PCR and sequencing results revealed that different regions that are amplified in *oex1-1* plants apparently are circular subgenomic molecules generated by recombination involving direct repeats (ex. repeats A, EE and L), and such circular subgenomes contain highly expressed genes. Similar results reporting a circular episome containing the highly expressed *ATP1* gene were recently reported, for the *reG1* mutant (Wallet, Le Ret et al. 2015). The hypothesis that the developmental phenotypes of *oex1* plant are due to reduced expression of oxidative phosphorylation genes because of mtDNA recombination still needs to be confirmed by other methods, such as whole mitochondria transcriptomic analysis by qRT-PCR and western blots. However, such analysis is difficult, because changes are different from plant to plant, and little material can be recovered from single severely affected plants.

OEX1 is targeted to both mitochondria and chloroplast as shown by expression of a GFP fusion protein. It was thus expected that OEX1 have similar roles in both organelles and should affect the maintenance of both the mtDNA and cpDNA. But surprisingly in all *oex1-1* mutants plants tested no effect was detected in the stoichiometry of the different cpDNA regions as compared to the wild type, apart a minor increase of some sequences. Similarly, RECG1 is targeted to both organelles, but no effect on the copy number of cpDNA regions was observed in *recg1* plants (Wallet, Le Ret et al. 2015).

Arabidopsis thaliana POLIA and POLIB are also dual targeted and T-DNA insertion in these genes has a minor effect on the cpDNA: and overall slight reduction in copy number (Cupp and Nielsen 2013). In contrast double mutants of *why1 why3* have rearrangements in the cpDNA due to recombination involving microhomologies (Marechal, Parent et al. 2009). One possible reason for the absence of detectable effects in the cpDNA of *oex1-1* plants is that dual targeting proteins have more drastic effects on one location as compare to the other (Carrie and Whelan 2013). The second possibility could be that the deficiency in OEX1 is complemented by OEX2, which possibly is redundant with OEX1 for chloroplastic functions. Finally, it is possible that the effects of OEX1 on the cpDNA are not revealed because the cpDNA of Arabidopsis has no interspersed repeats that could be major sites of homologous recombination.

The *OEX1* gene is expressed as two alternative proteins by alternative splicing, OEX1a and OEX1b, which differ by the presence or absence of 18 amino acids (Figure 27). The shortest

protein is better aligned with the exonucleases domain of bacterial DNA pol I, but the extension is conserved in the OEX1 sequence of other plant species. It would be interesting to test if the two proteins have differential effects in genome maintenance. The preliminary *in vitro* results didn't reveal any differences, but is possible that the proteins interact with other factors that differentially modulate their activities. Complementation of the *oex1* mutant with OEX1a or OEX1b sequences are planned fused or not to a tag epitope, and the studies will be continued with proteomic identification of potential protein partners by co-immunoprecipitation.

Double mutant plants were also obtained and analyzed. The double homozygous *pol1A-1 oex1-1* mutants showed severer phenotypes than the individual *oex1-1* mutants, suggesting a synergetic effect of the *polIA* mutation on *oex1*. Same types of phenotypes have been observed in double mutants of *recA3 msh1, osb1 msh1* and *recG1-1 recA3-2* (Arrieta-Montiel, Shedge et al. 2009; Wallet, Le Ret et al. 2015) respectively. As is could not be possible to obtain *pol1A pol1B* double mutants, it's convincing that these proteins are essential and have redundant functions, possibly in the replication of the organellar genomes.

Several double heterozygous *pol1A-1 oex1-1* mutants showed a "bushy" phenotype. Up to now no such phenotype has been described associated to organellar dysfunction. Molecular analysis of these plants revealed that they have reduced copy numbers of the mtDNA and cpDNA. At present it is not possible to affirm that this change in organellar copy number is responsible for the phenotype, because the phenotype was not always observed in double heterozygous plants. Therefore it cannot be excluded that the developmental phenotype results from a mutation that is not linked to *polIA* or *oex1*. The study of the segregation of the bushy phenotype in the progeny of these plants will eventually answer that question.

MATERIALS AND METHODS

Materials

Arabidopsis plants *(Arabidopsis thaliana* ecotype *Colombia* Col-0) were used for agro-transformation by floral dip (Zhang, Henriques et al. 2006). The selected transformants were 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 days to synchronize germination.

Germination medium: 4.3 g/L micro and macroelements M0255 (DUCHEFA), 10 g/L agar, 1% (w/v) sucrose, pH 5.7

I.1 Bacterial strains

- Escherichia coli

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

Rosetta^{TM}(DE3): *F- ompT hsdS_B(r_B m_B) gal dcm (DE3) pRARE (Cam^R)*

The *E. coli* strains were cultivated in **LB medium**.

- Agrobacterium tumefaciens

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, Hirsch 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.2 Plasmid vectors

- pUCAP

This 2.7 kb plasmid derives from plasmid pUC18 following the addition of the rare restriction sites AscI and PacI 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 is a 3 kb high copy number plasmid used for cloning and sequencing. 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. The vector is linearized and has 3'-terminal protruding thymidines at both ends. This greatly improves the ligation efficiency of a PCR product into the plasmid and prevents recircularization of the vector. Bacteria transformed with this vector are resistant to ampicillin. The vector contains T7 and SP6 RNA polymerase promoters flanking the multiple cloning region, allowing *in vitro* transcription of the cloned sequences.

- pER8

The binary plasmid pER8 of 11.5 kb is used for transformation of *A. thaliana* via *A. tumefaciens*. This vector has the cassette for inducible expression of a 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 β-estradiol (Zuo, Niu et al. 2000). The presence of the spectinomycin resistance gene permits the selection of transformed *E. coli* and *A. tumefaciens*. The hygromycin phosphotransferase gene (*hpt*) under the control of the promoter of the nopaline synthase gene (NOS) lets the selection of the transformed plant cells.

- pET-28a

The pET system is a powerful system developed for the cloning and expression of recombinant proteins in *E.coli*. The *E.coli* strains DH5α cell (DE3) cells were transformed with the recombinant plasmid pET28a vector containing the gene of interest. The vector pET28a containing the sequence coding for the protein OEX (pET28a -OEX) has been used for the experiments of expression of the protein. This vector allows expression of a protein with a

pattern hexa histidine amino-terminal, under the control of the promoter of RNA polymerase of the T7 phage. It provides resistance to kanamycin and carries the gene coding for a repressor of the Lac promoter, LacI allowing to inhibit the basal expression of the gene encoding RNA polymerase T7 in the bacterial strain Rosetta.

I.3 Primers

Oligonucleotides were synthesized by Sigma-Aldrich and Eurofins. Primer sequences are presented in respective tables. Their fusion temperatures (Tm) are given by the manufacturer and can be also calculated with the MacVector® software. For high salt concentrations the Tm can be estimated by the following formula:

Tm (°C) = 2 x (A+T) + 4 x (C+G).

I.4 Informatics tools

Sequence alignments

Sequence alignments were realized with MacVector® (MacVector Inc.) with the ClustalW algorithm, implemented in the MacVector® software.

Phylogenetic analysis

Bacterial and plant sequences were identified in the databases using the *E. coli* DNA polymerase I sequence as query. An alignment was constructed with ClustalW implemented in the MacVector package using the GonneT matrix. Phylogenetic trees were built with the PhyML v3.1 online software (www.phylogeny.fr) using the neighbor-joining method implemented in the BioNJ program and 100 bootstrap support (Dereeper, Guignon et al. 2008).

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/

- The European Bioinformatics Institute: https://www.ebi.ac.uk/gxa/home

- http://LinRegPCR.nl

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/

Organellar Genome DRAW (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 Gene Bank format entries. The program is specially optimized for the display of mitochondrial genomes (Lohse, Drechsel et al. 2007; Lohse, Drechsel et al. 2013). We provided the Gene Bank accession numbers.

The site used to retrieve RNA expression level is:

https://www.ebi.ac.uk/gxa/about.html

The Expression Atlas provides information on gene expression patterns under different biological conditions such as a gene knock out, a plant treated with a compound, or in a particular organism part or cell. It includes both microarray and RNA-seq data. The data is re-analysed in-house to detect interesting expression patterns under the conditions of the original experiment.

Designing primers for quantitative PCR

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

Quantification of DNA sequences and genes expression by qPCR and RT-qPCR

The second derivative maximum method was used to determine Cp values, and PCR efficiencies were determined from DNA serial dilution curves or using LinRegPCR software (http://LinRegPCR.nl). Three technical replicates were performed for each experiment. For quantification of mtDNA and cpDNA copy numbers, a set of primers located along the organellar genomes was used, and results were normalized against nuclear genes ACT1

(At2G37620) and UBQ10 (At4G05320). For RT-qPCR, results were normalized against the expression of *ACT2* (At3g18780) and *GAPDH* (At1g13440).

Evaluating genes expression by GraphPad Prism version 7.01

- https://graphpad.com/

GraphPad is a statistical software that was used to calculate the significant and non-significant difference in expression of genes, from RT-qPCR results. Correlation analyses, one-way analysis of variance (ANOVA) or one-way ANOVA (with multiple comparison tests) were used. *p*-values <0.05 were considered statistically significant.

Methods

I.5 Techniques related to nucleic acids

I.5.1 Extraction of mitochondrial nucleic acids

To extract mitochondrial nucleic acids, 150 µL of Trizol reagent (Invitrogen) are added to a quantity of mitochondria equivalent to 200 µg of proteins. 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. After centrifugation and washing with 70% ethanol the pellet is resuspended with an appropriate volume of water.

I.5.2 Extraction of mitochondria

Every steps of the extraction are done at 4°C. The 25-30 days old seedling are filtered and then crushed in a mortar with 50 mL of grinding buffer. The suspension is filtered through a 50 µm mesh filter and one layer of pre-wet Miracloth into a conical flask. The remaining solid material is crushed a second time in 30 mL of grinding buffer and then filtered. The homogenate obtained is distributed between 50 mL of Falcon tubes and centrifuged 5 min at 2450 g to remove the cell debris. The supernatant is transferred into new tubes and centrifuged again at 17400 g. The crude mitochondria pellet containing the mitochondria and a number of contaminations (broken mitochondria, peroxisomes etc.), is suspended and both centrifugation steps are repeated. The pellet is resuspend in 1 mL of 1x washing buffer with BSA and carefully layered over a percoll gradient and centrifuged for 40 minutes at 40000 g with break off. Mitochondria is carefully recovered from the 50% percoll interphase. The collected mitochondria is distributed into 4 tubes and diluted with 30 mL of 1x washing buffer with BSA and centrifuged at 31000 g for 15

minutes. The final mitochondria pellets are suspended in two tubes filled with 1x bushing buffer without BSA and centrifuged at 31000 g for 15 minutes. Mitochondria is finally recovered in Eppendorf tubes in a very small volume.

Grinding buffer: sucrose 0.3 M, Tetrasodium pyrophosphate 25 mM, EDTA 2 mM, KH_2PO_4 10 mM, PVP-40 1% (w/v), BSA 1% (w/v), pH 7.5, Sodium ascorbate 20 mM, L-cyctein 5 mM.

Washing buffer with BSA: Sucrose 0.6 M, MOPS 20 mM, BSA 0.2% (w/v), pH 7.5.

Washing buffer without BSA: Sucrose 0.3 M, MOPS 20 mM, pH 7.5.

Percoll Gradients

For 50%: Wash Buffer 2 X 5 mL, percoll 5 mL

For 25%: Wash Buffer 2 X 7.5 mL, percoll 3.75 mL, water 3.75mL

For 18%: Wash buffer 2 X 2.5mL, percoll 0.9 mL, water 1.6 mL

Heavy solution PVP-40 : Washing buffer with BSA 2X 17,5 mL, Percoll 9,8 mL , PVP-40 20% (P/v) 7.7 mL.

Light solution PVP-40 : Washing buffer with BSA 2X 17.5 mL, Percoll 9.8 mL, H_2O 7.7 mL.

I.5.3 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

I.5.4 Separation of nucleic acids on agarose gels

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 100-150 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 sizes are 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

I.6 Specific DNA techniques

I.6.1 Extraction of total plant DNA by the CTAB method

Approximately 3 g of plant tissue 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 an equal volume of chloroform, the suspension is mixed gently and centrifuged at 3000 g for 7 min. The supernatant is mixed gently with an equal volume of isopropanol. After incubating 10 min at room temperature, the DNA is pelleted by centrifugation 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

I.6.2 Extraction of total DNA by Edwards method

Approximately 20 mg of plant tissues in 96-well plates are used for extraction of total DNA using Edwards method (Edwards, Johnstone et al. 1991). Take half of a young dry leaf and cut it into small pieces then were ground in liquid nitrogen in 96 well plates with beads in tissuelyser machine until became powder then added 400 µL of the extraction buffer in each well. Spin at 15000 g for 3 minutes. Transfer 300 µL of supernatant into a new 96-well plate and add an equal volume of isopropanol and mix gently by inversion. Place the plate at RT for 5 min for incubation. Spin the plate at 15000 g for 5 minutes and discard the supernatant. Wash the pellet with 500 µL of 70% ethanol and spin for 2 minutes. Discard the ethanol and dry the pellet at RT and resuspend pellet in 50 µL of water. 0.5-1 µL can be used for standard PCR.

Extraction buffer: (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS).

I.6.3 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.

I.6.4 DNA purification after gel electrophoresis

After electrophoretic fractionation of the DNA, slices containing the DNA fragments of interest are excised from the agarose gels. The gel piece is solubilized and the DNA purified, with a kit based on the binding of the DNA to a silica membrane spin column (Nucleospin System®, Macherey-Nagel).

I.6.5 Amplification by Polymerase Chain Reaction (PCR)

PCR sets in motion a chain reaction in which the DNA template is exponentially amplified by a thermo resistant DNA polymerase (usually *Taq* DNA polymerase), using two primers specific to the extremities of the fragment of interest.

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 (10 pg - 10 ng of plasmid DNA or 1-10 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 Biometra T3000 Thermocycler.

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)

I.6.6 Amplification by Phire Hot Start II DNA Polymerase

For genotyping of plant mutants the Phire Hot Start II DNA Polymerase was used.

For 10 µL PCR set up:

DNA template (1 pg–10 ng)

0.5 µM of each primer

0.2 µL of dNTPs

2 µL of 5X PCR buffer

0.05 µL Phire Hot Start II DNA Polymerase

The reaction is carried out for 30 cycles in a Master Cycler (Eppendorf):

Step 1 (1 cycle):	98°C, 30 sec (template denaturation)
Step 2 (29 cycles):	95°C, 5 sec (template denaturation)
	58°C, 10 sec (primer hybridization)
	72°C, 90 sec (DNA elongation)
Step 3 (1 cycle):	72°C, 1 min (DNA elongation)

I.6.7 Reverse transcriptase Quantitative PCR (RT-qPCR)

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is performed in 384-well optical plates LightCycler 480 II (Roche) 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 cDNA template. 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.

I.6.8 Quantitative Polymerase Chain (qPCR) Reaction

qPCR assays were performed in a LightCycler 480 II (Roche), in 6 µL of reaction containing 3 µL LightCycler 480 SYBR Green Master Mix (Roche) and 0.5 µM of each primer. For each sample, qPCR is performed in triplicate using fixed amounts of DNA template. The thermo cycling program was as follows: a 7-min denaturing step at 95°C, then 40 cycles of 10s at 95°C, 15s at 58°C, and 15s at 72°C.

I.6.9 Absolute quantification of specific DNA

Purified plasmid clones (plasmid **pER8** and **PKTLS** gene) were quantified using the Qubit 3.0 fluorometer and Qubit dsDNA HS quantification kit. With the molecular weight of the plasmid and insert known, it is possible to calculate the number of copies as follows:

Number of copies = (amount (ng)*$6.022x10^{23}$) / (length*$1x10^{9}$*650)

- ng is the amount of DNA (plasmid)
- 6.022×10^{23} = Avogadro's number
- Length is the length of DNA fragment and PKTLS gene in base pairs.
- Multiply by 1×10^{9} to convert our answer to nanogram
- Number = (ng* number/moles) / (bp* ng/g*g/ mole of bp)

Knowing the copy number and concentration of plasmid DNA, the precise number of molecules added to subsequent real-time PCR runs can be calculated, thus providing a standard for specific cDNA quantification.

Real-time PCR runs were performed in 384 well optical plates in triplicate (each containing 2× PCR Sybrgreen master mix (Roche), 0.25 pmol/µL of forward primer, reverse primer and 1 µL template DNA (plasmid DNA of PKTLS dilutions ranging from $1:10^1$ to $1:10^6$ in a final volume of 10 µL), for 45 cycles using a LC480-II instrument (Roche). Default LC480-II cycle conditions were as follows; 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. A standard curve was drawn by plotting the threshold cycle (C_T) against the natural log of the template concentration (i.e. number of molecules). The C_T was defined as the cycle at which a statistically significant increase in the magnitude of the signal generated by the PCR reaction was first detected. C_T was calculated under default settings for the real-time LC480 software 1.5.1 (Roche) and the 2^{nd} maximal derivative method. The equation drawn from the graph was used to calculate the precise number of copies of PKTLS in our total RNA fraction and mitochondrial RNA fraction, tested in the same reaction plate than the standard used for the construction of the standard curve ("Standard curve in run").

PKTLS DNA standard was diluted within the wells of 384 optical plates in triplicate to a concentration range of 10^{-4} to 10^{-9}ng.µL^{-1}. Each plotted point is the average of triplicate fluorescence values for every standard concentration measured. Log concentration corresponds to the DNA dilution and crossing point indicates the CT-values.

Linear equation: $y= 0.0327x-1.8695$, $R^2=0.9988$

I.6.10 Statistically analysis

Statistically analysis is carried out using GraphPad Prism version 7.01.

One way ANOVA, multiple comparison and Tukey test were used to calculate the P values (Simonsen, Cumming et al. 2008).

I.6.11 Cloning techniques

I.6.11.1 Preparation of plasmid DNA

After overnight culture of bacteria at 37°C in **LB medium**, plasmid DNA is extracted from 2 mL of culture using the NucleoSpin system® (Macherey-Nagel) kit. By this method, up to 40 µg of

plasmid can be obtained. The technique is based on the optimization of the purification by alkaline lysis method (Birnboim and Doly 1979). DNA is eluted from 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

I.6.11.2 Digestion of DNA by restriction enzymes

To linearize or fragmentize DNA, restriction enzymes such as XhoI, SacI, SalI and SpeI 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.

I.6.11.3 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 volume 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.

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

I.6.11.4 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 purified from gel 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.

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

I.6.11.5 Traditional cloning by restriction enzymes and ligase

Restriction enzyme DNA digestions are performed following manufacturer recommendations. After digestion, DNA fragments of interest are eluted from agarose gels after electrophoresis,

using the "Extract II" kit (Macherey-Nagel). The purified DNA fragments are cloned into digested vectors using the "Rapid DNA Ligation Kit" (Fermentas) as described above.

I.6.11.6 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 bacteria transformation is induced by heat shock of 45 sec at 42°C, in a water bath. After addition of 1 mL **LB medium**, the bacteria suspension is incubated 1h at 37°C, to regeneration of the bacterial wall and expression of antibiotic resistance genes coded by the vector. Bacteria are spread on petri dishes, on LB medium containing 1.5% (w/v) agar and the appropriate antibiotic. For blue/white selection of bacterial colonies transformed with some vectors, IPTG (0.08 mM) and Xgal (10 µg/mL) are added to the media. After one night incubation 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 strain) 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 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 bacterial pellet 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.

I.6.12 Sequencing of DNA

The constructs (*orf77)* prepared are verified by sequencing using an automatic sequencer (Applied Biosystems, IBMP sequencing platform). The sequencing method used derived from that of (Sanger, Nicklen et al. 1992). The alignment of sequences is done with the MacVector® software.

I.7 Specific techniques for RNA

I.7.1 Extraction of total RNA

To extract total RNA from whole plants, the tissues are ground to a fine powder in liquid nitrogen and added of 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 vortex for 2 min. Incubation at room temperature for 5 min is followed by centrifugation at 16,000 *g* for 15 min at 4°C. The upper aqueous phase is recovered and 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.

I.7.2 Elimination of DNA from RNA samples

Before reverse transcription reactions, RNA samples are treated with RNase free DNase to eliminate contaminating genomic DNA. One µg of total RNA is 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, followed by ethanol precipitation. Two successive DNase treatments are usually necessary to eliminate the totality of contaminating DNA.

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

I.7.3 Reverse transcription (RT)

Reverse transcription is performed using different conditions described below.

I.7.3.1 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 random hexamer primers in a total volume of

12 µL in 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 100 mM DTT, 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. The reaction is then stopped by incubating at 70°C for 10 min. The samples are directly used for RT-PCR or RT-qPCR reactions.

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

I.7.3.2 RT using RevertAid™ Premium First Strand cDNA Synthesis Kit

The protocol recommended by the manufacturer (Fermentas) is followed. The reaction mix is as described above, except that 200 units of RevertAid™ Premium Reverse Transcriptase (Fermentas) is used. The samples are incubated for 10 min at 25°C, followed by 30 min at 50°C. The reaction is inactivated by incubating at 85°C for 5 min. The samples are directly used for RT-PCR or RT-qPCR reactions.

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

I.8 Specific techniques for proteins

I.8.1 Protein production and extraction

After heat shock transformation of bacterial Rosetta (DE3) strain, 50 mL cultures were grown. When the OD at 600nm was 0.4, protein expression was induced with 1 mM IPTG and cultures further incubated at 37°C for 2h. The cultures were centrifuged to obtain the bacterial pellet, which was then resuspended in lysis buffer. The suspended pellet was sonicated in a beaker, while keeping the beaker on ice. Sonication was performed for 30 sec at 20% amplitude followed by sonication at 30% and 40% amplitude until the sample became transparent. The sample was then centrifuged at 17000 g for 20 minutes and the supernatant was used as the soluble protein fraction.

Solution; 15 mL buffer 50 mM Tris-HCL pH 8.0, 300 mM NaCl, 5% glycerol (v/v)

I.8.2 Separation of proteins under denaturing conditions: SDS-PAGE

The theoretical molecular weight of a protein can be easily calculated from the amino acid composition in the case of a known sequence. This information is useful when the expression levels of recombinant proteins are estimated on polyacrylamide gels after electrophoresis under denaturing conditions in the presence of SDS (SDS-PAGE). By SDS-PAGE the proteins are fractionated according to their sizes, because the binding of SDS to the proteins gives them a uniform negative charge. The matrix meshes are defined by the ratio of polyacrylamide / bis-acrylamide (37:1) depending on the desired resolution and the size of the proteins of interest. The concentration gel at the top is at a concentration of 5% (w/v) acrylamide in 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS. This gel makes it possible to concentrate the proteins so that they enter uniformly into the separation gel. This is composed of acrylamide with a concentration between 8 and 15% (w/v) in a 375 mM Tris-HCl pH 8.8 buffer, 0.1% (w/v) SDS. The protein samples are supplemented with 1 volume of Laemmli 2x buffer and incubated for 5 min at 96°C before loading on gel. The migration is carried out in Tris-Glycine-SDS buffer with constant current of 25 mA for 30 minutes and then 1 h for 30 mA (for gels of 12 × 10 × 0.1 cm). After migration, the proteins can be visualized on the gel by staining for 30 min with Coomassie blue. The gel is then washed with the decolorizing solution until the background is clear. Prestained protein marker page ruler plus (Thermo Scientific) of 10 to 170 kDa was used to estimate protein sizes.

Laemmli 2x buffer: 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl, pH 6.8.

Coomassie blue staining: 10% acetic acid, 40% methanol, 0.04% bromophenol blue.

Wash buffer: 50% methanol, 7% acetic acid.

I.8.3 Protein quantification

The amount of protein is measured by Bradford test (Bradford 1976). This colorimetric method relies on the change in absorbance of the Bradford reagent from 450 nm to 595 nm when it binds to proteins. A commercial reagent (Bio-Rad protein assay dye reagent concentrate) was used. The test consists of mixing 200 µL of the commercial solution with 800 µL of protein solution diluted in water. The blank is made with the buffer used for protein extraction. After 5 min

incubation the protein concentration is estimated by measuring the absorbance at 595 nm and comparison to a standard curve established with known concentrations of BSA.

I.8.4 Protein purification by Immobilized metal ion affinity chromatography (IMAC) and gel filtration (GF)

The combination of IMAC and GF is often used for the purification of histidine-tagged recombinant proteins expressed in *E. coli*. IMAC is based in the interaction of proteins with histidine residues (or Trp and Cys) on their surface with divalent metal ions (e.g., Ni^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}) immobilized on chromatography beads via a chelating ligand. Hexa-histidine-tagged proteins have higher affinity in IMAC because of the multiple histidine residues and are usually the strongest binders among all the proteins present in a crude sample extract (e.g., a bacterial lysate), while other cellular proteins will not bind or will bind weakly.

OEX1a and OEX1b proteins were purified by IMAC and GF on HisTrap FF Crude 1ml column (GE healthcare) as follows:

The bacterial lysate was loaded on the column pre-equilibrated with Buffer A (50 mM Tris-HCl pH8, 300mM NaCl, 5%glycerol) containing 25mM imidazole. The column was washed with 15 mL of Buffer A plus 25mM imidazole and the protein was eluted by applying a 20 mL 25-500 mM linear gradient of imidazole, in 50 mM Tris-HCl pH8, 300mM NaCl, 15%glycerol. The cleanest fractions were pooled and concentrated by ultrafiltration on AMICON ULTRA MWCO 3kDa (Millipore). The concentrated protein were further purified on a Superdex 200 10/300 GL column (GE healthcare) run in Buffer A. Fractions corresponding to the major protein peak, at Ve=13,22 mL were pooled, analyzed by SDS-PAGE, aliquoted and stored at -80°C.

I.9 Stable transformation of *A. thaliana* plants by *A. tumefaciens*

I.9.1 Preparation of agrobacteria cultures

Agrobacteria strain GV-3101 is 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. The agrobacteria transformed with the constructions in binary vectors are further selected according to the antibiotic resistance given by the vector. A bacterial preculture is grown from a colony of transformed agrobacteria, cultivated for 12 to 20 h at 28°C in 3 mL of LB medium. The preculture is used to inoculate 500 mL of media, and grown at 28°C till an absorbance of 0.8 at

600nm. After centrifugation at 1500 g for 10 min the bacteria are resuspended in 10 mM $MgCl_2$. After a second sedimentation at 1500 g for 10 min the bacteria are resuspend 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

I.9.2 Transformation of *A. thaliana* plants by floral dip

The plant inflorescences are dipped for 90 seconds in the bacterial suspension in agro-infiltration medium. The plants are then placed in small green houses and put under obscurity for 48 hours. Then they are transferred to green houses (25°C, 16 h light and 8 h dark) till harvesting of the seeds.

I.9.3 Harvesting and selection of transformed seeds

Six to ten weeks later, it is possible to harvest the seeds. These are then sterilized, before growth in vitro and selection of transformants. For this, about 100 mL of 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 solution is removed and seeds are washed 5 times with absolute ethanol. The seeds are then dried in a laminar flow hood. The next morning, they are placed on large plates containing a **selection medium**.

Sterilization solution: 70% (v/v) ethanol, 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

I.9.4 Selection and regeneration of transformed plants

Seeds on plates are stratified in the dark at 4°C for 2-days to synchronize germination. They are then grown *in vitro* in a 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 big enough. Two weeks in mini growth chambers (25°C, 12 h light and 12 h dark) are needed to maintain humidity and

limit losses due to their adaptation from *in vitro* to green house conditions. When plants are large enough, to select the correctly transformed plants, a leaf is taken for DNA extraction and analysis by PCR and sequencing. 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. Four lines of construct-*orf77* and three lines of control plants were selected for further studies. For heterozygous plant lines, 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, 40 µg/mL hygromycin, pH 5.7

I.9.5 Crosses between two Arabidopsis plants

Arabidopsis is a self-pollinating plant. To obtain double mutants *pol1A oex1, pol1B oex1* a cross between the two mutant lines was performed. Since organelles are maternally transmitted in most plants the choice of the pollen donor is important. It allows studying the effect of the integration of a protein on mitochondria from mutant plants. For crosses, only flowers near about to open are kept on the floral stem. These flowers are emasculated taking care not to damage the pistil, and the mutants (*polIA-2* and *polIB-2*) are kept in the greenhouse at night so that the pistil finishes its maturation. In the morning, the pistil is brought into contact with a mature flower of the other mutant (*oex1-1*), to allow pollen deposition on the pistil. The siliques are collected as soon as they are matured and finally dry in an open Eppendorf tube.

I.10 Transfection by biolistic method

Biolistic transfection uses a particle gun device to project into the plant material tungsten or gold particles on which DNA is precipitated (Sanford, Smith et al. 1993; Klein 2010). Transfections are carried out on young leaves of *Nicotiana benthamiana* cut at the petiole and deposited with the ventral surface up in a petri dish containing solid germination medium. Twenty µL of a suspension of tungsten beads (mean diameter of 1 µm) at 30 mg/ml in 50% glycerol are supplemented with 10 µg of plasmid DNA. The whole mixture is vortexed energetically and added with 20 µL of 2.5 M $CaCl_2$ and 8 µL of 0.1 M spermidine. The miux is further vortexed for 30 min at 4°C. The particles are washed 3 times with 200 µL of absolute cold ethanol and finally taken up in 30 uL of ethanol. Biolistic transfection is carried out using a PDS-1000 / He™ device (Bio-Rad). The leaf on the petri dish plate containing germinating medium is placed in the

chamber of the apparatus, under vacuum (0.1 bar). Six µL of DNA-bearing particles suspension are deposited on a "macrocarrier" disc and projected onto the leaf at 1100 psi (calibrated rupture discs). The plates are then incubated in the dark overnight at RT. To observe the cells under the microscope, the areas of the leaf intact but close to the impacts of the bombardment are cut and placed on microscope slides, the upper face against the lamella. A drop of water is deposited on the underside and the leaf is gently scraped with the lamella to pierce the cuticle and facilitate observation. For these observations, the confocal microscope Zeiss LSM 700 was used.

Solid germination medium: MS0255 (Duchefa) pH 5.7; Agarose 0.9% (w / v); Sucrose 1% (w / v).

Table 7: Primers used in this work

Primers mtDNA

Coordinates on Col-0 mtDNA		Forward	Reverse
4234	4329	TCCTCCGGTCGAGTCATCTTTC	CGGTTTCGGACAGCTCATGATG
7816	7912	CACCTTTCTTTGTTTCAGGCTC	AGGAATCGCAAGAATTGAGAAG
12410	12515	GACCTTGAATGCGCTCTTGGAG	AGAGGACGAAGGCTACTTTCG
17273	17382	AACTTTATGCAATCCGCCATAC	GGGCTTGGAGTTAATCATCTTG
22005	22096	AACTAGACCCGGAAAGGAGAAG	CTTCTGTATCGTCGCTTGAATG
26152	26244	TGCTCGCAGTCGTCTCATAGAG	GACCTTAGCAACCCGAAACGTG
29683	29774	TACTATCCAGAGGCGAAGAAGC	AGGACCCTAAAGAAGGATCGTC
32274	32383	AGTCAGTGGGAAATCGGTAGC	AATGGGCCAGCTGCATAATCAC
34904	35000	CATTGAGGAGAGAGGAGAGAG	CGCCCATACTCTTAACTTTTCG
39428	39536	TAAGCCTGCCTGACTGTGAGAC	GCGTACCTTTGATCCGTTGAGC
43283	43378	CTTGAATGGGAGTTTCTTGGTC	TAGCTTTCGCTTCTTCAGATCC
48567	48661	ATTACGTCTGCCTCCCAGGATG	AGGTGATGCGGGAACTAAGTCC
54799	54917	CCTTGGCGGATAACTAGAAGTG	CCCGAATGTCTTTAATCGAGAG
59534	59642	GGCTCTTCCCTTGGGTCATAGG	ACCCAGCTCAGGAACCTAAACC
63647	63760	ATTCTTGGTGGGAATCATCAAC	AACAAACCAATTACCAAATCCAC
68782	68892	TCACTTCGACGACGGAAGACTC	ATGACCCGGATCGAGTACACTG
73964	74053	AAGATGACAAGCGGAGTAAAGC	GGACGATAGTAGTTTGCCCTTG
79716	79828	AGACCTCCTGAGCTGTTGCAAG	TTCCCAGACTATGATGCCTGG
84857	84974	TATTCGTACTGGTTTCTCATGTG	TTGCCGTAATGCTGAAACTATG
90211	90297	GAGCTTGACCTGCACACACTTG	TCACAGCAGCCAGACAGAAGAC
94846	94954	TTTAAGGAGTTAGCTGCATTTCC	TCCAATCTTGTTGCTAATGTGC
99214	99312	CGACCCTGCCCTTCTTACTCAG	AGGTAGCTAAGCCTGCCGAATC
104322	104436	GAGATGGGGTGACTAAACCTTG	AGTTTTGACTTGCTTTGGCTTC
107511	107614	CCAAGAATAAGGATTCTAAAGG	GCTTTGAGTGCTCCTTAATAG
112496	112595	AGGCTGACAGAAGTACCGAAAC	TTCGATCACAGAATCCATTGAC
113547	113645	GTCTTTCTCGTCAGGTCTATG	TAAGCTAGAGGAGACTTGTTTG
114471	114564	AGCATAGCATAGTAGTGCAAG	CGCTACTTTGTTATCTAAGGTC
119849	119943	GGAGTTTGCTTTACCCAACAAC	GGGCACTCTTCATTCTTCATTC
123165	123271	TGCCTCTCTAGCTGGCTTGATC	CGGAGACATCAGAGGAAGGAGC
128104	128210	CATCGTCTGATGTAGCCTTTTG	TGGAATTGATTGTCGTGAACTC
132432	132549	CGCAGGCATGTGATTGTAAGG	ACTTACTTGACCGCTAGCCAGG
137615	137698	ACAGGATTCGAACCTATGGC	GCAATTACAGTGAGACGCGAGG
142333	142437	AAGGTCTGGCGAAGTCTTGAGG	CATCTTAGCAGCAGGGCTTTCG
149136	149238	CCCTTATGAATGGATCTTGCTC	AGTTCACCCCGCTGTTAGATAG
154724	154823	GAAGAGCTGGTAGGACGGACTG	AGGCCTTCTGTTCCAGTGACAG
159570	159689	AACCGCTAAGAATGTGGAGAAG	CTTGCATAGAAGAACGCAACTG
164577	164690	TCTCGCTACCATACCACAAGG	TTCGATCTTGTACCCACGGAGC
169363	169479	ATTGGTGACCTGTTCGATTACC	TAAACCCTGGTGAATCTTTTGG
175463	175551	AGGATTTCTGGTCTCCTGCAGG	GAGTGGTTCTAGTCTCCCTGG
181437	181541	TACTGTAGCTCCCCAAAAGCTC	TTTGTTTGGTGTCTTGGAGTTG
186252	186347	TCCGTTTGGAGGGACAGATGAG	AGGACTTCGCCTTGACCTTCTG
190999	191099	AGGACTCTCTTCTGTCCGTTTG	CTTTCATAGATGTCGTGCGAAG
197765	197855	ATGGCTACAAGTGGGCTACGAG	TCGCAATGAAGTCTTCCCGTTG
201598	201690	AAGCCTGACACGAAGAATAAGC	CTTCTCTCGACCTCTCTCCTTG
208193	208300	TCTCCTCGTGCTCTGCCATAAG	AGCCAATAACTCGAACGCGTTC
213948	214056	CGGTTCTTCTTAGTTGGATTGG	CGTCCGTCGTCGTAAAGTAGTC
218611	218726	AGGCAGGGTAAGAAAGGGTGAC	AAGTTCGAATCCGTTCCGTTCG
223926	224015	AAATTCCACATCCTGACATTCC	TATGTTTCTTACCCACCCGAAC
231026	231115	TCTGGCCTGAACCTTTGGGTAG	TTCCCGAATGACCGTACCAAGG
237257	237348	GTTTCCGTCCCAGTACCTGTAG	TGTCACTAAGTTGCCAATCGAC
242471	242579	AGGCGCTGTCATCTATCTCGAC	ACCCTTCCTCCTCTGCTTTGAG
245308	245427	GACATGTTCTGCCGCATACCAC	CATAGCCTTCAACCCGCAAAGG
246842	246960	AAGACAGAGAGCTTGGCAGAG	GGGCTGTTTCCCTCTCCTAGAG
251558	251649	ACTTCGATTCCAAAGAAAGCAG	GATTACCATTAGGCGAAAGCAG
255737	255846	GACGGTTGGCTAAAGACATGG	TGGCAATGTCGGAGATCCTAC
262023	262125	ATGGAAGACCCCTCTCCTTATC	GAATCTCGGAAAACGACAAATC
265093	265190	TGATTGATCCAACACGCCAAGC	TCCTGACTAAAGCACCTCTCC
267468	267574	AGCAGCACCAACCTAGCTCTAC	GCTTATTTCGATACCGCTTCTG
270478	270578	CGAGAGCACCAGATACACCATG	ACTAGCTCGCCTTCTTTCCTCG
274140	274247	ATCCAATCTTCCTTGAGAGCTG	TGATTTCTTGATGGTTGACTGC
277941	278045	CGACAAGTCGTGCCTGAAGTTG	GAATTCACTGCAAGGTGGCGAG
282177	282273	TTTGAAACATTTGTGAGATGCC	TTCCTACCAATTCCATTTCGAG
286792	286911	TGGAAGGACCTCCAAGAAACC	AAAGGCTTGCAGACCTTATGC
292828	292948	CTTTCCCCACCTCACATAGAAG	GAGTTGACATGAAATGGATCAC
299589	299691	GTTCAGTTGTTGCCTCCGGTTG	AGCAGAGAACCACAGGGAAGAC
306260	306369	TGGCAACTGATTTGGTTTTATG	GGGTAGAACTGAGAAATGGCAC
310532	310649	TTAGCTCGTCCAGGATCGCAAG	TGCCCTTGTGTTCTAGACCTCG
314566	314674	ATCTCCAAGCCCCTTTACTAGG	ACAAGGACCAGCTTACTTTTCG
319855	319968	TCCGGATCCTTCAGACTTTCG	GTAGGCTCATGCATTCGCTGAC
324834	324942	TCCCTTTACTGTTGACATGGTG	CTTGACACAGGTTGTTCTTTCG
328085	328165	GTAGATGGCGCTCAACTTCCTG	GAGGTCATGTGCGATGCAAGTG
332522	332640	GTCTGAGCAGCAAACGTACAAC	GATGTTGAGGAGAAAGCAGGAG
337297	337401	TCGTCGCAAAGGTTCAAGCAAG	ATCGGACGCTAACCTGGAACTG

341942	342038	GTGGACTTCCCAAGACTTTCAG	TCTGGAACATGGAAATTGATTG
346953	347035	TAGTCCAACGCTCTCGCTATGG	TAAGCACACAAAGCAGGGATGC
352185	352280	GACATAAGCCGTTTCCTTGTTC	AAGCAGCAATACACTCAAGCAC
356004	356123	GATGTTGTTCTCGACTCCGTG	CGGATTGGAGGTCTTGGGAATG
360635	360742	GGGGAATCCTCCTTAATAGACG	CATCCGAAGAAACGGAAAATAC
363962	364069	GCGACAAGAAGATCCTGCATCG	TATCGGGAGGGTCGCAGATTTG
366597	366694	GTTGATCCATTGTGATGTTTGG	AAGTCCCATCTTGCCTATGTTG

Primers cpDNA

Coordinates on Col-0 cpDNA		Forward	Reverse
1002	1120	GAATATGCAACAGCAATCCAAG	GGCGGTCCTTATGAACTAATTG
6058	6168	CGAGCCAAGAGCACCTATATTC	ACGATGTGGTAGAAAGCAACG
11104	11219	CTCGACCATCAATAGGGTTAGC	GATGATCCAAGAAGGAAGTTCAG
15777	15894	CGATGGAACCTTCTCTTGTACC	TTGCTGTAGTCATTTGCTTTCG
19090	19206	ATTGACCCGCGATAATACCTAC	GAACTCCCTTTACTTGTCGGAG
24016	24141	AAGTCGATACCTGAATGCCAAG	TAGGATCGTGGGTTGAAACAG
33265	33362	TTTCCAAGGGTTTCATAATTGG	TAGCACCATGAATAGCGCATAG
37469	37575	TGCACGATTGAAAGAGCTACTG	GGTTATTGGCAGGAATTGATTG
41520	41636	TGAGCACTAGGTCCAATGTGAG	TTTCGGCCAACTCTCTATCATC
46929	47054	AATTGGATTGAGCCTTGGTATG	TTGTAAACTCCGGTTTGTTCG
51198	51327	TTAGGGCTATACGGACTCGAAC	AGTTAATGAAAGAGCCCAATGC
56158	56270	CAATTCGGTGGAGGAACTTTAG	GCAAGATCACGTCCCTCATTAC
60199	60303	ATATGGAAATTCGAGGTCAAGG	GCAAGAAGTAAGCCAATTCAGC
66049	66176	TTGGCTGGATTATTCGTAACTG	CTGAATTAGACCTCCCGCATAG
70683	70808	TGCTCCTCCGACTAGGATAAAG	CCTGGTGGATGGGTAATATCTG
76165	76289	GTGTTGGACAATCCACTTTGAC	TAAGGGACCAGAAATACCTTGC
81419	81535	GTCGTACATTTCGTGTCATTGC	CTTCTCGTGGGAATCGTATTTG
85937	86047	TGTATGTCCCAGAATAGGTCC	ACAGAAATAAAGCATTGGGTCG
91180	91290	TGAACAACCGGGAACAATTTAC	GCAAGGAATATCCGTCTTTCTG
96056	96181	ACGGATGCTCCTATTACACTCG	ATTACGGGTAGTTCCTGCAAAG
101430	101550	AAGAAGCAATGACGGTATCTGG	AAGCCACCTACAGACGCTTTAC
105328	105429	TAGCGAAAGCGAGTCTTCATAG	CTCCACTTAGTTTCACCCAAGC
110181	110290	TGGGACTAAACAGGACCAAGAG	CTCGGATTTCTTCCATTTCATC
114762	114881	TAAACACAATAACTGCGCCAAG	TCATTAACCACTGGGACTGGAG
118021	118122	GCCCTAACCATATTTCGACTTG	AAGGAGTTGCTGAATGATACTCG
122976	123095	AATAATCCCATCGCGTTACATAC	ATGGTGAGGATGTTGTTGACTG
127385	127499	GCCTTCCAATTCTTCCAACTC	AACTGATAGCAACGATCAAACG

Primer RT-qPCR

Target genes	Forward	Reverse
nad1b	TTCTCGTCTAGCAGAAACTAATCG	TTCTACATTATAGCCTGCAACTGATT
nad2b	GCTCTAGCCAAAACGAATCCT	GGGGTATTCCTGCGTATGAG
nad4	CCATATGAATCTGGTGACTATTGG	CGGTCATATAAAACACCAACACA
nad5a	CCACCTACGGCTTTGATTGT	GTTGCCGCAAGGAATGAC
nad5b	CAATTTTTGGGCCAATTCC	TGGTTGGAGCAGCAAACTC
nad6	GATTGGGGATTCAGTGGTGT	TGGTACGTCAAGATCCGATTG
nad7	GATTGGGGATTCAGTGGTGT	TGGTACGTCAAGATCCGATTG
nad9	AACTTCTCGCTCCCACCAG	AGTGCAGACGAAGTAACACGAA
cob	GAACACATTATGAGAGATGTTGAAGG	AACATACTTGCCCCATTAGCA
cox1	GTATGCCACGTCGTATTCCA	CGGATATATAAGAGCCAAAACTGG
cox2	TCCGATGAGCAGTCACTCAC	AATAAACGTGATTGACCCAATTCT
cox3	CCTTGGGAAATCCCTTTTCT	TTTCTTTCCCCGCGAGTAT
atp6-1	GCCTTACCCGTCATTGAAAC	CACATTGGTGGGAATATAGGC
atp4	CGTTTTCAGGACGATCTAGTCA	TTTCGAGATACAAGAGTAGGCAAA
atp9	CGGAGCTGCTACAATTGCTT	CGCCACAGAATGAATCAAAG
rps3	CTCGACCAGCGAGAAAAAGT	CGAATGAAGTGGGTCAACCT
rps4	TCGGATCCAAACTTCTTTGTTC	CCCGGTTTTTGCAACAGT
rps7	GCACGTAGGAAAAGGGAGAAT	ATCTGAAATGCGCGAAACTT
rpl2	TCTGCCTTCTCCTCTCCAAA	GCTATCCTTGGGAAACCAAA
rpl5	CTCGATGGAAACGGAGTTTT	CATTGAACCCCCGAATATGT
rrn18	CACACTGGGACTGAGACACG	GCCCATTGTCCAAGATTCC
rrn26	GGGAAGGTTTTTGGTGACAA	TCGTTACTCATGTCAGCATTCTC
ccmFN1	GATTGTGGCACTCCACTCG	ATCCAGCAGAGCGAAGCA
ccmFN2	GTGGTTTTTAACCGTAGGCATC	CCACCCCGACCTAATTCAT
ccmB	CGGAATGGATCGGTTAAACA	CCGAACGAGAATGAATACCAC
mttB	CGCTTCTTCTTGTTCCTGTTC	GCACCCACGAAGTATGGAAA
matR	CGCGACAACCTTTATCAAGTC	GGCTAGGGTGAATATTGCAGA
ccmFC	TGGCTGTTGGTCACGACTAC	GGCAATCGTTGAGTGAACTG
aox1a	TGGTTGTTCGTGCTGACG	CACGACCTTGGTAGTGAATATCAG
aox1d	CAAACTCTGAAAATACCGGTTCA	TCTCTAGCAACATCGCATGG
actin	GGATCTGTACGGTAACATTGTGC	TGCTCATACGGTCAGCGATA
gapdh	TTGGTGACAACAGGTCAAGCA	AAACTTGTCGCTCAATGCAATC
64PKTLS	TCGCCAGTTAGCGAGGTCT	GTTCCGATGACCCTCGGAAG

PKTLS construction

	Forward	Reverse
orf77	TGCATCGTTTGACTCAATGATG	GAGTCAAAAGACACAGTGAAAG
orf77-PKTLS-cHDV	CATTTGGAGAGGACACGCTGAAG	ACGTTGTCGAAACCGATGATACGG
orf77-PKTLS	CTGAAGCTAGTCGACTCTAGC	TGGTTCCGATGACCCTCGGA
cHDV-Ter	TGGCATCTCCACCTCCTC	ATTCTGGTGTGTGGGCAAT

Primer used for genotyping

	Forward	Reverse
OEX1-1	CAGACATTCCATGAAGACAG	GTAGCCTTGACACAACTGAG
787	CGCCTATAAATACGACGGATCGTA	Verification of T-DNA insertion in OEX1-1
Pol1A-1	AGAAAGTAGCCAAAGCGGAAC	TCCGTTGACAGTGGGCTATCA
319	TGGTTCACGTAGTGGGCCATCG	erification of T-DNA insertion in Pol1A-1
Pol1A-2	CAAATCGTGTACTTACAGTTGCC	AGAAAGTAGCCAAAGCGGAAC
319	TGGTTCACGTAGTGGGCCATCG	erification of T-DNA insertion in Pol1A-2
Pol1B-2	AGCAAGATGTGCCAGAATCC	GTTAGCTTGAATGGAGATGCAATC
452	ATATTGACCATCATACTCATTGC	erification of T-DNA insertion in Pol1B-2

Primer for OEX used in PCR

	Forward	Reverse
Nested PCR1 OEX1	GCAATAGTCTTCCTCTTCTGT	GAGTTTCAGACAGCATTTTAGA
Nested PCR2 OEX1	GTAGGATCCGTGAATGTGTTTCCTCCGCTA	GTACTCGAGTGCTTCCAGTTTTTCCCACA
Nested PCR1 OEX2	AGAGGAAATCGAAATGAAAAGA	CAGCCATGTCCATAGTGATAA
Nested PCR 2 OEX2	GTAGGATCCACAGTAGGATTTATTCAACCCAA	GTACTCGAGTCATCCATGCAATGTTGAGA
OEX1 coding sequences	GTA GAG CTC GTG AAT GTG TTT CCT CCG CTA	GTA CAG CTG TCA TGG CAC TGC TTC CAG TT
OEX2 coding sequences	GTAGAATTCACAGTAGGATTTATTCAACCCAA	GTA CTC GAG TCA TCC ATG CAA TGT TGA GA
pGEMT before T7	GCCAGTGAATTGTAATACGA	
pGEMT after T7	TAGAATACTCAAGCTATGCA	
EX1 primer for sequencing	CATCTACAGGTTATCGTTCT	
	CATTGCCTTGATGGACGCTT	
OEX1 forsequencing	GTACATATGTCCCTTGCACGCTCTGCGA	GTAGGATCCTCATGGCACTGCTTCCAGT
OEX2 for sequencing	GTAGGATCCATGTCTTCCGTTGAGACATTTCA	GTACTCGAGTCATCCATGCAATGTTGAGA
Repeat EE-1	CAGCATGGTGAAAAGTAAGC	TTTAATCAAAGCTAGCTACAGGG
Repeat EE-2	AGGCTTCAGGGTAGCTTCTG	CACAAGGAGCAGTTGAGACAG
Repeat L-1	GTTATGCCATTTTGGGCTTTGCTC	CGCTCGACCGAAGAAATGAGTAAC
Repeat L-2	CCGGTTGAAAGCTAAGCCGGAG	CCCTCACTGAACCGACTTGAATCTG

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SYNTHESE EN FRANCAIS

I. Introduction

Dans une cellule végétale, nous trouvons trois génomes : nucléaire, mitochondrial et chloroplastique. Les trois opèrent de façon coordonnée afin d'assurer les fonctions centrales de la cellule telles que la photosynthèse ou la respiration. Dans les cellules des organismes eucaryotes, les mitochondries assurent des fonctions vitales telles que la production d'énergie ou la régulation des potentiels redox, et participent aux voies métaboliques ou à la mort cellulaire programmée. Chez l'homme, des mutations dans le génome mitochondrial peuvent amener à des maladies neurodégénératives. Chez les plantes supérieures, les réarrangements affectant le génome mitochondrial conduisent souvent à la stérilité mâle cytoplasmique (CMS), un caractère qui est largement utilisé en agronomie pour la production de lignées hybrides.

L'ADN mitochondrial (ADNmt) de plante est riche en petites séquences répétées. Ces répétitions recombinent entre elles à de faibles fréquences et causent des réarrangements de l'ADNmt conduisant à une diversité génétique qui contribue à l'évolution du génome mitochondrial (Gualberto *et al.*, 2014). Les mécanismes de recombinaison sont également à la base des voies de réplication et de réparation de l'ADNmt des plantes. Les processus complexes qui contrôlent l'expression et la dynamique du génome mitochondrial ne peuvent être étudiés à travers une approche génétique classique car, jusqu'à présent, seules les mitochondries des levures et de l'algue verte unicellulaire *Chlamydomonas reinhardtii* peuvent être transformées par des méthodes conventionnelles (Bonnefoy *et al.*, 2007).

Pour la manipulation génétique des mitochondries des plantes, deux stratégies alternatives ont été développées dans le laboratoire d'accueil. La première cible les ARN et la seconde les réarrangements de l'ADNmt. La stratégie ARN est basée sur le mécanisme naturel d'import d'ARN de transfert (ARNt) depuis le cytosol vers l'intérieur de la mitochondrie (Sieber *et al.*, 2011). C'est un fait que les mitochondries des plantes supérieures importent une partie de leurs ARNt depuis le cytosol, en particulier les ARNt spécifiques de la valine. Le laboratoire a développé une stratégie basée sur l'utilisation d'une séquence virale qui imite l'ARNt valine dans sa structure tridimensionnelle et ses propriétés fonctionnelles. Cette structure appelée TLS (*tRNA-like-structure*) correspond aux 82 derniers nucléotides à l'extrémité 3' de l'ARN génomique du virus de la mosaïque jaune

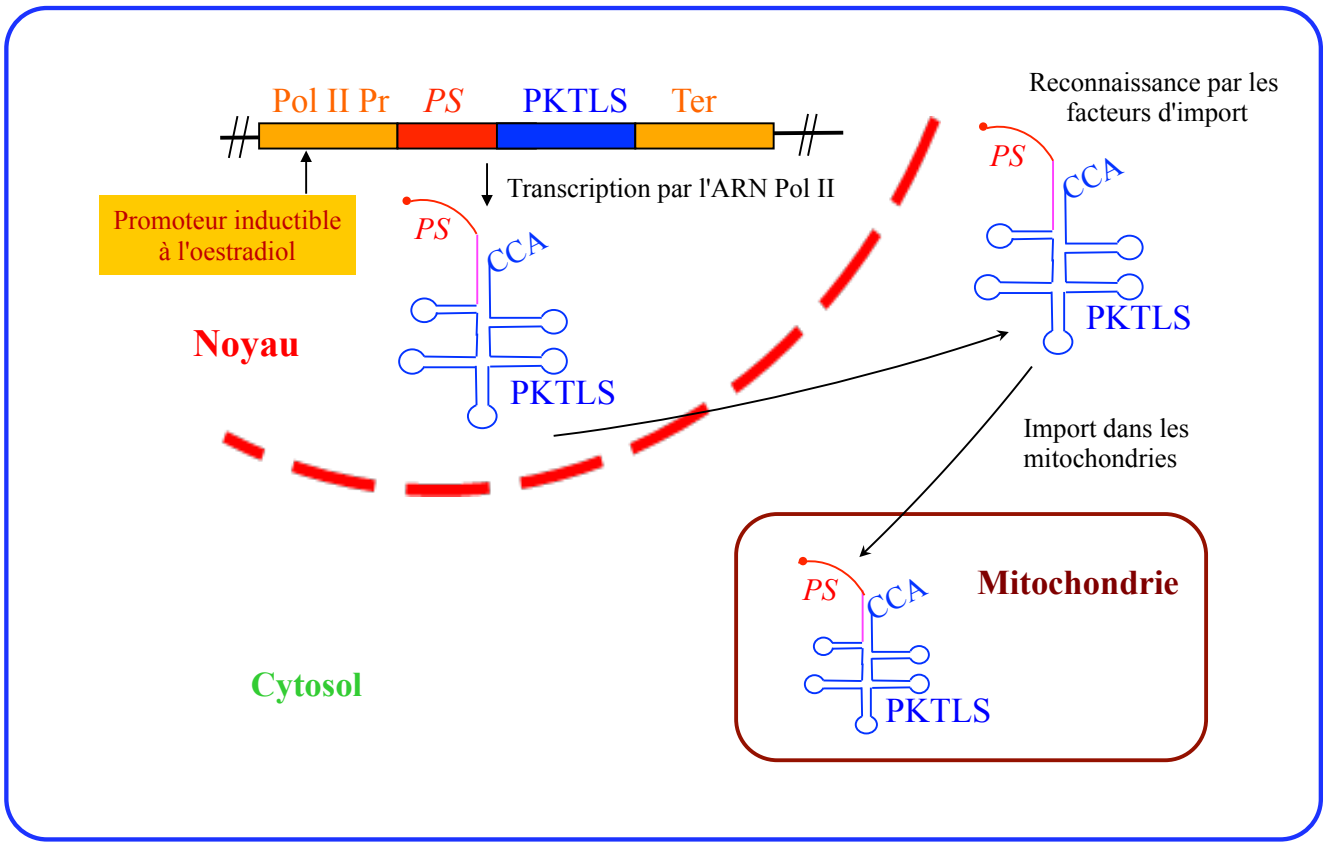


Figure 1: Stratégie expérimentale. L'ARN composite formé par la séquence passagère (PS) et la navette PKTLS est exprimé à partir d'un transgène nucléaire, transporté dans le cytosol, reconnu grâce à la partie PKTLS par les facteurs d'import des ARNt et importé dans les mitochondries (Val *et al.*, 2011). Pol II Pr : promoteur pour l'ARN polymérase II; Ter : terminateur.

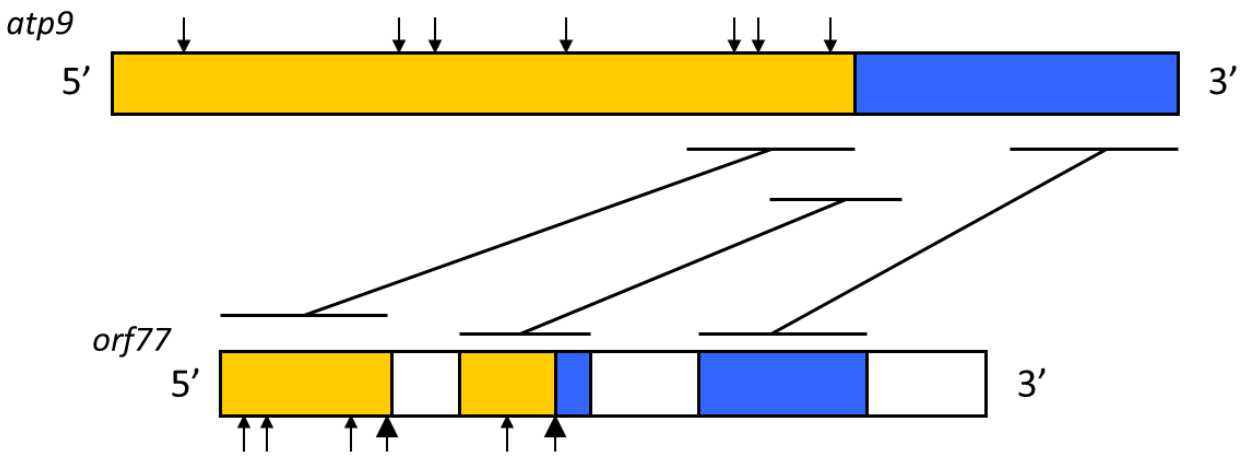


Figure 2: Régions communes entre *atp9* et *orf77*. Les flèches indiquent des sites d'édition de l'ARN.

du navet (*Turnip yellow mosaic virus* ou TYMV). La séquence juste en amont de la TLS adopte une structure en pseudo-nœud appelée UPSK, qui aide à la reconnaissance de la TLS par la valyl-ARNt synthétase. Cette reconnaissance est naturellement importante pour l'import d'un ARNt du cytosol dans la mitochondrie. Nous utilisons la combinaison de la TLS et de l'UPSK, appelée PKTLS, comme une navette pour importer des séquences attachées à l'extrémité 5' dans la mitochondrie (Val *et al.*, 2011). L'équipe de notre laboratoire a montré qu'avec cet outil il est possible d'importer des ARN d'intérêt dans la mitochondrie suite à une transformation nucléaire stable de plantes avec les constructions adéquates. Le développement de cette stratégie de manipulation dirigée du transcriptome mitochondrial ouvre la possibilité d'étudier d'une manière directe les mécanismes de la régulation génétique mitochondriale et les processus de coordination avec le noyau et les plastes.

La deuxième stratégie de manipulation génétique des mitochondries repose sur les facteurs codés par le génome nucléaire qui contrôlent les processus de recombinaison de l'ADNmt. Il s'est avéré que la mutation de certains facteurs relâche ce contrôle, ce qui entraîne une augmentation importante de la recombinaison ectopique entre les petites séquences répétées et génère des configurations alternatives du génome mitochondrial issues de réarrangements. D'abord présentes en faible proportion, de telles configurations alternatives peuvent devenir dominantes dans certaines plantes des générations suivantes par des mécanismes de ségrégation qui sont encore peu connus. L'utilisation de cette approche de mutation nucléaire permet par conséquent de créer de la diversité génétique mitochondriale et de ségréger de nouveaux phénotypes. Son application à des espèces d'intérêt économique pourra ouvrir des perspectives agronomiques.

II. Résultats et Discussion

II.1. Régulation de l'expression génétique mitochondriale

Dans mon projet de thèse, j'ai utilisé la stratégie d'adressage d'ARN basée sur la navette PKTLS (Figure 1) pour analyser la régulation du transcriptome mitochondrial et pour contribuer à une meilleure compréhension des mécanismes de la stérilité mâle cytoplasmique. Chez le maïs, la stérilité mâle cytoplasmique de type S (CMS-S) est associée à la présence de transcrits mitochondriaux spécifiques comportant deux phases de lecture ouverte, l'*orf355* et l'*orf77*. Remarquablement, l'*orf77* est une chimère qui combine des parties codantes et non-codantes du gène mitochondrial *atp9* avec des séquences d'origine

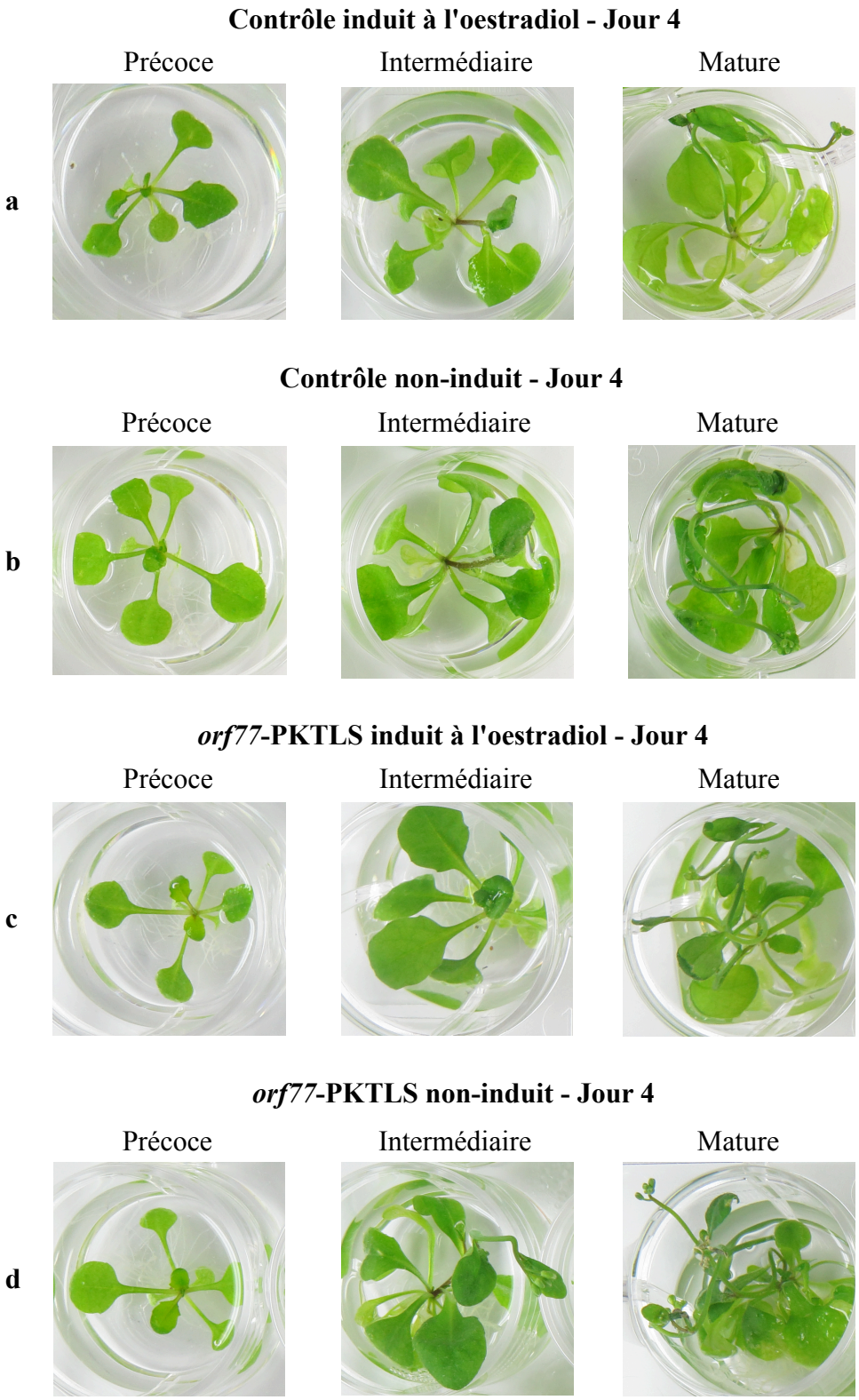


Figure 3: Stades de développement des lignées transformées d'*A. thaliana* utilisés pour l'induction de l'expression de l'ARN *orf77*-PKTLS. Les graines ont été semées sur milieu agar dans des boîtes de Petri, puis transférées au stade de développement approprié à la lumière (précoce, intermédiaire ou mature) dans les puits de boîtes de culture contenant du milieu liquide complémenté avec de l'oestradiol ou du milieu de culture simple. Les images ont été prises au jour 4 après induction. Ni l'expression de l'ARN *orf77*-PKTLS (**a** versus **c**), ni le traitement à l'oestradiol par lui-même (**a** versus **b**, **c** versus **d**) n'ont montré d'effet phénotypique.

inconnue (Figure 2). Du fait de cette identité partielle avec *atp9*, l'hypothèse que l'*orf77* serait la cause principale de la CMS-S chez le maïs est souvent émise, potentiellement à travers une compétition avec *atp9* au niveau de l'ARN ou de la protéine (Su *et al.*, 2016).

Afin d'explorer ces mécanismes et de déterminer s'il est possible de générer de la CMS dirigée, nous avons couplé la séquence de l'*orf77*, additionnée d'une séquence codant pour une étiquette de six histidines, au mime d'ARNt PKTLS. La construction a été placée dans le vecteur pER8 sous le contrôle d'un promoteur inductible à l'œstradiol et utilisée pour générer des transformants nucléaires stables d'*Arabidopsis thaliana*. La présence de la construction a été confirmée par PCR. Quatre lignées distinctes ont été sélectionnées pour la suite des travaux. Pour les contrôles, trois lignées transformées avec le vecteur vide ont été sélectionnées. Les plantes d'*A. thaliana* transformées ont été mises à pousser sur milieu de culture gélosé supplémenté de β-œstradiol pour l'induction du transgène. En parallèle, des plantes ayant poussé sur agar ont été transférées dans des boîtes de culture contenant du milieu liquide supplémenté avec du β-œstradiol, et les racines ont été plongées dans le milieu. Dans toutes les conditions, des plantes ont été récoltées tous les jours jusqu'au jour 4 après l'induction. Ces protocoles ont été appliqués à différents stades de développement à la lumière et à des plantes poussant à l'obscurité. Quelles que soient les conditions de croissance, les transformants n'ont pas montré de phénotype particulier sur la période d'étude (Figures 3 et 4). Une induction efficace de l'expression de l'ARN chimérique *orf77*-PKTLS a été montrée aux différents stades de développement et dans les différentes conditions de culture, avec généralement un niveau maximal un jour après induction, et une décroissance progressive au fur et à mesure de la métabolisation de l'oestradiol par les cellules (Figure 5). L'import mitochondrial de l'ARN *orf77*-PKTLS a été confirmé en purifiant des mitochondries à partir de plantes induites. Nous avons par la suite analysé l'effet de la présence de l'*orf77* sur le transcriptome mitochondrial. Pour la détermination d'effets significatifs ou non-significatifs, les données de RT-qPCR ont été analysées avec le logiciel de statistiques Graph Pad Prism (version 7.01).

Les résultats sont synthétisés dans le Tableau 1. Au stade précoce du développement à la lumière (4-6 feuilles), nous n'avons observé quasiment aucun effet de l'expression de l'ARN *orf77*-PKTLS sur le transcriptome mitochondrial, seuls 3 gènes voyant leur expression affectée et uniquement au jour 2. L'effet est également resté très limité au stade intermédiaire de croissance (8-10 feuilles), avec en tout une dizaine de transcrits

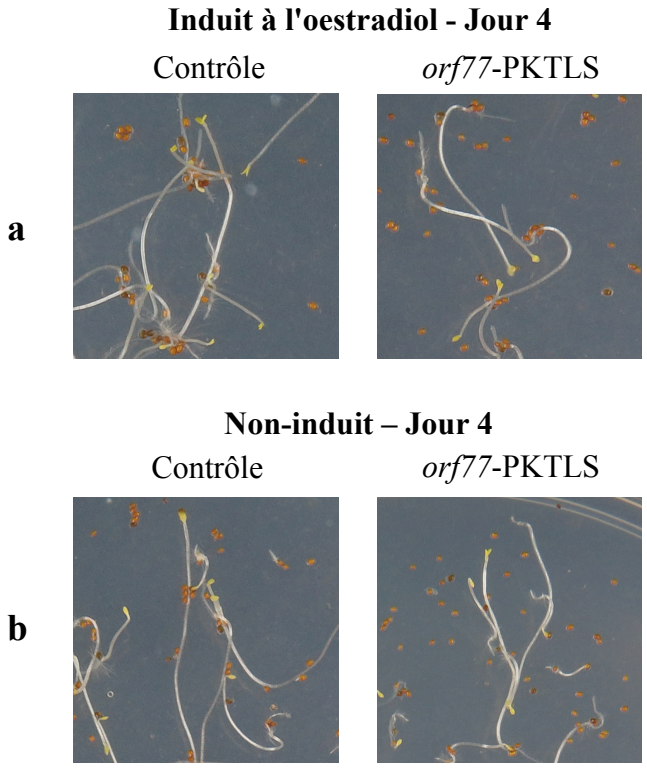


Figure 4: Plantes étiolées d'*A. thaliana* utilisées pour l'induction de l'expression de l'ARN *orf77*-PKTLS. Les graines ont été semées sur milieu agar dans des boîtes de Petri. Après croissance des pousses à l'obscurité, le milieu solide a été recouvert par du milieu liquide complémenté avec de l'oestradiol ou du milieu de culture simple. Les images ont été prises au jour 4 après induction. Ni l'expression de l'ARN *orf77*-PKTLS (**a**, droite versus gauche), ni le traitement à l'oestradiol par lui-même (**a** versus **b**) n'ont montré d'effet phénotypique.

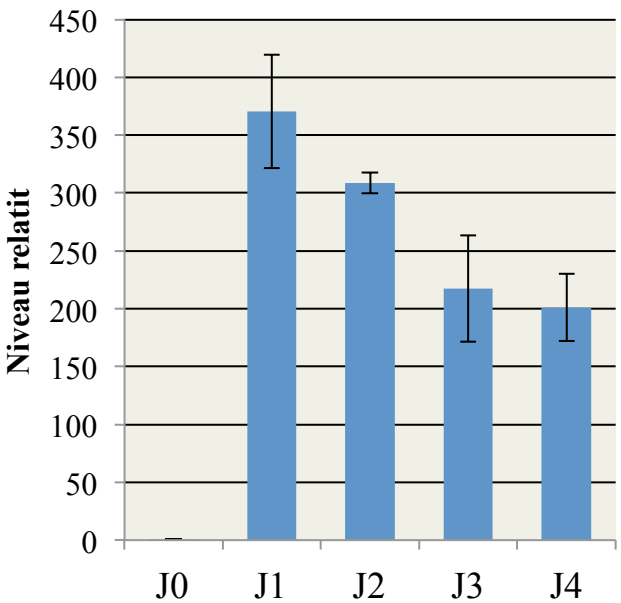


Figure 5: Cinétique d'expression de l'ARN *orf77*-PKTLS au stade précoce de développement des plantes transformées d'*A. thaliana* poussées à la lumière. Analyse par RT-qPCR avec de l'ARN total. Pour l'induction du transgène, l'oestradiol a été ajouté au jour 0 et les échantillons ont été recoltés jusqu'au jour 4. J0 : avant l'addition de l'oestradiol; J1 à J4: jours après le début de l'induction. Niveau d'expression relatif de l'ARN *orf77*-PKTLS par rapport à l'expression au jour 0.

Conditions et stade de développement	Jour 1	Jour 2	Jour 3	Jour 4
Lumière *Stade précoce*		↓nad5a∗ ↓atp4∗∗ ↑rps7∗ ↑AOX1d∗	 ↓AOX1d∗∗∗	 ↓AOX1d∗∗∗
Lumière *Stade intermédiaire*	↑nad5b∗∗ ↓ccmFN2∗∗	↑nad5a∗∗ ↑atp4∗ ↑rpl2∗ ↑rpl5∗ ↑AOX1d∗∗∗	↑cob∗∗ ↓ccmFN2∗∗∗ ↑AOX1d∗∗∗	↑rps7∗ ↑rpl2∗∗ ↑rpl5∗ ↑AOX1d∗∗∗
Lumière *Stade mature*	↓nad2b∗∗ ↑nad5a∗∗ ↓nad5b∗∗ ↓nad9∗∗ ↓cox1∗∗ ↓ccmB∗∗ ↓mttB∗ ↓rpl5∗ ↓AOX1d∗∗	↓atp4∗ ↓atp9∗∗∗∗ ↓rps4∗∗ ↓rps7∗∗∗∗ ↓rpl2∗∗∗∗ ↓rpl5∗∗ ↓rrn18∗∗ ↓AOX1d∗	↑nad1b∗ ↓nad2b∗ ↓nad4∗∗ ↑nad5a∗∗∗ ↓nad5b∗ ↓nad6∗ ↓nad9∗∗ ↓cob∗∗∗ ↓cox1∗ ↓cox2∗∗ ↓cox3∗∗∗∗ ↑atp9∗ ↓ccmB∗∗∗ ↓ccmFC∗ ↓ccmFN1∗∗∗ ↓ccmFN2∗∗ ↓mttB∗∗ ↓rrn18∗∗∗ ↓rrn26∗∗ ↓AOX1a∗ ↓AOX1d∗	↓cox1∗∗∗∗ ↓atp9∗ ↓ccmFC∗∗∗ ↑mttB∗ ↑rpl5∗∗ ↓rrn26∗
Obscurité	↑nad2b∗∗ ↓nad5a∗∗ ↑nad5b∗∗∗∗ ↑nad9∗ ↓cox2∗ ↓matR∗ ↑rps7∗∗ ↓rpl5∗ ↓rrn26∗	↑nad4∗ ↑nad5b∗ ↑cox1∗ ↓ccmFN2∗∗ ↑AOX1d∗∗	↓nad1b∗∗∗ ↓nad2b∗∗ ↑nad5b∗∗ ↓ccmFN2∗∗ ↓mttB∗∗ ↓rrn26∗∗	↓ccmFC∗∗∗ ↓matR∗ ↓mttB∗ ↓rrn18∗∗∗∗ ↑rrn26∗∗

Tableau 1. ARN mitochondriaux dont le niveau augmente (en noir et ↑) ou diminue (en violet et ↓) significativement, en relation avec l'expression et l'import mitochondrial de l'ARN *orf77*-PKTLS. Les variations significatives des ARN nucléaires codant pour l'oxidase alternative (AOX1, soulignée), enzyme adressée dans les mitochondries, sont également incluses. L'expression du transgène *orf77*-PKTLS a été induite au jour 0 et des échantillons ont été recueillis chaque jour jusqu'au jour 4. Les analyses ont été faites par RT-qPCR avec de l'ARN total. Les résultats de 3 réplicats biologiques ont été analysés avec le logiciel Graph Pad Prism; ∗=p≤0.05, ∗∗=p≤0.01, ∗∗∗=p≤0.001, ∗∗∗∗=p≤0.0001.

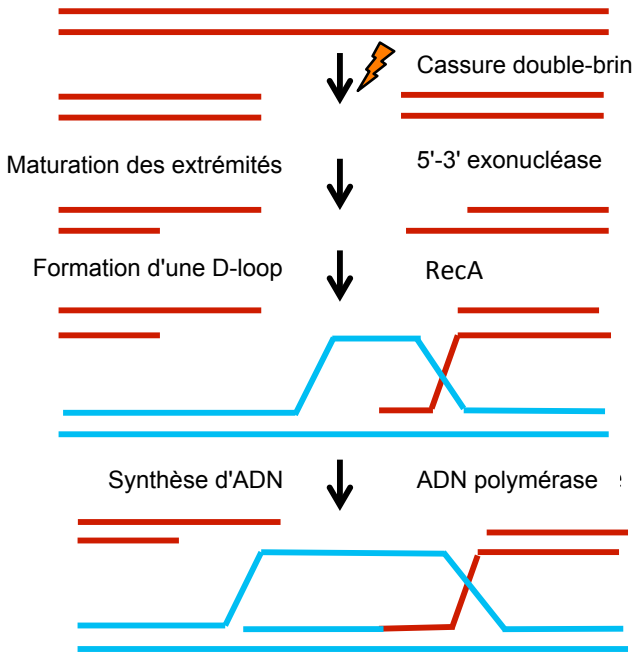


Figure 6: Réparation des cassures double-brin de l'ADN par recombinaison homologue. L'activité 5'-3'exonucléase est requise pour générer des extrémités simple-brin.

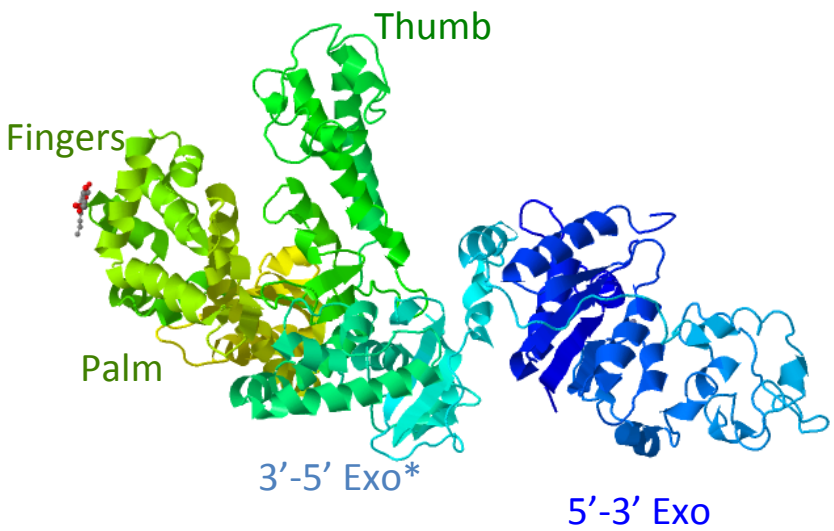


Figure 7: Structure de l'ADN polymérase I bactérienne. Les différents domaines sont indiqués.

mitochondriaux affectés et répartis sur l'ensemble de la cinétique de 4 jours. En revanche, les plantes matures (16-18 feuilles) ont montré une réponse importante et générale à l'expression et à l'adressage mitochondrial de l'ARN *orf77*-PKTLS. Dans ce cas, le nombre d'ARN mitochondriaux affectés est allé de 6 au jour 4 jusqu'à 19 au jour 3, sur un total de 30 transcrits testés, touchant tous les complexes mitochondriaux (en dehors du complexe II dont les sous-unités ne sont pas codées par le génome mitochondrial chez *A. thaliana*), ainsi que le système de synthèse protéique mitochondrial (ARNm codant pour des protéines ribosomiques et ARN ribosomiques eux-mêmes). Finalement, les plantes étiolées ayant poussé à l'obscurité ont développé une réponse intermédiaire, avec un maximum de 9 ARN affectés au jour 1. Pour les plantes au stade intermédiaire ayant poussé à la lumière et pour les plantes étiolées, les variations de niveau d'expression des ARN mises en évidence se sont montrées relativement équilibrées entre augmentations et diminutions. Au contraire, dans les plantes matures ayant poussé à la lumière, la vaste majorité des ARN affectés ont vu leur niveau d'expression diminuer, ceci quel que soit le jour considéré.

Ces résultats mettent en évidence que la présence de l'ARN CMS *orf77* dans les mitochondries est effectivement capable de perturber le transcriptome des organelles, mais essentiellement dans les plantes matures. La réponse est donc fortement dépendante du stade de développement et des conditions de culture, ce qui implique des mécanismes de régulation appropriés. Le niveau de l'ARNm *atp9*, qui présente des séquences communes avec *l'orf77*, n'a été affecté significativement que dans le cadre d'une réponse maximale dans les plantes matures. De façon intéressante, les plantes qui expriment l'ARN *orf77*-PKTLS ont montré dans toutes les conditions testées une variation, positive ou négative selon le cas, du niveau des ARN nucléaires codant pour l'oxidase alternative mitochondriale. L'introduction de *l'orf77* dans les mitochondries semble donc déclencher également une réaction rétrograde et une réponse antérograde.

II.2. Recombinaison et diversification de l'ADNmt

L'équipe a caractérisé antérieurement différents gènes nucléaires codant pour des facteurs impliqués dans la recombinaison de l'ADN dans les mitochondries des plantes. Ces gènes semblent avoir une origine procaryotique et ne sont pas présents chez la levure ou chez les mammifères (Gualberto *et al.*, 2014). Cependant, l'un des facteurs clés des mécanismes de recombinaison (Figure 6), l'exonucléase 5'-3' nécessaire pour générer des régions simple-brin aux extrémités d'une cassure double brin de l'ADN, n'avait pas encore été identifié dans

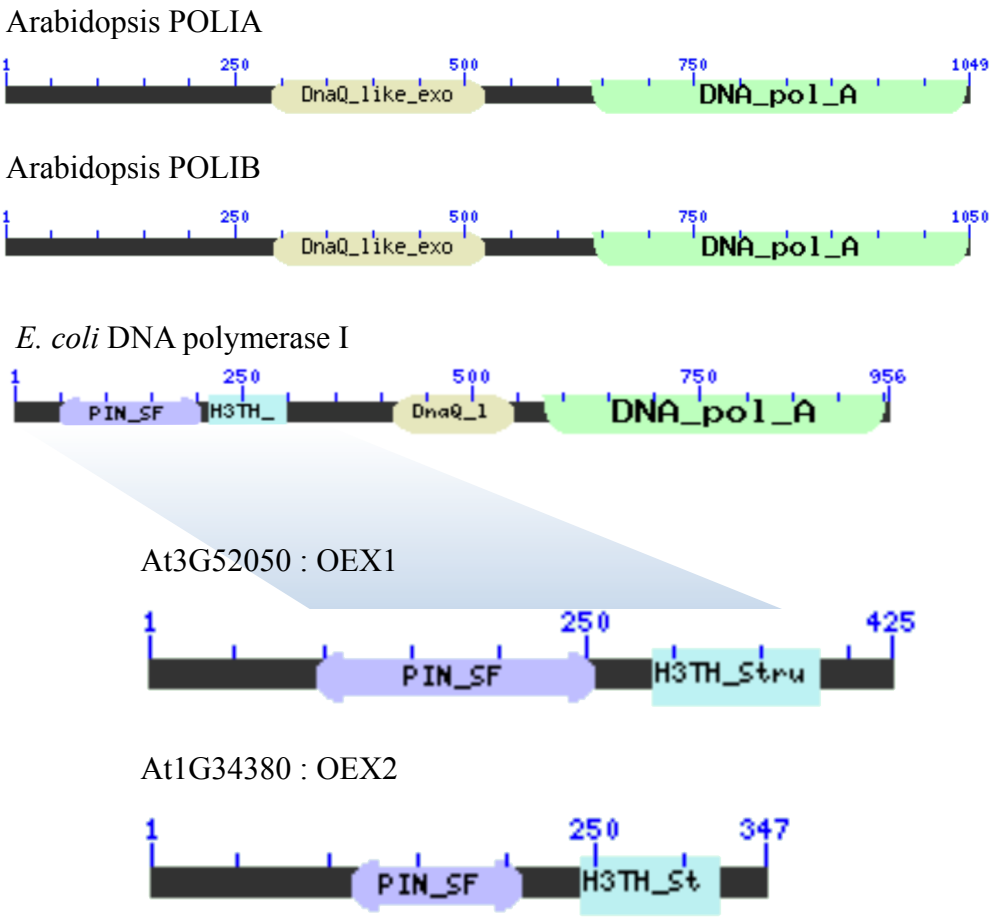


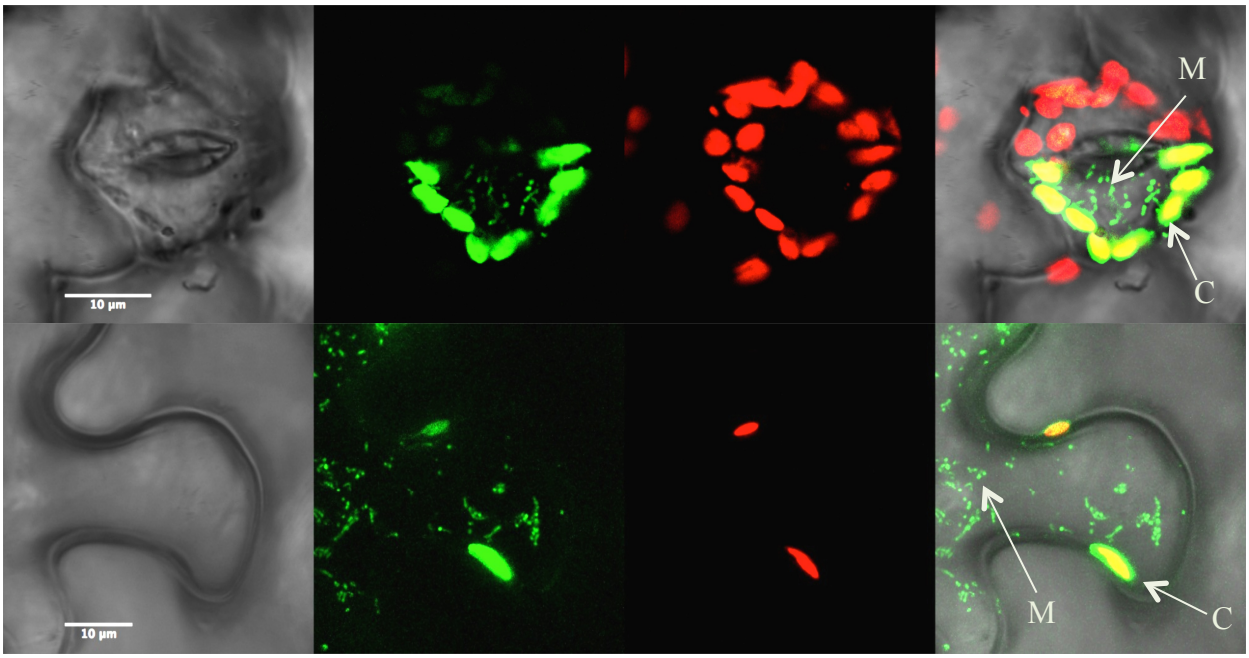
Figure 8: Le domaine 5'-3' exonucléase de l'ADN polymérase I bactérienne est absent des ADN polymérases POLIA et POLIB des organelles d'*A. thaliana*. Les protéines OEX1 et OEX2 sont similaires à ce domaine.

les mitochondries végétales. Le domaine exonucléase 5'-3' présent dans l'ADN polymérase I d'*E. coli* (Figure 7) n'a pas été retrouvé dans les ADN polymérases mitochondriales POLIA et POLIB des plantes, mais nous avons identifié chez *A. thaliana* deux gènes nucléaires codant potentiellement pour une telle enzyme. Ils ont été nommés "Organellar Exonuclease" (OEX) 1 et 2 (Figure 8). La localisation subcellulaire des protéines OEX1 et OEX2 fusionnées à la GFP a été analysée par microscopie confocale après transformation par biolistique dans *Nicotiana benthamiana*. La protéine OEX1 est adressée dans les chloroplastes et dans les mitochondries, tandis qu'OEX2 est localisée uniquement dans les chloroplastes (Figure 9).

Nous avons analysé différentes lignées mutantes d'OEX1 sur les générations F1 à F3. Les plantes *oex1* mutantes ont montré des phénotypes de développement altéré dans des conditions de croissance normales (Figure 10). Afin de comprendre les raisons génétiques de ces altérations, nous avons étudié les événements de recombinaison dans le génome mitochondrial des mutants, en liaison avec les petites séquences répétées (Figure 11). Le nombre relatif de copies des différentes régions de l'ADNmt a ainsi été analysé par qPCR. Dans toutes les plantes *oex1*, des variations significatives du nombre relatif de copies des différentes régions de l'ADNmt ont été détectées en comparaison avec l'ADNmt des plantes sauvages (Figure 12). Pour certaines régions, nous avons détecté une augmentation de 1,5 à 4,5 fois du nombre relatif de copies, tandis que pour d'autres régions, nous avons observé une diminution de moitié, voire la perte de la séquence. Ces variations étaient différentes pour chaque plante individuelle, tant pour les régions concernées que pour les niveaux de variation.

Une analyse détaillée a été réalisée en utilisant à la fois la PCR directe et la PCR inverse suivie d'un séquençage. Les résultats ont révélé que les différentes régions amplifiées dans les plantes *oex1* étaient sous la forme de molécules d'ADN circulaires subgénomiques résultant d'événements de recombinaison entre des petites séquences répétées en orientation directe (Figure 12). Toutes ces molécules circulaires contenaient des gènes hautement exprimés. La protéine OEX1 étant adressée également dans les chloroplastes, l'ADN chloroplastique a été scanné en parallèle, mais aucun changement notable du nombre de copies des différentes régions n'a été détecté dans les mutants *oex1*.

Des analyses phylogénétiques suggèrent que OEX1 et OEX2 sont apparentées au domaine 5'-3' exonucléase de l'ADN polymérase I bactérienne. Comme mentionné, ce



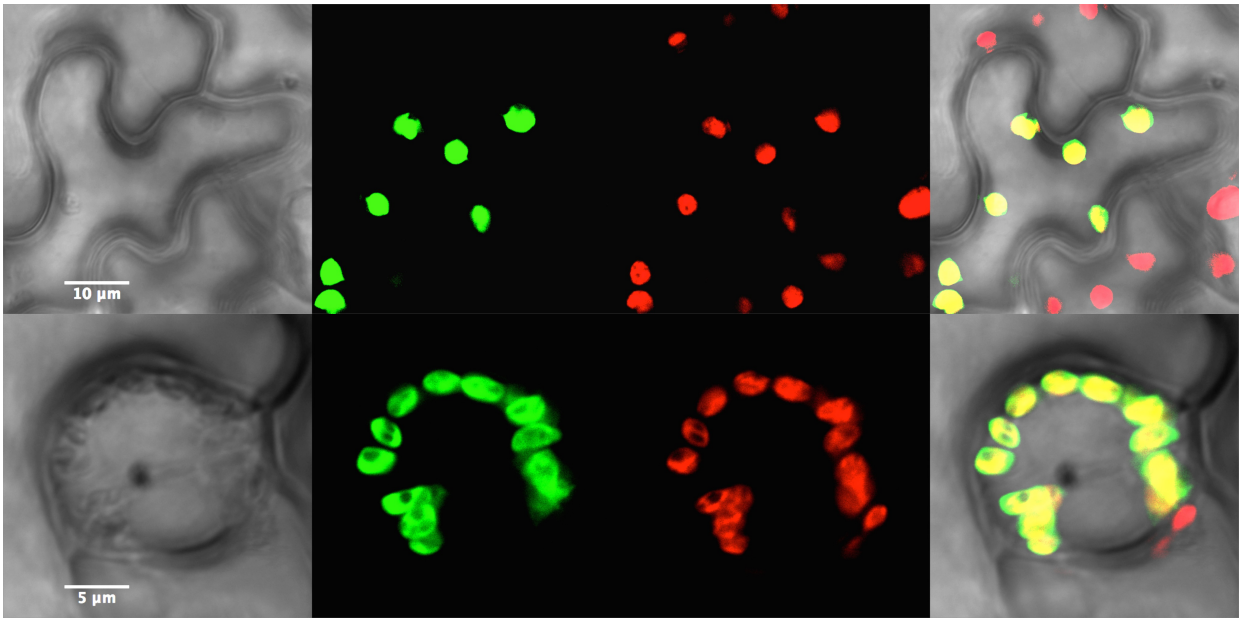


Figure 9: Expression transitoire des protéines de fusion OEX1-GFP et OEX2-GFP dans des cellules épidermiques de feuilles de *Nicotiana benthamiana.* Les images de microscopie confocale montrent le double adressage de OEX1 dans les mitochondries (M) et les chloroplastes (C), ainsi que l'adressage de OEX2 dans les chloroplastes. Les chloroplastes sont identifiés par l'autofluorescence de la chlorophylle. Les mitochondries sont reconnaissables en tant que petites structures rondes et mobiles.

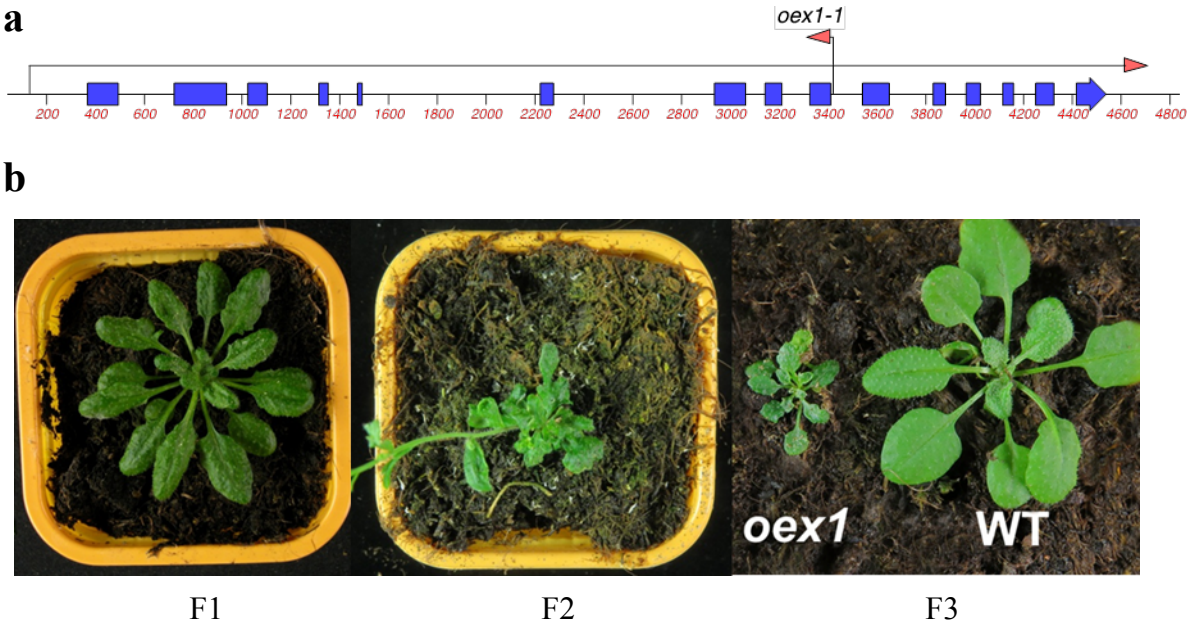


Figure 10: Analyse de lignées mutantes *oex1*. (**a**) Représentation du gène *OEX1* d'*A. thaliana*. Les exons correspondent aux boîtes bleues. La position de l'insertion T-DNA dans la lignée mutée est indiquée, la flèche montrant l'orientation de la bordure gauche du T-DNA. (**b**) Phénotype des plantes *oex1-1* cultivées sur terre. Les plantes étaient affectées plus sévèrement dans leur développement dans les générations F2 et F3. WT, lignée sauvage.

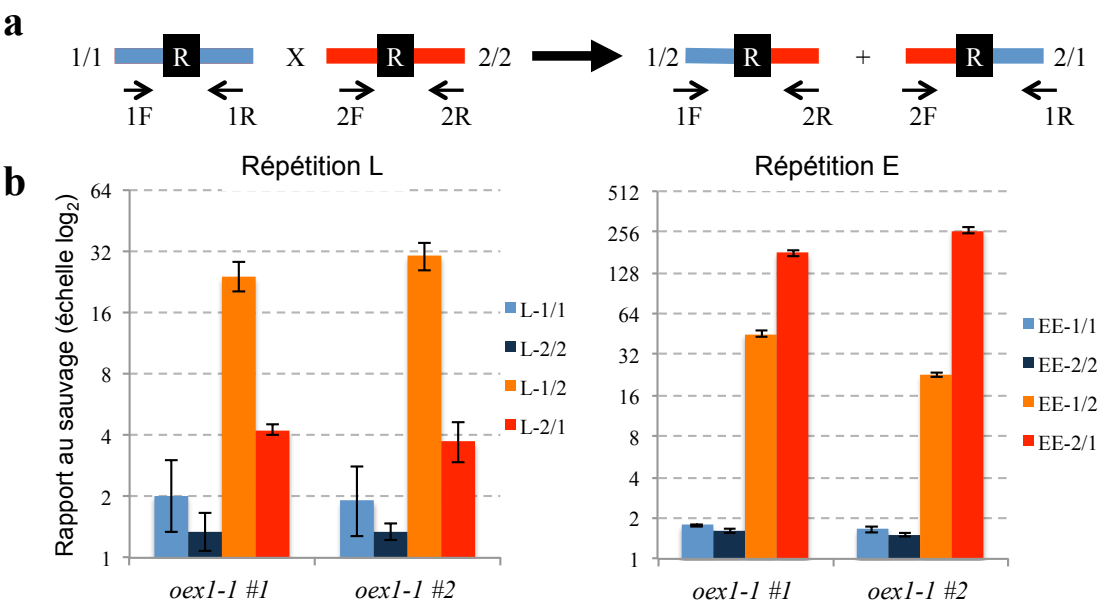


Figure 11: Analyse par qPCR de la recombinaison impliquant les répétitions L et EE. (**a**) Schéma de la quantification relative par qPCR des séquences parentales 1/1 et 2/2 comprenant une même répétition (boîte R) et des produits de crossover correspondants 1/2 et 2/1, en utilisant des combinaisons d'amorces directes (F) et réverses (R). (**b**) Analyse de l'accumulation de produits de crossover dans deux plantes individuelles *oex1-1* (*oex1-1 #1* et #2) par rapport à des plantes sauvages. Les résultats de qPCR pour les répétitions L et EE ont été normalisés contre les gènes de référence actine *ACT1* (At2G37620), polyubiquitine *UBQ10* (At4G05320) et 18S ARNr (18SRRNA). Les résultats sont présentés sur une échelle $\log_2$.

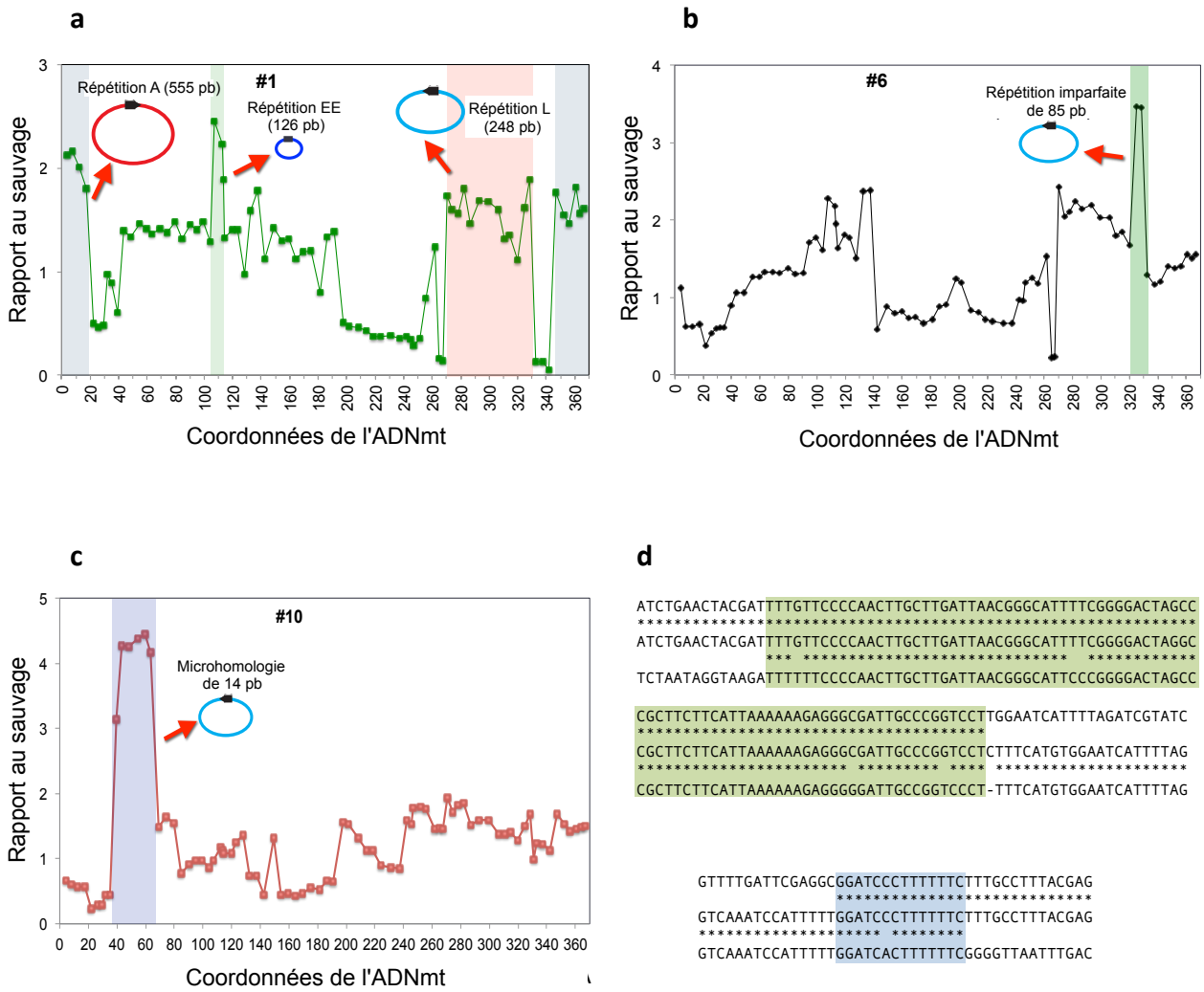


Figure 12: Changements du nombre de copies et de la stoichiométrie des régions de l'ADNmt dans les mutants *oex1*. Ces changements résultent partiellement de recombinaisons impliquant des répétitions en orientation directe qui génèrent des sous-genomes à réplication autonome. Les régions de l'ADNmt dont le nombre de copies était augmenté dans les plantes mutantes #1, #6 et #10 ont été analysées par PCR inverse et séquencées afin de déterminer leurs bordures. Elles contenaient les signatures de recombinaisons impliquant des répétitions directes : des répétitions de plus de 100 pb (répétitions A, EE et L) dans plusieurs régions de l'ADNmt de la plante *#1* (**a**), une courte répétition imparfaite dans un locus spécifique de l'ADNmt de la plante #6 (**b**) et une microhomologie dans la plante #10 (**c**). (**d**) Détail des répétitions imparfaites impliquées dans les événements de recombinaison en (**b**) et (**c**).

domaine est absent dans les deux ADN-polymérases des organelles des plantes. Nous avons préparé des doubles mutants avec ces ADN polymérases (*pol1A/oex1, pol1B/oex1*). Les doubles mutants hétérozygotes *pol1A/oex1* ont montré des phénotypes de feuilles hirsutes, alors que les homozygotes étaient affectés de phénotypes délétères allant jusqu'à la stérilité. Les plantes individuelles *pol1A/oex1* de la génération F2 ont à nouveau montré des changements importants dans la stœchiométrie des régions de l'ADNmt par rapport à des plantes de type sauvage. Mais ces phénotypes moléculaires n'étaient pas plus sévères que ceux des simple mutants *oex1* individuels. De l'ensemble des analyses, OEX1 apparaît comme un facteur impliqué dans les processus de recombinaison mitochondriaux qui est essentiel à la stabilité de l'ADNmt des plantes.

Deux formes de la protéine OEX1, OEX1a et OEX1b, sont produites dans les plantes en raison d'un épissage alternatif des transcrits (Figure 13). Les deux formes ont été surexprimées et purifiées (Figure 14). Les activités biochimiques des protéines purifiées ont été testées sur différents substrats. Les premiers tests ont montré que l'ADN simple brin n'est pas reconnu comme un substrat de dégradation par les deux formes alternatives de OEX1. Mais toutes les structures d'ADN contenant de l'ADN double brin sont reconnues comme substrats et clivées par les deux formes alternatives d'OEX1 (Figure 15). Cette protéine semble donc bien être une nucléase et une candidate pour assurer la résection des extrémités double-brin dans les mécanismes de recombinaison mitochondriaux.

III. Conclusion et perspectives

Nos expériences montrent que le transcriptome mitochondrial d'*A. thaliana* n'est que très peu perturbé par l'adressage de l'ARN *orf77* dans les organelles aux premiers stades du développement des plantes. Ces résultats renforcent les conclusions d'autres travaux réalisés par Adnan Khan Niazi dans notre équipe. Dans ce cas, ce sont des *trans*-ribozymes destinés à invalider spécifiquement des ARN individuels qui ont été adressés dans les mitochondries de plantes transformées d'*A. thaliana* grâce à la navette PKTLS. L'invalidation attendue s'est avérée impossible au stade précoce du développement et rapidement contrecarrée au stade intermédiaire. En revanche, une invalidation efficace a été obtenue à partir du stade plante mature et au delà. Il apparaît donc que le transcriptome mitochondrial des plantes est contrôlé par un mécanisme "tampon" aux premiers stades de développement, les fonctions mitochondriales étant alors essentielles.

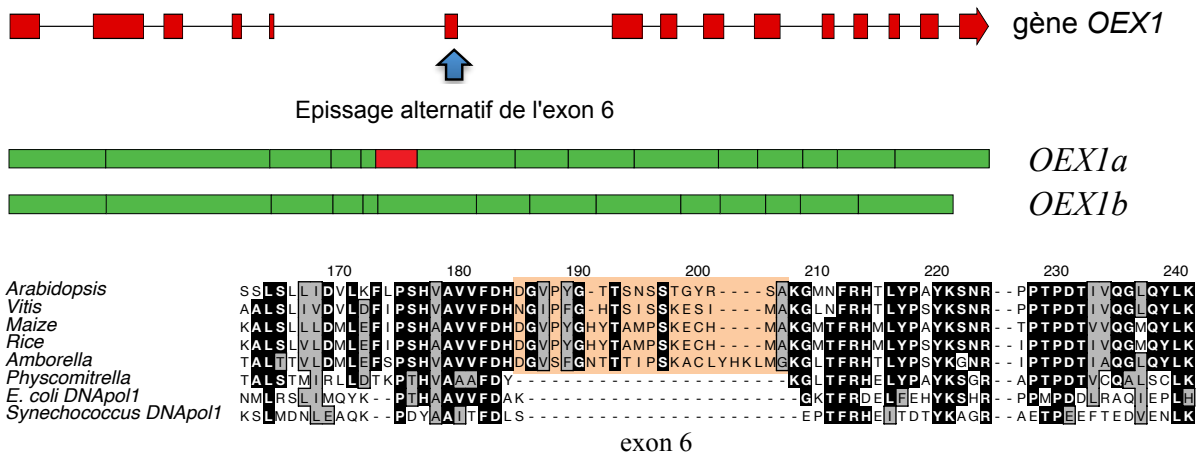


Figure 13: Epissage alternatif de l'exon 6 de *OEX*. Le processus génère deux protéines.

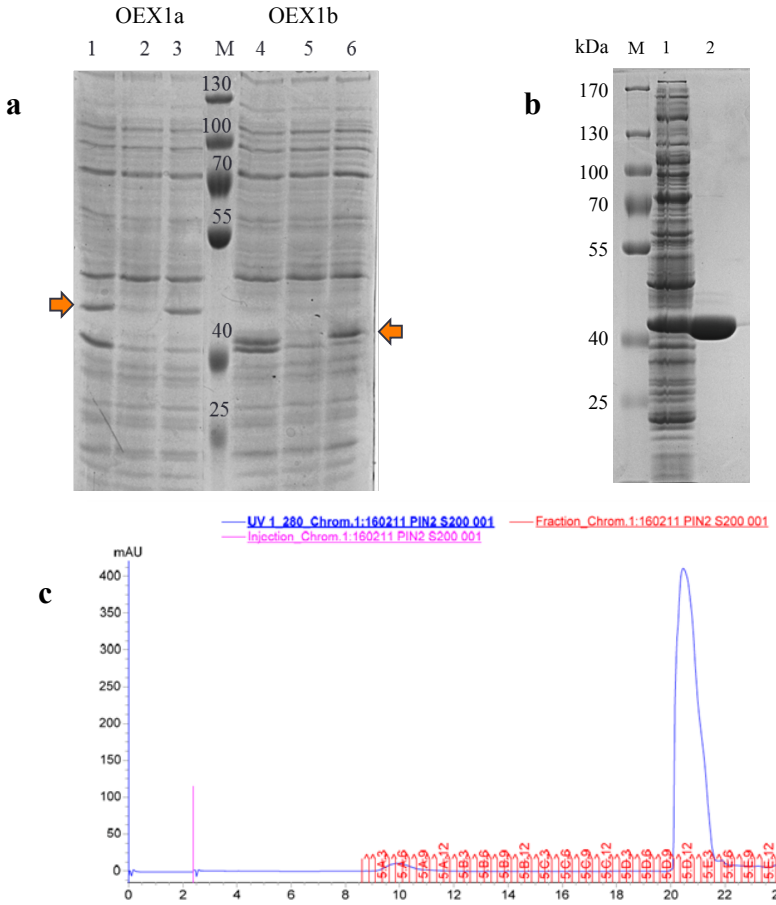


Figure 14: Expression et purification des protéines OEX1. (**a**) OEX1a et OEX1b ont été purifiées à partir d'*E. coli* recombinant par une combinaison de chromatographies IMAC et gel filtration. Pistes 1 et 4, extrait protéique bactérien total après induction à l'IPTG; pistes 2 et 5, protéines solubles totales avant induction à l'IPTG; pistes 3 et 6, protéines solubles totales après induction à l'IPTG; M, marqueur de poids moléculaire. Les protéines recombinantes sont indiquées par des flèches. (**b**) Pureté de OEX1b évaluée par coloration au Bleu de Coomassie. M, marqueur de poids moléculaire; piste 1, fraction soluble totale; piste 2, protéine OEX1b purifiée après IMAC et gel filtration. (**c**) Chromatogramme de gel filtration de OEX1b montrant la monodispersion de la protéine.

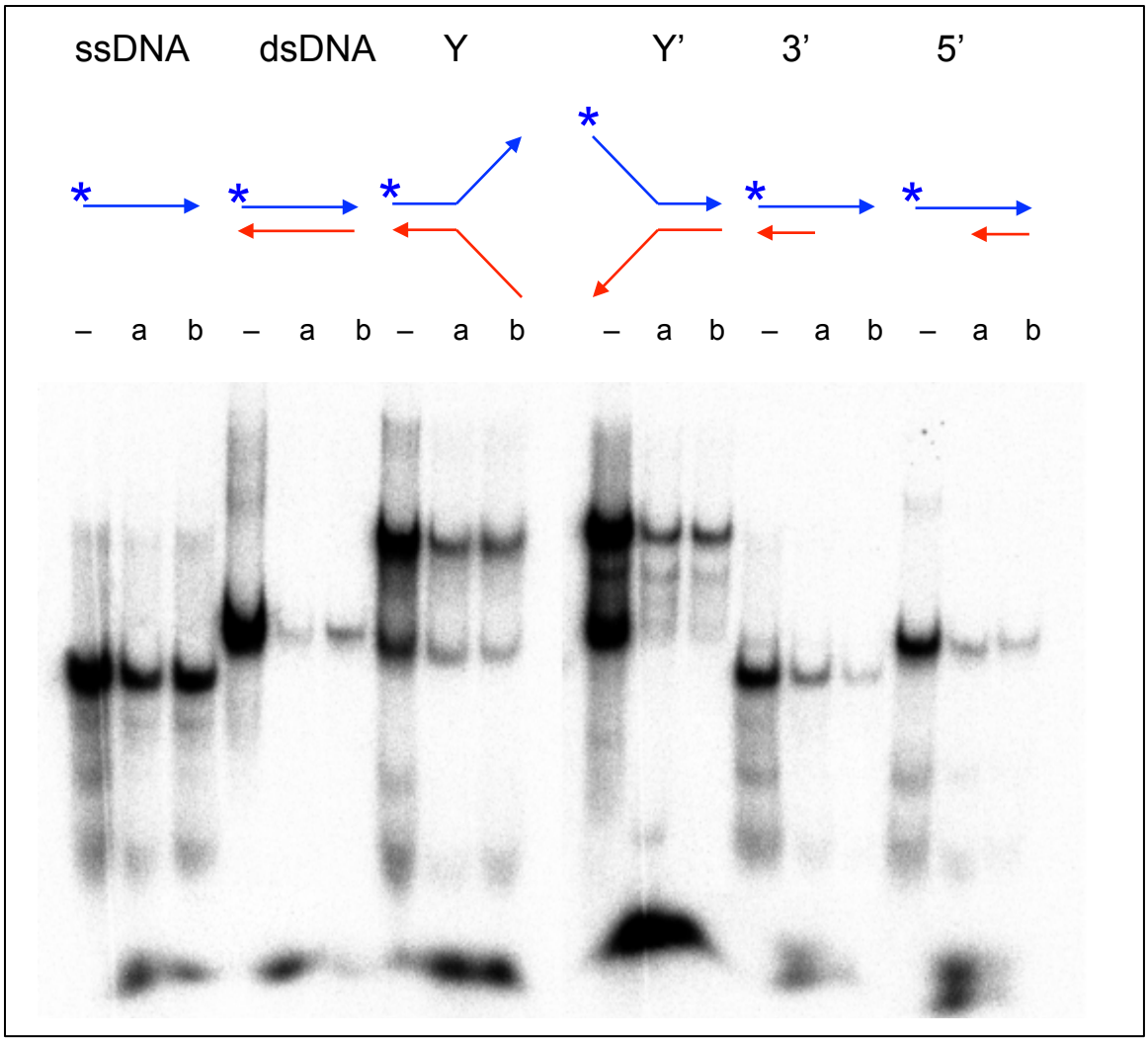


Figure 15: Tests biochimiques de l'activité nucléase des deux formes alternatives de OEX1. Les substrats étaient constitués d'ADN simple-brin (ssDNA), d'ADN double-brin (dsDNA), d'ADN branché (Y et Y'), d'ADN à extrémité 3′-sortante (3') et d'ADN à extrémité 5′-sortante (5'). Les étoiles indiquent le site de radiomarquage 5'. (-) Contrôle du substrat sans addition d'enzyme, (a) addition de protéine OEX1a, (b) addition de protéine OEX1b. Les produits de réaction ont été analysés sur gel de polyacrylamide à 8% et révélés par Phosphorimager.

La suite de ces expériences est de déterminer si l'adressage mitochondrial de l'ARN CMS *orf77* est susceptible de générer une stérilité mâle. Dans un premier temps, nous avons maintenu l'expression de l'ARN *orf77*-PKTLS dans les plantes transformées en ré-additionnant régulièrement de l'oestradiol jusqu'au stade de la floraison. Les plantes *orf77*-PKTLS ainsi traitées ont eu un rendement pondéral inférieur aux plantes de contrôle et ont produit moins de siliques, ce qui suggère un effet possible de l'*orf77* sur la fertilité. Nous préparons actuellement des plantes transgéniques d'*A. thaliana* exprimant l'ARN *orf77*-PKTLS sous promoteur constitutif.

En lien avec la problématique de la diversité génétique mitochondriale, nous avons montré dans la seconde partie du projet que la mutation du gène nucléaire *OEX1* provoque des réarrangements importants de l'ADNmt et affecte la fertilité. Cette approche est donc susceptible d'être appliquée à des espèces d'intérêt économique afin de générer de la variabilité génétique mitochondriale, et à terme des lignées CMS inédites utilisables pour produire des variétés hybrides. D'un point de vue fondamental, les épisomes circulaires générés par la mutation *oex1* seront des substrats pertinents pour l'étude des mécanismes de réplication mitochondriaux. Par contraste, les mutants *oex1* n'ont pas montré de changements notables dans la structure de l'ADN chloroplastique. Les fonctions de la protéine OEX1 dans les chloroplastes sont donc autres que dans les mitochondries et restent à élucider.

IV. Références

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APPROCHES BIOTECHNOLOGIQUES DE L'EXPRESSION ET DE LA DIVERSITE DU GENOME MITOCHONDRIAL DES PLANTES

Résumé

L'ADN mitochondrial des plantes est dynamique et son expression est complexe. Par la voie naturelle d'import d'ARN de transfert codés par le noyau, nous avons adressé dans les mitochondries d'*Arabidopsis* l'ARN *orf77* caractéristique de la S-CMS du maïs et nous avons analysé les effets sur le transcriptome mitochondrial. Celui-ci s'est avéré strictement régulé durant le développement et fortement tamponné aux stades précoces. L'adressage mitochondrial de l'*orf77* a aussi promu un cross-talk avec le noyau. D'autre part, la réplication et la réparation de l'ADN dans les mitochondries de plante impliquent une recombinaison active contrôlée par des facteurs codés par le noyau. Nous avons identifié l'exonucléase 5'-3' potentiellement responsable de la résection des extrémités de l'ADN dans la réparation par recombinaison des cassures double-brin. Nos résultats ouvrent des perspectives pour la génération de diversité génétique mitochondriale et la création de lignées CMS d'intérêt agronomique.

Mots-clés : mitochondries des plantes, régulation génétique, recombinaison de l'ADN, réparation de l'ADN, CMS, exonucléase, trafic des ARN

Résumé en anglais

The mitochondrial DNA of plants is dynamic and its expression is complex. Using a strategy based on the natural import of nuclear-encoded transfer RNAs from the cytosol, we targeted to mitochondria in *Arabidopsis thaliana* the *orf77* RNA characteristic for S-CMS in maize and we analyzed the effects on the transcriptome. The results showed that the mitochondrial transcriptome is tighly regulated during plant development and is strongly buffered at early stages. Mitochondrial targeting of *orf77* also triggered a cross-talk with the nucleus. On the other hand, DNA replication and repair in plant mitochondria involve active recombination controled by nuclear-encoded factors. We identified a new member of this set of factors, the 5'-3' exonuclease potentially responsible for the resection of DNA ends in recombination-mediated repair of double-strand breaks. As a whole, the results open prospects for generating mitochondrial genetic diversity and creating CMS lines with agronomical interest.

Keywords: plant mitochondria, genetic regulation, DNA recombination, DNA repair, CMS, exonuclease, RNA trafficking