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THE MAIZE *EMPTY PERICARP* MUTANTS, A TOOL TO STUDY THE GENETIC CONTROL OF DEVELOPMENT AND THE INTERACTION BETWEEN NUCLEAR AND MITOCHONDRIAL GENOME.

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Ai tanti che mi hanno aiutato
e agli altri che hanno finto di farlo.

LUPUS ET AGNUS

(*Phaedrus*)

Ad rivum eundem lupus et agnus venerant, siti compulsi.
Superior stabat lupus, longeque inferior agnus.
Tunc fauce improba latro incitatus iurgii causam intulit:
"Cur - inquit - turbulentam fecisti mihi aquam bibenti?"

Laniger contra timens :
"Qui possum - quaeso - facere quod quereris, lupe? A te decurrit ad meos haustus liquor."
Repulsus ille veritatis viribus:
"Ante hos sex menses male - ait - dixisti mihi".

Respondit agnus:
"Equidem natus non eram!"
"Pater, hercle, tuus - ille inquit - male dixit mihi!"
Atque ita correptum lacerat iniusta nece.

Haec propter illos scripta est homines fabula qui fictis causis innocentes opprimunt.

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1. ABSTRACT

The empty pericarp4 (*emp4*) gene encodes a mitochondrion-targeted PentatricoPeptide Repeat (PPR) protein that is necessary for the correct regulation of mitochondrial gene expression in the endosperm. The two main objectives of this thesis are to understand the role exerted by EMP4 during plant development, and to study the action of *emp4* at the molecular level.

Homozygous mutant *emp4* embryos are retarded in their development and unable to germinate; therefore to examine the role of the *emp4* gene during seedling development homozygous mutant seedlings were obtained from the cultivation of excised immature embryos on a synthetic medium. When exposed to light, after 30 days of culture, 44% of mutant embryos germinated and only few of them produced a seedling. In contrast, in dark condition mutant embryos have a germination rate of 83% and the number of plants that reached the first and the second leaf stage were almost doubled.

A genetic stock carrying an *Ac* transposon and a double *Ds* element located on the long arm of chromosome one, where *emp4* resides, had been crossed with +/*emp4* plants. Chromosome breakages, induced by *Ds* in somatic tissues, are expected to produce in heterozygous plants clonal sectors hemizygous for the *emp4* mutation. Clearly distinguishable yellow sectors were isolated from wild-type green leaves and their hemizygous genotype was confirmed by PCR. Hemyzigous and heterozygous tissues as well as mutant first leaves and primary roots recovered from both light and dark treatment, were analyzed at light microscopy and by TEM analysis.

Typical root anatomy with the peculiar root cell layers was observed in both wild-type and in mutant samples, however in mutant epidermis and hypodermis cell layers were less ordered and alterations in the cell shape were evident, particularly in light. Moreover, comparison between mutant and wild-type root morphology, indicated that mutant roots either grown in the dark or exposed to light were less developed then wild-types and the cell layers epidermis and hypodermis were not properly organized.

All the mesophyll compartments (epidermis, vessels and parenchyma) were clearly detectable in both mutant and wild-type tissues in both conditions. In the dark, mutant tissues were composed by smaller cells with abnormal shapes, moreover in both genotypes, chloroplasts, that were distinguishable in wild-type leaf grown in light treatment, could not be observed. In the light, mutant leaves were smaller in comparison with wild-type and displayed a pale green color. Abnormalities were founded in sections of homozygous mutant plants, like alteration in cell shape and size, a smaller population of chloroplasts and lack of nuclei.

Changes in the subcellular structure were highlighted from the comparison of wild-type and mutant tissues by means of transmission electron microscopy. In *emp4* mutant leaves, mitochondria as well as chloroplast populations were significantly reduced and both organelles displayed a less organized structure. In particular, the main alteration were observed in leaf tissues exposed to light while dark-grown tissues seem to be less affected by the effect of *emp4* mutation.
Many alterations were detected from the comparison of hemyzigous and heterozygous sectors. Such alterations are similar to those observed in homozygous mutant leaf tissues derived from embryo rescue. Thus, we concluded that *emp4* gene is essential to leaf tissues in order to develop and maintain a proper cellular organization. These alterations resemble (for some aspects) in part the macro-autophagy model and in part the model for senescence. Light exposure had a deep effect on cell morphology of *emp4* cells, as if a magnification in the senescence process was triggered by this signal, particularly visible in the deterioration of the whole cellular components within the mutant cells.

Despite the growing number of studies in PPR field the mechanism of action of PPR proteins is still elusive. In addition it is not clear if PPR proteins act alone or with some molecular partners. There are few well documented cases in which PPR proteins are confirmed to be associated in protein complexes where PPR could act as adapters, recruit some additional factors on the RNA target or, in addition, work in a dimeric state like the *Arabidopsis* HCF152.

A complementation test was conducted to address the genetic relationship between emp4 and emp9475 mutants. The test showed controversial results; one gene was inferred from the lack of complementation observed in F1 and two genes based on the observation of a segregation close to the 9 to 7 ratio, expected when the heterozygous *emp* F1 plants identify two genes.

Two hypotheses have been postulated. The first one is that the two mutants are allelic and a third *emp* gene segregates in the F2/F3 progenies. The second is that the two mutants are ascribable to two linked genes whose product interact. If the second hypothesis were correct, *emp9475* would have been a good candidate for the isolation of a partner of *emp4*.

Heterozygous females were crossed with heterozygous or hyperploid B-A translocation males with the aim of establishing the chromosomal arm location of *emp9475*. The F1 revealed the mutant was obtained from crosses involving the TB-1L-a male parent, thus indicating that *emp9475* lies on the long arm of chromosome 1. A more refined position for *emp9475* was achieved by analysis of simple sequence repeat (SSR) marker distribution in a segregating population obtained by crossing heterozygous females with B73, Mo17 and LEL (Large Embryo Line) inbred male parents. A polymorphism for the marker bnlg1347 established a distance of about 1 cM (1 recombinant out of 13) between this marker and *emp9475* on the long arm of chromosome 1 at 1.10 bin. These data confirmed the linkage between the two genes. Different molecular analyses were performed on emp9475 mutants, however the origin of the mutation was not revealed and its function remains to be elucidated.

A second approach was conducted in parallel to elucidate the mechanism of action of EMP4. The full EMP4 protein as well as two fragment of EMP4 comprising the N and C terminal domains respectively were produced in *E. coli* using pBAD-Thio-TOPO expression vector and LMG194 strain. The amount of the putative EMP4-thioredoxin fusion protein, produced in *E. coli*, tend to increase during time of induction until the 70 hours. Prolonged inductions (over 70 hours) seem to had no effect on further accumulation of the protein. Low growing temperature (29 °C) and arabinose concentration at 2%, during the induction period, increase the amount of protein produced by the bacteria.

2. INTRODUCTION

2.1 THE SUPER-FAMILY OF PPR PROTEINS

Pentatricopeptide repeat proteins (PPR) are a family of proteins discovered by Small and Peters (2000) by mean of computation analysis on the *Arabidopsis* genome. The first study on a PPR protein dated 1995, performed by Manthey and McEwen, described the *Pet309* gene of *Saccharomyces cerevisiae* whose product is required for the stability and transcription of the mitochondrial *cox1* gene. PPR proteins have been isolated in every eukaryote analyzed by man to the *Chlamydomonas reinhardii* algae (**Figure 1**; Lurin et al. 2004).

Bioinformatic analysis showed that PPRs are mainly targeted to organelles and over 50% of the PPR gene products have a N-terminal signal that routes the fully functional protein within the mitochondria (Lurin et al. 2004); however no PPR genes have been founded in *Rickettsia prowazekii* and *Synechocystis* spp. that are considered the closest ancestors to mitochondria and chloroplasts indicating that the PPR gene family have evolved after the two endosymbiotic events that generates these organelles (Margulis 1981). Outside the plant kingdom PPR proteins are, without exception, mitochondrial proteins although some may also have roles in other compartments (Mili and Pinol-Roma 2003; McArthur et al. 2000). The main feature of PPR proteins is a tandem array of 35 aminoacids that forms two antiparallele alpha elices; this is called PPR motif (Small and Peters 2000). PPR proteins have from 2 to 26 motif; PPRs have been classified on the basis of the PPR motifs present in the protein sequence (Lurin et al. 2004). This classification showed that some PPRs possess ulterior domains (E, E+ and DYW) that could have catalytic functions (**Figure 2**; Aubourg et al. 2000; Lurin et al. 2004). PPR motif is very similar to TPR (TetratricoPeptide Repeat) motif that is known for the capacity to bind other proteins (Small and Peters 2000) therefore PPR are suspected to be proteins involved in interaction with other macromolecules like DNA, RNA or proteins. The functional characterization of PPR in different species (**Figure 4**; man, *Drosophila*, protozoan, algae and land plants) suggests that PPRs play an active role as RNA interacting protein but so many PPRs exist especially in plants and a general model for their functions is yet to be understood (reviewed in Woodson and Choury 2008). The vast majority of studies propose that PPRs are effectors of organellar transcription; however, some authors raise the question that some PPR could bind the DNA (Ding et al. 2006;

Organism	Sequences	PPR Hits
Homo sapiens	37,490	6
Drosophila melanogaster	17,087	2
Caenorhabditis elegans	20,673	2
Schizosaccharomyces pombe	5,010	6
Saccharomyces cerevisiae	6,304	5
Trypanosoma brucei	16,757	19
Cyanidioschyzon merolae	4,772	10
Arabidopsis thaliana	28,581	470
Oryza sativa	74,385	655
Ralstonia solanacearum	5,118	1
Rickettsia prowazekii	834	0
Synechocystis sp	3,169	0

FIGURE 1 Diffusion of PPR proteins in different organisms (adapted from Lurin et al. 2004).

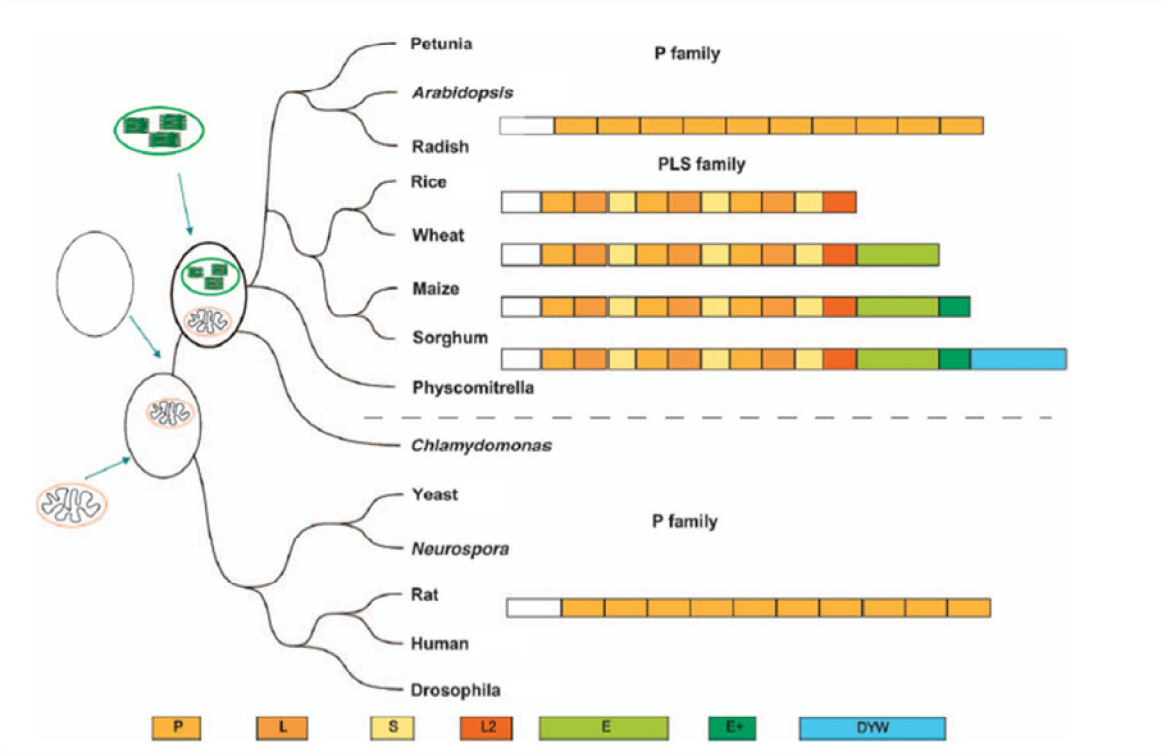


FIGURE 2 The PPR family. Typical structure of proteins from each of the principal sub-families and sub-groups. P, L, S, L2, E, E+, and DYW are the specific domains that are assembled in PPR family to originate the different PPR proteins (adapted from Andres et al. 2006).

Pfalz et al. 2005; Shadel 2004). In the transcription world the number of processes in which PPRs are involved varies from splicing of plastidial mRNA (Hattori et al. 2007), accumulation of chloroplastic transcript (Lown et al. 2001, Yamazaki et al. 2004; Meierhoff et al. 2003), cleavage of plastidial mRNAs (Hashimoto et al. 2003) and editing (**Figure 3;** Kazama et al. 2003; Kotera et al. 2005; Okuda et al. 2006). Mutations in PPR genes are associated with emb phenotypes in *Arabidopsis* and maize or to defective kernel phenotypes in maize (Cushing et al. 2005; Gutierrez-Marcos et al. 2007).

Cytoplasmic male sterility is a maternally inherited trait leading to pollen abortion and thus converts normally hermaphroditic plants into females (reviewed in Budaret al. 2003 and Chase 2006). In the vast majority of cases, the sterility-inducing factor is a novel open reading frame (ORF) in the mitochondrial genome that codes for a hydrophobic protein capable in some way of altering mitochondrial function leading to pollen abortion. CSM can be suppressed by the action of nuclear genes known as restorer of fertility (*Rf*), with the single exception of *Rf2* of maize, encoding an aldehyde dehydrogenase (Cui et al. 1996). All the identified *Rf* correspond to PPR proteins (Bentolila et al. 2002; Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003; Akagi et al. 2004; Oguchi et al. 2004; Klein et al. 2005; Shmitz-Linneweber et al. 2005). The mechanism of fertility restoration is not clearly understood but it has been proposed that PPRs could stabilize the mitochondrial transcription in CSM through abolishing the expression of chimeric ORFs that causes the fertility failure (Chase 2006).

The origin of PPR gene family is unclear and the wide colonization of plant genome, where PPR are one of the most abundant super-family of proteins, remains to be addressed. Some authors suggest that PPR have evolved with a mechanism like “Birth and Death” initially described for immunoglobulins (Nei et al. 1997) and adapted for the resistance genes by Michelmore and Meyers (1998). Also divergence and duplication may be the possible mechanisms by which the PPR family has expanded in plants (Geddy and Brown 2007). Geddy and Brown (2007) also suggested that PPR are under a great selective pressure to alter their sequences, thus creating changes that will diversify the PPR population. This evidence supports the hypothesis that PPR proteins act as sequence specific binding proteins, requiring changes in their own sequence to match the sequence they will bind.

The position of PPR gene in the genome of *Arabidopsis* (Lurin et al. 2004), petunia (Bentolila et al. 2002), radish (Brown et al. 2003) and rice (Kazama et al. 2003; Komori et al. 2004; Akagi et al. 2004) has been extensively studied. No clusters of PPR genes have been discovered apart from the chromosome 1 of *Arabidopsis* that bears 19 putative PPR genes (or pseudogenes) in approximately a 1 Mb region (Lurin et al. 2004).

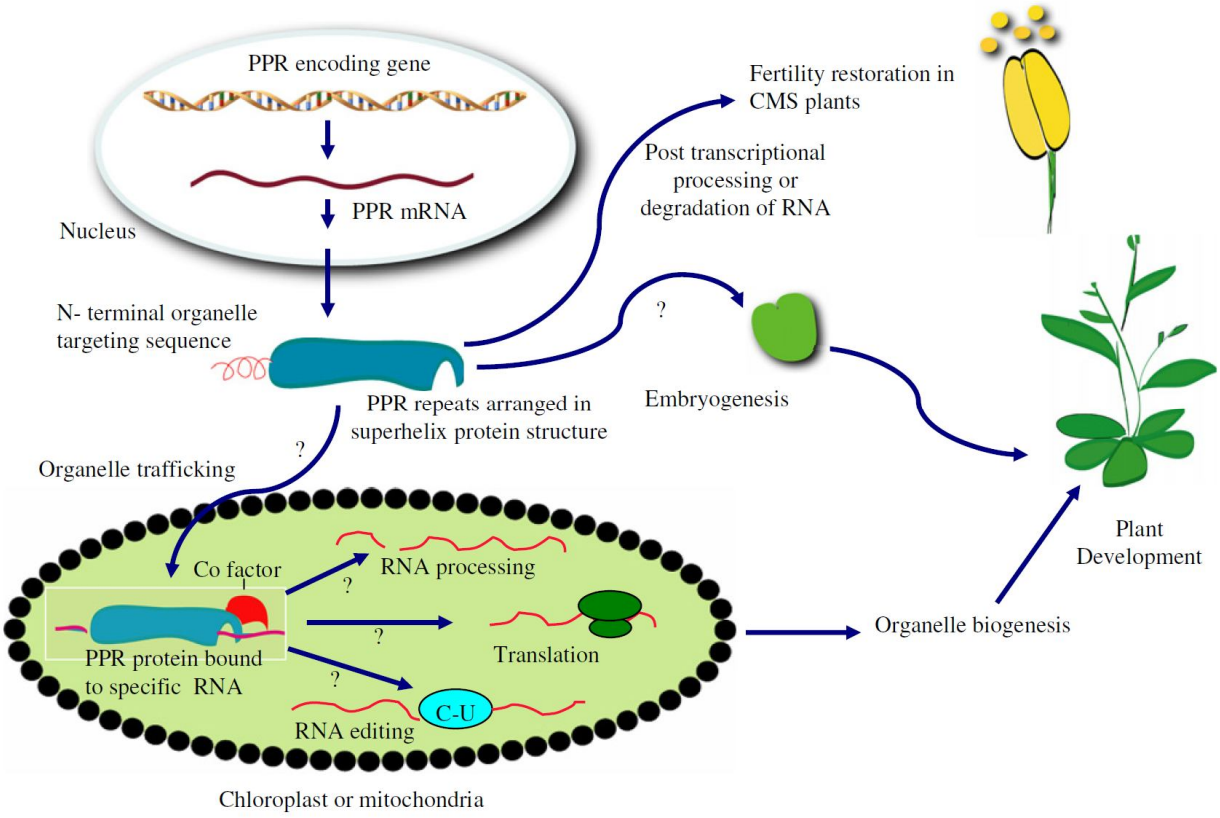


FIGURE 3 Functions proposed for PPR proteins (from Saha et al. 2007).

PPR protein-encoding gene	Organism	Localization (Predicted, Confirmed)/ target RNA (Predicted, *in vivo* association, *in vitro* confirmation)	Possible function or involvement	Loss of function phenotype
Plants				
CRP1	Maize	Chloroplast stroma (C) /*psaC* 5' UTR (*in vivo*), *petA* 5' UTR (*in vivo*), *petD* (P)	Translation of *petA*, *psaC* mRNA, processing of *petD* mRNA	Reduced cytochrome *f* (*petA*) and photosystem I subunit (*psaC*) protein, unprocessed polycistronic *petD* mRNA, loss of cytochrome b_6f complex, decrease of photosystem I activity
CRR2 (*At3g46790*)	*Arabidopsis*	Chlorplast (P) /*rps7-ndhB* pre-mRNA (P)	Controls *ndhB* expression by regulating intragenic processing of *rps7-ndhB* transcript	Decreased NdhB protein levels, reduced chloroplast NA(P)DH dehydrogenase complex activity
CRR4 (*At2g45350*)	*Arabidopsis*	Chloroplast (C) /*ndhH* (*in vitro*)	Binds *ndhD* mRNA to recruit RNA editing machinery	Reduced NdhH protein, reduced chloroplast NA(P)DH dehydrogenase complex activity
EMP4	Maize	Mitochondria (C) /unknown	Mitochondria development, seed development	Lethal loss of endosperm development in seeds, reduced levels of mitochondrial *rps2A*/*rp2B*, *rps3*/*rpl13*, *mttb* RNA's
Grp23 (*At1g10270*)	*Arabidopsis*	Nucleus (C) /RNA polymerase II, subunit III (*in vivo*)	Transcriptionally regulates early embryo development through interactions with RNA polymerase II	Loss of proper embryonic development
GUN1 (*At2g31400*)	*Arabidopsis*	Chloroplast (C) /unknown	Chloroplast development, Retrograde signaling	*gun* mutant, does not repress photosynthesis-related nuclear genes after chloroplast photo-oxidative damage or translation inhibition
HCF152 (*At3g09650*)	*Arabidopsis*	Chloroplast stroma (C) /*petB* pre-mRNA(*in vitro*)	Controls processing of *psbB-psbT-psbH-petB-petD* polycicstronic RNA	Impaired *petB* intron splicing/stabilization, impaired endonucleolytic cleavage of *psbH-petB* mRNA transcript, reduced cytochrome b_6f complex activity
LOI1 (*At4g14850*)	*Arabidopsis*	Mitochondria (P) /unknown	Post-transcriptionally regulates 3-hydroxy-3-methylglutaryl coenzyme A reductase and isoprenoid biosynthesis	Insensitive to isoprenoid biosynthesis inhibitors lovastatin and clomazone
MCA1	*Chlamydomonas reinhardtii*, (eukaryotic algae)	Chloroplast (C) /*petA* (P)	Regulates *petA* mRNA transcript stability	Reduced levels of *petA* mRNA transcript, reduced levels of cytochrome f (*petA*) protein, reduced activity of cytochrome b_6f complex
OTP3 (*At1g74910*)	*Arabidopsis*	Mitochondria (P) /*nad1* (*in vivo*)	Required for *trans*-splicing of intron 1 of *nad1* mRNA	Reduced levels of Complex I activity. Defects in seed development and germination
PGR3 (*At4g31850*)	*Arabidopsis*	Chloroplast (P) /*petL* (P), *ndhD* (P)	Regulates stabliziation/translation of *petL* mRNA and translation of *ndhD* mRNA	Reduced *petL*, *petG* mRNA, reduced NdhD protein, reduced level of cytochrome b_6f and NA(P)DH dehydrogenase complex activities
LRPPRC, *LRP130*	Human	Mitochondria (C), nucleus (C) /*coxI*, *coxIII* (P)	Regulates the stability/ translation of *coxI*, *coxIII* mRNA's	Leigh syndrome French Canadian, cytochrome *c* oxidase deficiency
Protists				
TbPPR1-TbPPR8	*Trypanosoma brucei* (protist causes African Sleeping Sickness)	TBPPR1-7: Mitochondria (C) TBbPPR8: Cytosol (C) /TbPPR5: 12S rRNA (*in vivo*)	TbPPR2-7 involved in mitochondrial ribosome assembly/function	Defects in oxidative phosphorylation and growth in glucose medium, *TbPPR2-7* mutants have reduced mitochondrial rRNA levels
Fungi				
Cya-5	*Neurospora crassa*	Mitochondria (P) /*coxI* (P)	Required for the stabilization/translation *cox1* mRNA	Reduced cytochrome *c* oxidase activity
PET309	*Saccharomyces cerevisiae*	Mitochondrial inner membrane (C) /*coxI* 5' UTR(P)	Required for the normal processing/ stabilization of *coxI* pre mRNA and its translation	Reduced cytochrome *c* oxidase activity, reduced levels of mature *coxI* mRNA and COXI protein

PPR2	Maize	Chloroplast stroma (C)/ unknown	Required for the synthesis or assembly of chloroplast translation machinery	Chloroplasts lack ribosomes, albino seedlings
PPR4	Maize	Chloroplast stroma (C) /1st intron of *rps12* pre-mRNA (*in vivo*)	Regulates *rps12 trans*-splicing and chloroplast ribosome biogenesis	Defective in *rsp12 trans*-splicing, albino seedlings lacking chloroplast ribosomes
PPR531-11	*Physcomitrella patens* (moss)	Chloroplast (P) /*clpP* (P)	Regulates intergenic cleavage between *clpP* and 5'-*rps12* mRNA and splicing of *clpP* mRNA	Reduced levels of photosystem II reaction center protein D1 (*clpP*), abnormal chloroplast morphology
PTAC2 (*At1g74850*)	*Arabidopsis*	Chloroplast (C) /unknown	Required for normal chloroplast encoded RNA polymerase-dependent transcription	Reduction of chloroplast transcription, albino seedlings
Tbc2	*Chlamydomonas reinhardtii*, (eukaryotic algae)	Chloroplast stroma (C) /*psbC* 5' UTR (P)	Regulates translation of *psbC* mRNA as part of a protein complex	Reduced levels of chlorophyll-binding photosystem II reaction center subunit P6 protein (*psbC*), reduced photosystem II activity
Plant CMS restorers				
Rf1a, PPR791, PPR8-1	Rice	Mitochondria (P) /B-*atp6*/*orf79* (*urf-rmc*) (P)	Dominant restorer of BT-type CMS, reduces levels of aberrant CMS-associated protein, promotes endonucleolytic cleavage of *urf-rmc* RNA, role in *atp6* mRNA editing	Male sterility, accumulation of aberrant *urf-rmc* protein product
Rf1b	Rice	Mitochondria (P) /B-*atp6*/*orf79* (*urf-rmc*) (P)	Dominant restorer of BT-type CMS, reduces levels of aberrant CMS-associated protein, destabilizes *urf-rmc* mRNA	Male sterility, accumulation of aberrant *urf-rmc* protein product
Rfk1, ORF687	Radish	Mitochondria (P) /*orf125* (P)	Dominant Kosena-CMS restorer of fertility, decreases accumulation of CMS-associated protein ORF125 but does not decrease transcript levels	Male sterility, accumulation of ORF125 protein product
Rfo	Radish	Mitochondria (P) /*orf138* (P)	Dominant Ogura (*ogu*)-CMS restorer of fertility, decreases accumulation of CMS-associated protein ORF138 but does not decrease transcript levels	Male sterility, accumulation of ORF138 protein product
Rf-PPR592	Petunia	Mitochondria (C) /*pcf* (*in vivo*)	Dominant CMS restorer of fertility, decreases accumulation of CMS-associated protein PCF	Male sterility (CMS), accumulation of CMS associated protein PCF
Animals				
BSF	*Drosophila melanogaster*	Cytoplasm (C) /*IV/V* RNA (3' UTR of bicoid (*bcd*) mRNA) (*in vitro*)	Regulates stability of *IV/V* RNA during oogenesis	Reduced levels of IV/V RNA

FIGURE 4 List of PPR proteins characterized so far (adapted from Woodson and Choury 2006).

2.2 EMP4 IS A MAIZE MITOCHONDRIAL PPR PROTEIN

The *empty pericarp4* gene of maize encodes a PPR protein composed by a signal peptide that lead the protein within the mitochondria, two domains with unknown function (at the N and C-termini), nine PPR motifs and one PPR-like motif (**Figure 5**; Gutierrez-Marcos et al. 2007). The *emp4* gene was cloned by means of a gene-tag approach achieved with *Mutator* transposon. Homozygous mutant seeds are lethal and impaired in the germination; mutant seeds are clearly distinguishable in a segregating ear as early as 10 DAP because of the pale, translucent, and collapsed appearance of mutant caryopses. Mutant seeds are clearly altered in the Basal Embryo Transfer Layer (BETL) cells both morphologically and functionally. In fact the expression of BETL specific markers is altered in *emp4* seeds. In addition, the accumulation of starch is reduced in the central starchy endosperm of homozygous kernels (Gutierrez-Marcos et al. 2007). The *emp4* gene is expressed in almost every tissue analyzed with the single exception of anthers in which the *emp4* transcript levels are low. However, *emp4* mRNA could not be detected by RNA gel blot analysis, indicating that it is expressed at very low levels. EMP4 protein is targeted in mitochondria were it regulates a small sub-set of mitochondrial transcript, that are down-regulated in mutant kernels (Gutierrez-Marcos et al. 2007). Thus, *emp4* gene action is needed within the mitochondria, and its absence is much more evident especially in those cells whose metabolic activity is higher during the seed development. One of the most metabolic active tissues in the seed is the BETL layer; in the mutant seeds the BETL cells are much more vacuolated and their mitochondrial population is reduced (**Figure 6**). Therefore, it has been concluded that *emp4* is essential for the development of maize kernel linking the mitochondrial functionality to the proper development of the seed (Gutierrez-Marcos et al. 2007).

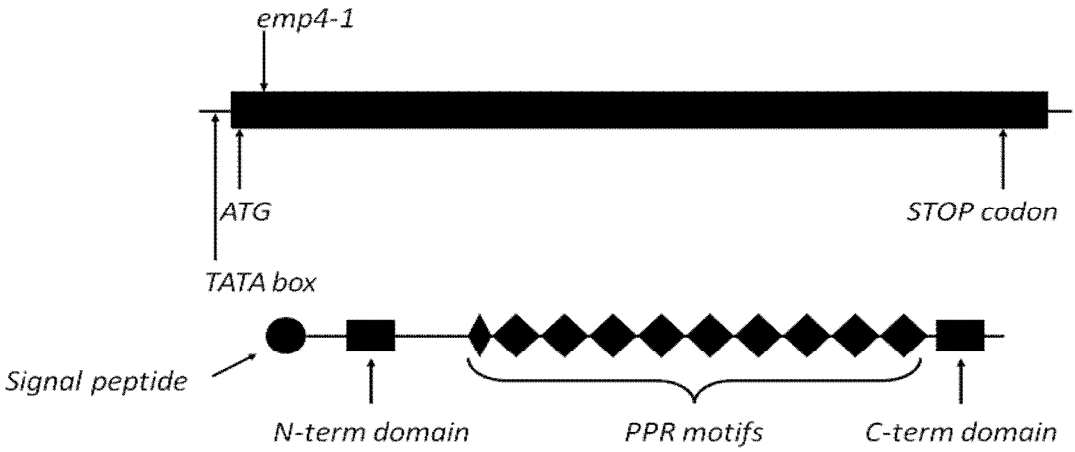


FIGURE 5 Organization of *emp4* gene and its protein structure (adapted from Gutierrez-Marcos et al. 2007).

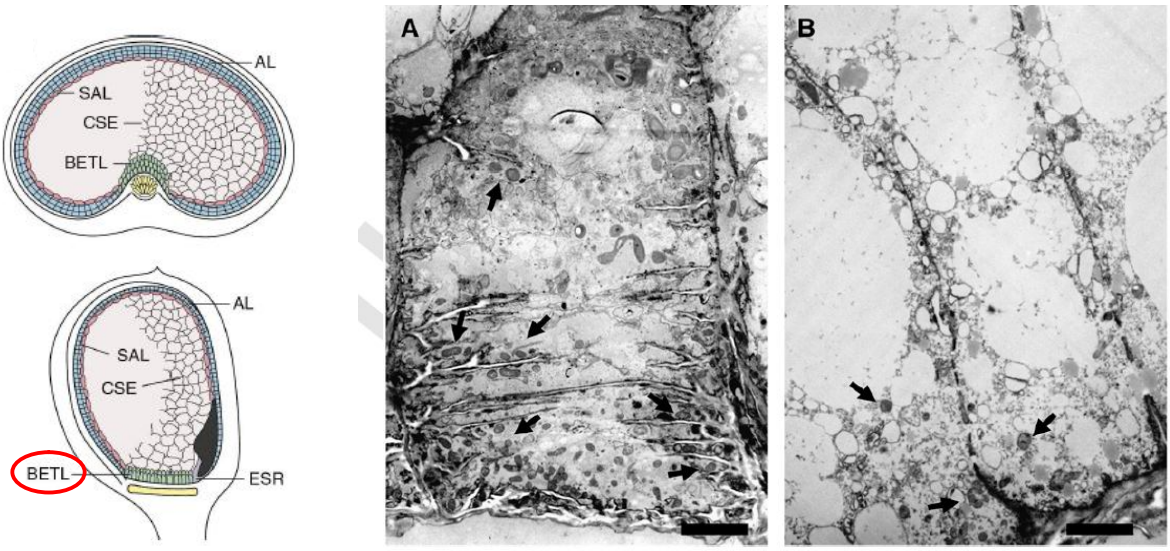


FIGURE 6 Morphology of BETL cells. On the left: schematic representation of the main compartments of maize kernel. On the right electron microscopy images of wild-type (A) and mutant (B) transfer cells, arrows indicates the mitochondria. Bar = 2 μm (adapted from Gutierrez-Marcos et al. 2007).

2.3 THE MITOCHONDRIAL COMPARTMENT

Mitochondria are semiautonomous organelles that perform a variety of fundamental functions in the cell ranging from energy production to program cell death. The vast majority of the ATP production in a plant cell is associated with energy-trasducing membranes, i.e. the inner mitochondria membrane and thylakoid membrane of chloroplasts.

The generation of ATP is coupled to the controlled dissipation of the proton electrochemical gradient; membranes allow compartimentalization of protons by the action of primary proton pumps (Mitchell 1979). In the mitochondria the primary proton pumps comprise complexes I, III and IV; they generate a high gradient of protons that triggers the action of the secondary proton pump, the ATP synthase. Any proton that leaks across the membranes would cause a short circuit that uncouples the proton motive force from the ATP synthase. The energy-transducing membranes must, therefore, be essentially closed and have a high resistance to proton flux (**Figure 7**; Mitchell 1979).

Palade's model (Palade, 1952), also called the baffle model, described the invaginations of the inner mitochondrial membranes, the cristae, as random wide in-folds of the membrane while Sjostrand (1953) suggested that the cristae were composed of a stack of independent membranous lamellae. The recent ultra-structural analysis through ultra-resolution scanning electron microscopy and electron tomography revealed that neither model was entirely correct (**Figure 8**; Mannella 2006). The inner mitochondrial membranes are mainly composed by tubular cristae that are structurally different from the rest of the inner mitochondrial membranes. In addition cristae are connected to the inner boundary membrane-by-membrane tubules called crista junctions. According to these findings mitochondria are composed by at least six compartments: outer membrane, inner boundary membrane, intermembrane space, crista membranes, intercristal space and matrix (Perkins and Frey 2000; Mannella2006). Mitochondria have a complex dynamic internal structure, the number of crista junctions and the morphology of the intercristal space change in relationship to the metabolic state of the mitochondria that is in turn tightly related to the metabolic condition of the whole cell (Hackenbrock 1968; Mannella et al. 1994, 1997).

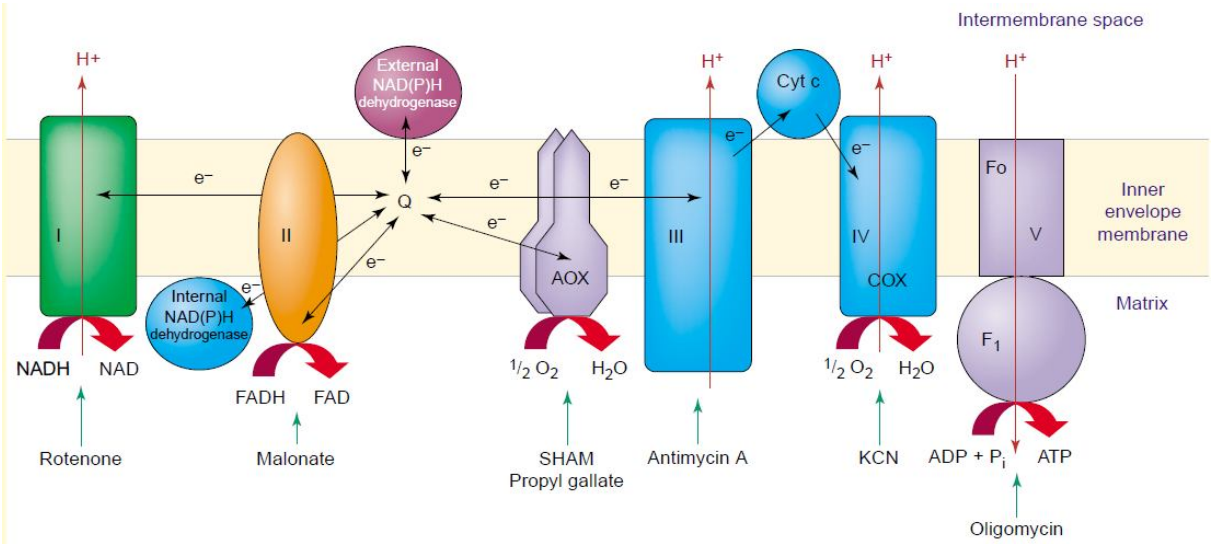


FIGURE 7 Mitochondrial oxidative electron transport chain and its typical inhibitors. Abbreviations: AOX = alternative oxidase; COX = cytochrome oxidase; Cyt c = cytochrome c; SHAM = salicylhydroxamic acid (from Raghavendra and Padmasree 2003).

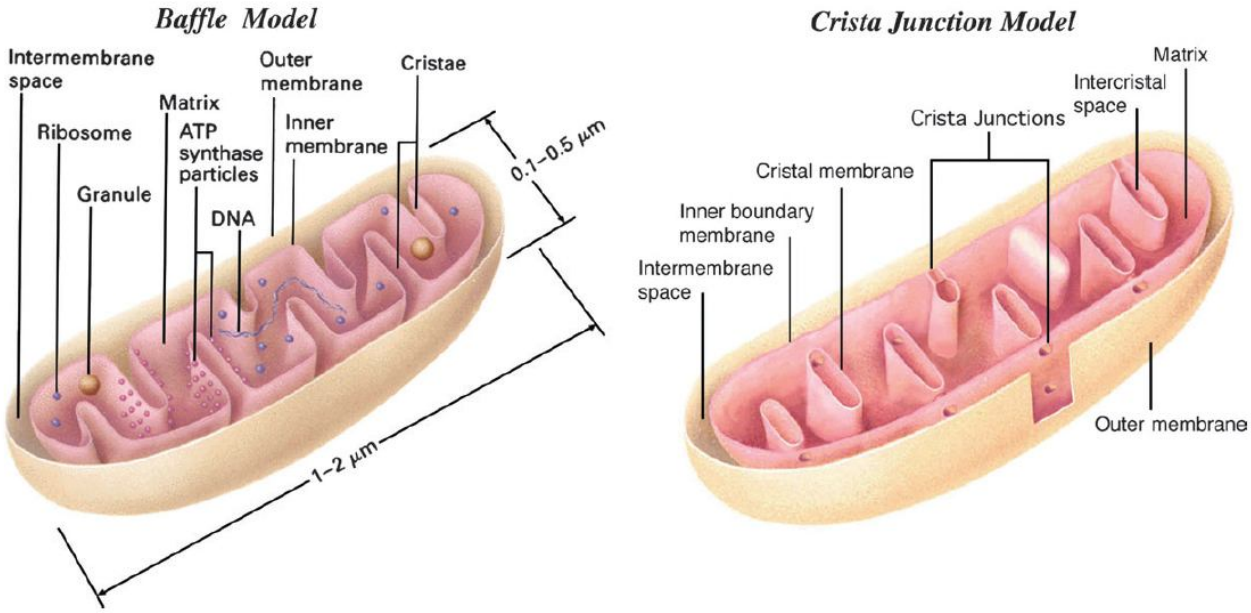


FIGURE 8 Models of mitochondrial membrane structure. On the left: baffle model. On the right: crista junction model (from Logan 2006)

In the orthodox state, corresponding to partial matrix expansion, the intercristal space is compressed and tubular with few crista junctions connecting the inner boundary membrane. In the condensed state, corresponding to partial matrix contraction, the intercristal spaces are dilated and there are numerous intercristal membrane connections and crista junctions. The switch from orthodox to condensed state, analyzed during imbibitions of maize kernels, is related to the changes in mitochondrial energy supply and the redox state of the cell (**Figure 9**; Logan et al. 2001). In addition, according to computational analysis and computer simulations, the condensed morphology of mitochondria is more favorable to the production of ATP (**Figure 10**; Mannella 2006; reviewed in Logan 2006).

Mitochondria inner membrane morphology is linked to the biochemical status of the cell, also it has been suggested that the motility of the organelle helps ensuring that mitochondria are located where they are needed. Physical association between mitochondria and either energy-requiring structures or organelles has been well described in a variety of systems (Munn 1974; Tyler 1992; Bereiter-Hahn and Voth 1994; Hales and Fuller 1996, 1997). Particularly in photosynthetic tissues mitochondria are generally associated to chloroplasts; this association is assumed to facilitate the biochemical exchanges between the two organelles (Stickens and Verbelen 1996; Logan and Leaver 2000; Foissner 2004). The association of mitochondria with endoplasmic reticulum is thought to be related the delicate equilibrium of the Ca^{2+} that is an important second messenger of the cell and may modulate the mitochondrial functionality (Rizzuto et al. 1998; Rutter and Rizzuto 2000).

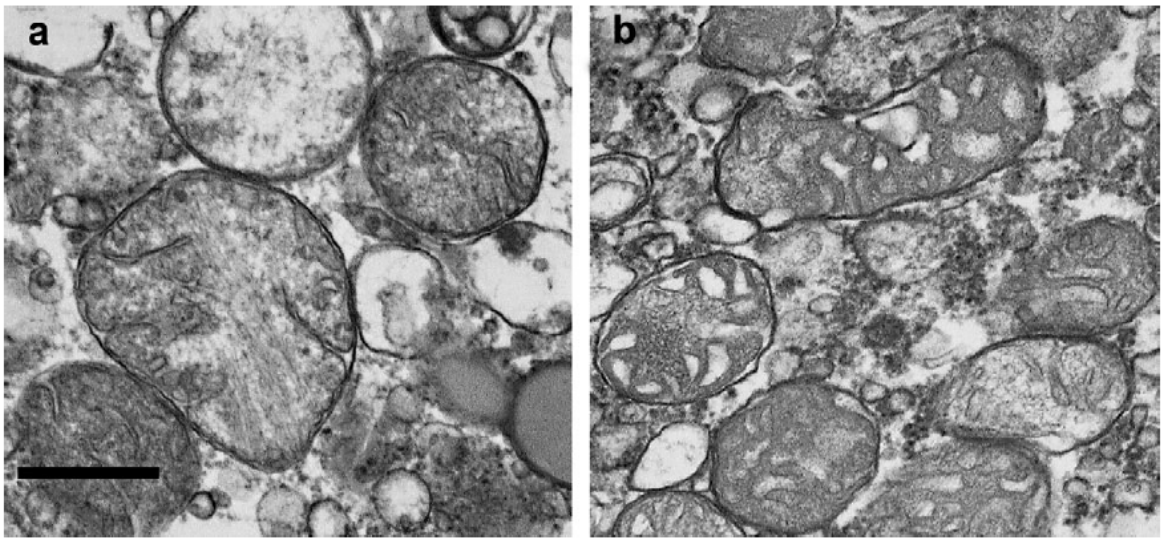


FIGURE 9 Conformation of internal structure in mitochondrial purified from germinating maize embryos. A) orthodox B) condensed. Bar = 500 nm (from Logan et al. 2001)

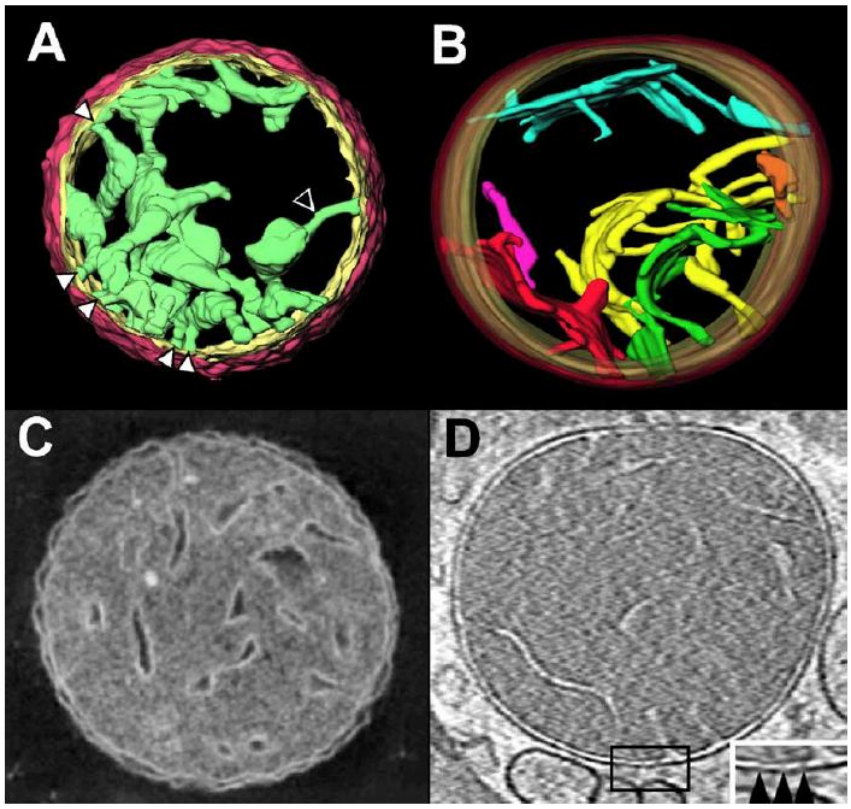


FIGURE 10 Tomographic reconstruction (A and B) of rat liver mitochondria in condensed (A and C) and orthodox (B and D) conformations (from Mannella 2006).

2.4 HIGHER PLANT MITOCHONDRIA

Mitochondria are organelles originated from a bacterial ancestor (Margulis, 1981); the presence of a small genome is one of the best proofs of their endosymbiotic origin. Thus, mitochondria cannot arise *de novo*; the only way to generate new mitochondria is from fission of existing ones (Gray et al., 1999). Mitochondria are semiautonomous organelles and each organelle needs 1000 to 2000 different types of proteins but it encodes only 13 to 66 genes (depending on species). The remaining part of the proteins, approximately 95% of the proteome, needed is encoded by nuclear-cytosolic system, than imported through specific pores (the TOM/TIM complexes) in the organelle (Herrmann, 2003).

A part from the specific characteristics of plants and animals (like autotrophy or the different ability to avoid stress factors and many more) there are many differences between plant and animal mitochondria. Even though they share many features like size, morphology, their energetic role inside the cell and their virtual uniparental inheritance, animal and plant mitochondria differ in many characteristics. Their genome is organized and controlled in different ways (Schuster and Brennike 1994, Kubo and Newton 2008). They have particular biosynthetic ability, and their role in some cellular processes deeply differ (reviewed in Kubo and Newton 2008). Although both plant and animal mitochondrial genomes include genes for ribosomal RNAs, tRNAs and several subunits of the oxidative phosphorilation complexes are only present in plants genes for some ribosomal proteins and some of the proteins involved in the biogenesis of cytochrome *c* (Clifton et al. 2004). Even the gene expression is quite different between plants and animals mitochondria. In plant mitochondrial genome, it has been reported, the presence of cis- and trans-splicing events (Schuster and Brennike 1994) and of co-trascriptional units particularly when intergenic regions are small (Unseld et al. 1997). As a result plant mitochondrial genomes are much larger and more complex than animal and fungal counterparts (Palmer, 1990; Wolstenholme and Fauron, 1995).

The presence of two enzymes, an additional NAD(P)H dehydrogenases and an alternative oxidase in the inner membrane (Vanlerberghe and McIntosh 1997; Moller and Rasmusson 1998), confer to the plant mitochondria more flexibility in controlling the energetic metabolism of the cell. Moreover, in photosynthetic tissues, mitochondria are partners of chloroplasts with respect to CO_2 light-dependent assimilation, due to their implication in the

photorespiratory pathway (Douce et al. 1994), and their contribution involves the oxidation of glycine, at very high rates, in the matrix space (Vauclare et al. 1996). Plant mitochondria play also a role in many different metabolisms and it has been reported that they are implicated in the synthesis of essential cofactors such as biotin (Baldet et al. 1997), folate (Rébeillé et al. 1997), lipoate (Gueguen et al. 2000) and vitamin C (Siendones et al. 1999). Therefore, the mitochondrial compartment is one of the greatest sites for biochemical reactions in the cell and works in a coordinated fashion with the cellular environment.

2.5 GENE EXPRESSION IN HIGHER PLANT MITOCHONDRIA

Plant mitochondrial genes encode 3 rRNAs, 15 to 20 tRNAs and about 30 proteins, which are the sub-units of respiratory chain complexes, cytochrome c-type maturation or ribosomal proteins. Thus, all mitochondrial genes are either directly or indirectly required for oxidative phosphorilation. The gene expression in plant mitochondria is a very complex process that differs from nuclear gene expression in many ways.

Here follows a summary of the main features of gene expression in plant mitochondria.

Promoters and cryptic transcripts. One of the most peculiar features of plant mitochondria is the structure and organization of their genome (reviewed by Kubo and Newton 2008). Mitochondrial genes are transcribed by nuclear encoded T7 phage-type RNA polymerases. These enzymes are capable of promoter recognition, initiation and elongation but require auxiliary factors to recognize all transcription initiation sites *in vivo* (Kuhn et al. 2007). In monocotyledonous plants the consensus motif of the conserved promoter type is centered on four conserved nucleotides just upstream from the first transcribed nucleotide (Rapp and Stern 1992). A second part of the element resides about 10 nucleotides further upstream and is characterized as an AT-rich region of six residues. In dicotyledonous plants, the promoter structures of this type are much better conserved, since a stretch of nine nucleotides shows considerable sequence identity between different genes as well as between different species (Hoffmann and Binder 2002). The conserved nonanucleotide region extends into the transcribed region with two nucleotides. Further downstream into the transcribed region, the identity of the next two nucleotides was also found to be

important for efficient promoter activation (Hoffmann and Binder 2002). Thus, many of the transcripts, at least those from this conserved type of promoter in dicotyledonous plants, will begin with the same nucleotides, characteristically the GA dinucleotide. This feature of the promoter overlapping the transcription initiation site has been observed in phage-type polymerases, and indeed RNA polymerases of this class have been identified for plant mitochondria as well as plastids (Binder and Brennicke 2003). Promoter motifs were identified by a combination of sequence analysis and functional studies using *in vitro* transcription systems (reviewed in Gagliardi and Binder 2007). Following the sequencing of the *Arabidopsis* mitochondrial genome, it became apparent that potential promoters exist in intergenic regions (Dombrowski et al. 1998). In addition, a number of genuine genes do not show the typical promoter consensus motifs raising the possibility that different sequences can initiate transcription (Binder et al. 1994; Dombrowski et al. 1998; Gagliardi and Binder 2007). This multiplicity of promoter sequences also results in the frequent initiation of transcription in intergenic regions or on the opposite strand of genuine genes. To make things worse, no efficient transcription termination mechanism seems to exist in plant mitochondria. Therefore, once initiated, transcription gives rise to RNAs with variable sizes and sometimes to extremely large transcripts. For instance, primary transcripts of *atp9* and *atp8* can reach more than ten times the size of the mature mRNA (Perrin et al. 2004). Thus, the lack of transcription termination mechanisms contributes significantly to the transcription of intergenic regions. The combination of large intergenic regions with the duplication of promoters by recombination, the relaxed promoter specificity, and the absence of termination mechanisms, account for the massive production of cryptic transcripts in plant mitochondria (**Figure 11;** Binder and Brennicke 2003). It cannot be excluded that some of these transcripts could correspond to functional non-coding RNA. However, intergenic transcripts are not conserved between closely related species and are often transcribed from recombined regions of various genetic origins.

It is therefore expected that most intergenic RNA correspond to illegitimate, cryptic transcripts (reviewed in Holec et al. 2008).

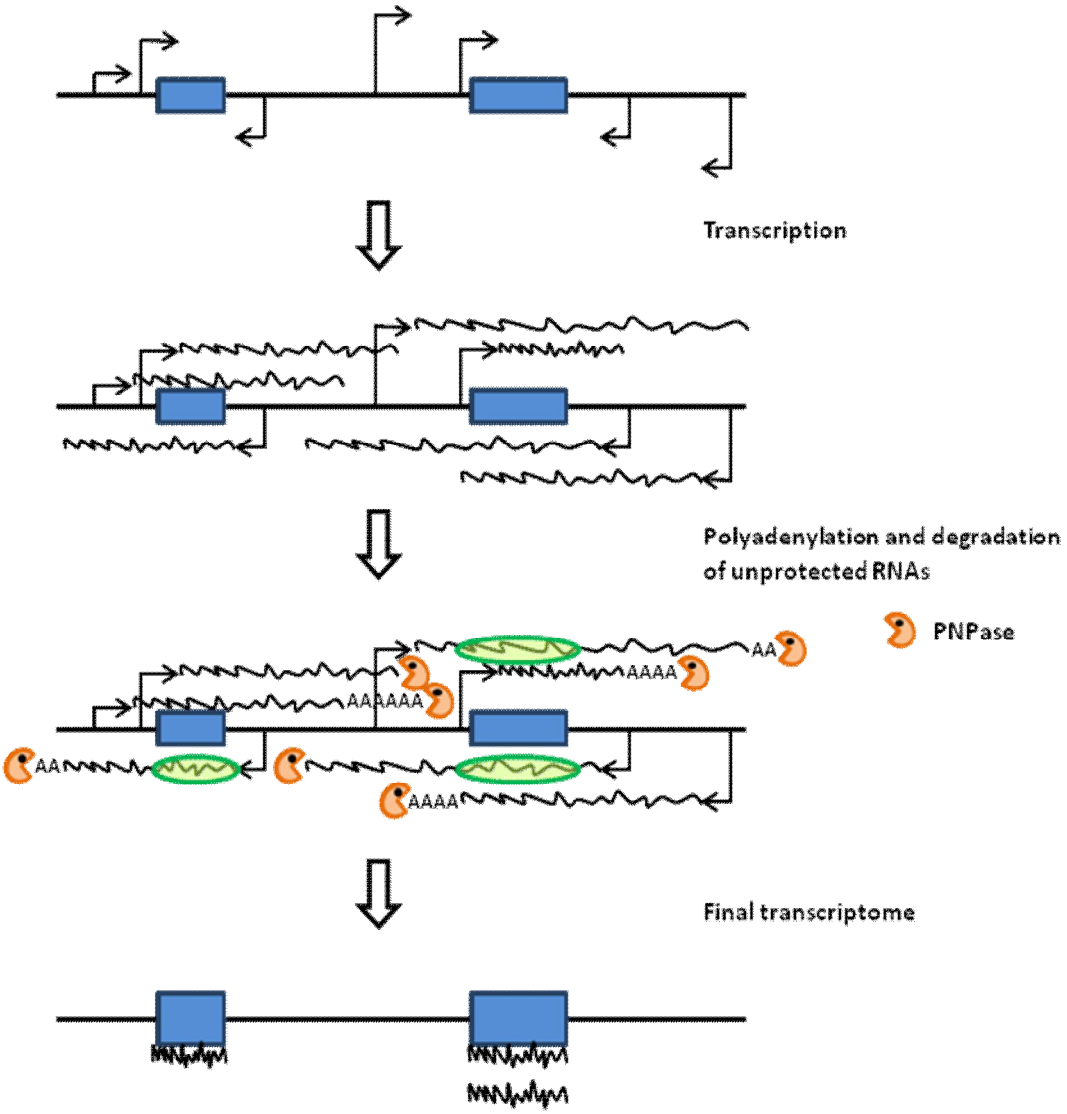


FIGURE 11 RNA surveillance in plant mitochondria. Cryptic transcripts result from transcription initiated in intergenic regions, or antisense to genuine genes, as well as lack of transcription termination. Transcripts are polyadenylated and degraded by PNPase unless stabilized by specific stability factors. Rectangles: mitochondrial genes; Bent arrows: promoters; Undulate lines: transcribed RNAs (modified from Giegé and Brennicke 2001).

Control of RNA stability. Mitochondrial RNA is abundantly transcribed from regions lacking bona fide genes (Finnegan et al. 1990). This extraordinary production of cryptic and potentially deleterious transcripts is apparently efficiently counterbalanced by mechanisms controlling RNA stability in plant mitochondria. tRNA and rRNA are protected from degradation by interacting with their specific protein partners. Molecules, which are misfolded, misprocessed or simply in excess, cannot interact with respective binding proteins and are degraded. In most mitochondria, mRNAs display common features: poly(A) tails in both humans and *Trypanosoma* or a conserved sequence in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Ojala et al. 1981; Lukes et al. 2005; Schafer 2005). In contrast, plant mitochondrial mRNAs lack any obvious generic feature that could be responsible for their stabilization. Initially, stem–loop structures located at 3' extremities of several mRNA were thought to act as common stability signals. Although these terminal structures can partially protect some transcripts against 3' to 5' exoribonucleases (Kaleikau et al. 1992; Dombrowski et al. 1997; Kuhn et al. 2001), a systematic mapping of 3' extremities in *Arabidopsis* revealed that actually only very few mRNAs terminate with such stable secondary structures (Forner et al. 2007). In addition, only ORF sequences are highly conserved between plant species, while both 5' and 3' UTR of any given mitochondrial mRNA are extremely divergent even between closely related species. These data indicate that no common primary or secondary structure elements are present at mRNA extremities in plant mitochondria. Mitochondrial mRNA stability is in fact controlled individually by specific nuclear-encoded factors. The existence of these factors was first shown by genetic approaches while studying CMS (Cytoplasmic Male Sterility) systems. The action of most mitochondrial genes responsible for CMS is restricted to a specific nuclear background and can be counterbalanced by nuclear genes called *Rf* for restorer of fertility (Hanson and Bentolila 2004). Expression of *Rf* genes restores fertility by preventing the expression of the CMS-linked gene in all known cases except one (Hanson and Bentolila 2004). The rice *Rf* gene that affects the stability of the *Boro II* CMS-associated mRNA has recently been identified and is a member of the PPR gene family (Wang et al. 2006). The majority of plant PPR proteins are targeted to chloroplasts and mitochondria. PPR proteins function as molecular adapters in which PPR motifs recognize a specific RNA sequence. Growing genetic

and biochemical evidence indicates that individual PPR proteins may be linked to specific post-transcriptional processes for a unique or limited number of transcripts (Lurin et al. 2004; Andres et al. 2006). These processes include editing, splicing, 3' and 5' end maturation, translation and stabilization of transcripts (Lurin et al. 2004; Andres et al. 2006). Thus, the amounts of mitochondrial mRNAs are not regulated by modulating the activities of mitochondrial promoters, but by the expression of nuclear proteins controlling the stability of mitochondrial messengers. In contrast, most cryptic transcripts probably lack the target sequences recognized by the specific stabilizing factors and therefore fail to accumulate. The particular mechanism of mRNA stabilization in plant mitochondria could thus explain why abundant transcription of cryptic transcripts is tolerated in plant mitochondria.

Editing. RNA editing can be broadly defined as any site-specific alteration in an RNA sequence that could have been copied from the template, excluding changes due to processes such as RNA splicing and polyadenylation. Changes in gene expression attributed to editing have been described in organisms from unicellular protozoa to man, and can affect the mRNAs, tRNAs, and rRNAs present in all cellular compartments (reviewed in Gott and Emerson 2000). The majority of RNA editing events that have been identified so far involve changes in mRNA sequences and result in the production of altered protein products (**Figure 12**). Creation of new start and stop codons by uridine insertion and cytidine to uridine (C-to-U) conversions has been observed in trypanosomatid protozoa, plant organelles and man (Chang et al. 1998; Steinhauser et al. 1999; Stuart et al. 1997). Stop codons are also subject to removal by U-to-C changes in plants, most frequently in hornworts (Yoshinaga et al. 1996). Internal changes include: creation of open reading frames (ORFs) by widespread nucleotide insertions (Benne 1994; Miller et al. 1993), frameshifting between alternative ORFs (Kolakofsky and Hausmann 1998), amino acid substitutions (Skuse et al. 1996; Nagalla et al. 1994; Sharma et al. 1994) and changes in introns (Wissinger et al. 1991). "Silent" codon changes are also observed, but more often editing creates codons for highly conserved or functionally essential amino acids. Alterations within 5' and 3' untranslated regions (UTRs) are rare (Covello and Gray 1990; Schuster et al. 1991; Shaw et al. 1988), but such changes could potentially affect mRNA stability, translatability, and processing.

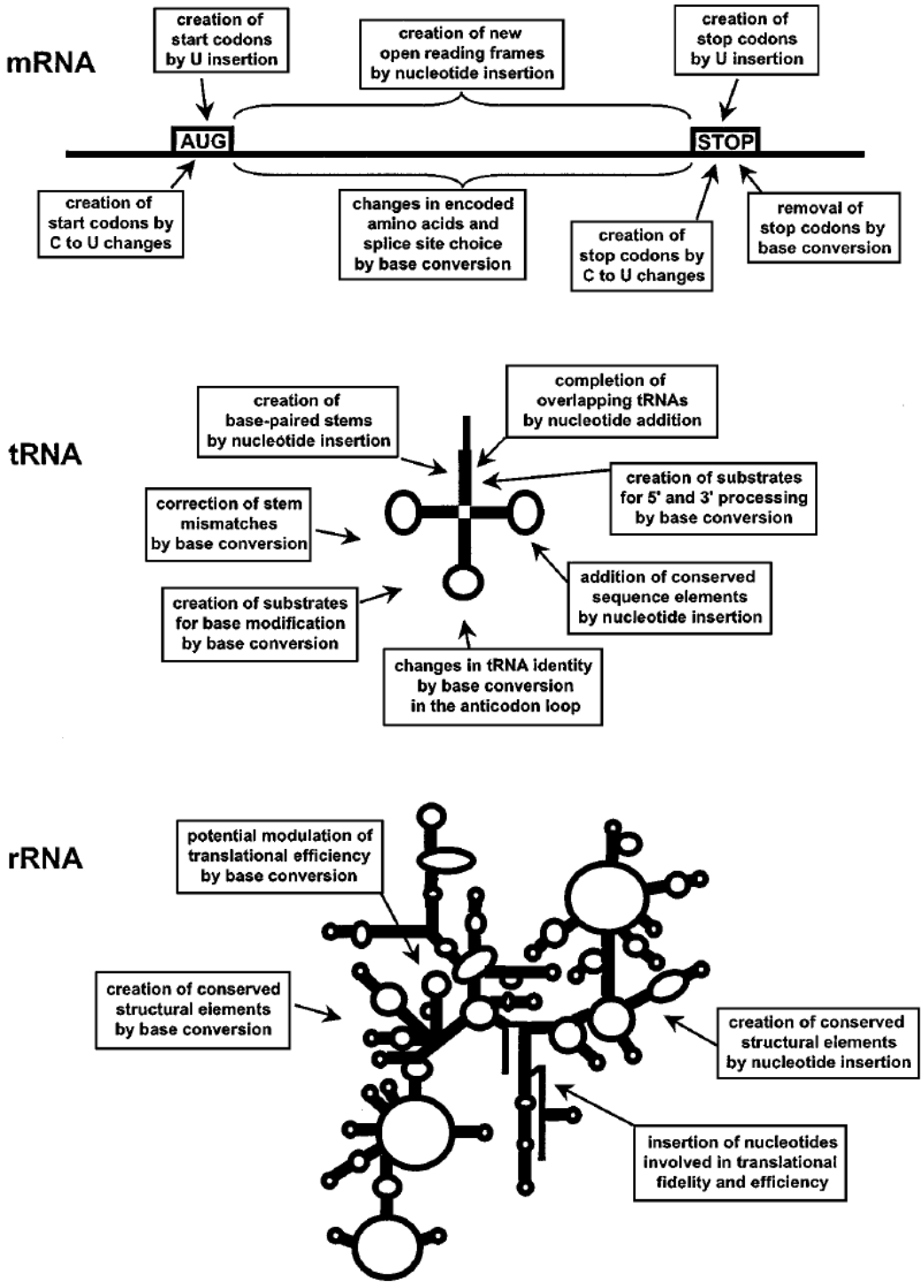


FIGURE 12 Functions of RNA editing (from Gott and Emarson 2000).

Degradation of transcripts. As transcripts fail to accumulate in absence of corresponding stabilizing factors, an efficient pathway for RNA degradation must operate by default in plant mitochondria. This process involves mtPNPase, a 3' to 5' exoribonuclease that shows sequence homology with bacterial PolyNucleotide Phosphorylase (PNPase). Downregulation of mtPNPase expression results in the accumulation of several classes of RNA species: mRNA and rRNA precursors, chimeric ORFs, rRNA and tRNA maturation byproducts, antisense RNA and intergenic transcripts (Perrin et al. 2004a; Perrin et al. 2004b; Holec et al. 2006). With the exception of precursors, these transcripts can be considered as illegitimate transcripts. PNPase rapidly degrades illegitimate transcripts. A remarkable feature of illegitimate transcripts is their polyadenylation status: up to 80% of cryptic transcripts are polyadenylated. Functional mRNAs are not constitutively polyadenylated in plant mitochondria. Nevertheless, the addition of poly(A) tails to a fraction of mitochondrial mRNAs was reported in different plant species such as sunflower, maize, pea, *Oenothera* and *A. thaliana*. Both in vivo and in vitro experiments demonstrated that poly(A) tails can trigger RNA degradation in plant mitochondria (**Figure 13**; Gagliardi et al. 2001; Perrin et al. 2004a; Perrin et al. 2004b; Khun et al. 2005; Holec et al. 2006). The destabilizing effect of polyadenylation was discovered in *Escherichia coli* and subsequently reported in chloroplasts (Dreyfus and Regnier 2002; Bollenbach et al. 2004); polyadenylation can also trigger the degradation of certain nuclear transcripts in yeast (Vanacova et al. 2005; LaCava et al. 2005; Wyers et al. 2005) and probably in other eukaryotes (West et al. 2006; Slomovic et al. 2006). Thus, the ancestral role of polyadenylation in triggering RNA degradation has been conserved throughout evolution. It is therefore somewhat surprising that polyadenylation plays diverse roles in mitochondria from different organisms (Gagliardi et al. 2004).

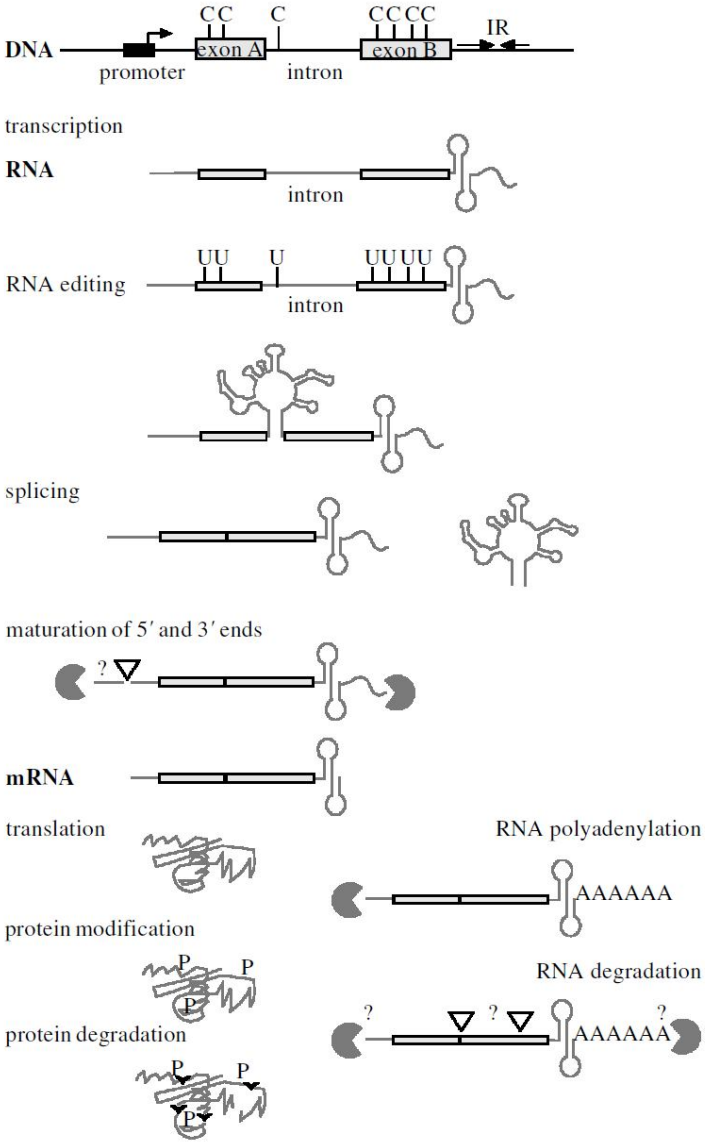


FIGURE 13 Transcription in higher plant mitochondria. The maturation process includes RNA editing, intron splicing, and trimming of 5' and 3' termini. Control of gene expression may be exercised at any of these steps. In addition, gene activity may be modulated at the promoters, and during and after translation of the mRNA. Gene expression is terminated by degradation of the mRNA, which can be enhanced by polyadenylation (from Binder and Brennicke 2003).

2.6 INTERACTIONS OF MITOCHONDRIA AND CHLOROPLAST IN ILLUMINATED LEAVES

Chloroplasts and mitochondria are traditionally considered to be autonomous organelles but they are not as independent as once thought to be. Photosynthesis and respiration produce virtually all the energy, in form of both ATP and redox equivalents in the plant cell. For a long time the biochemical interdependencies between mitochondria and chloroplast have been debated. Now we could say that the mitochondrial metabolism (oxidative electron transport and phosphorylation) is essential for sustaining the photosynthetic carbon assimilation (Kromer 1995; Gardestrom 1996; Raghavendra and Padmasree 2003). Four important aspects in the dynamic biochemical equilibrium between mitochondria and chloroplasts in illuminated leaves are: 1) dissipation of excess of reduced equivalents from chloroplast; 2) optimization of photosynthetic carbon assimilation; 3) protection of chloroplast from photoinibition; 4) photorespiratory pathway.

Dissipation of redox equivalents from chloroplasts. Photosynthesis is composed of photochemical reactions that produce ATP and NADPH as well as carbon assimilation that uses ATP and NADPH. However, there is a difference of several orders of magnitude between generation and use of reductants, therefore it is essential to dissipate the excess of redox equivalents to overcome the problem of over-reduction of the electron transport chains (Huner et al. 1998; Niyogi 1999, 2000). There are four major possibilities for exporting NADPH from chloroplast: 1) export of glycolate and import of glycerate involving photorespiratory reactions (Gardestrom et al 2002); 2) export of malate and operation through the so called “malate valve”(reviewed in Scheibe 2004); 3) import of oxoglutharate to form gluthamate (Atkin et al. 2000; Padmasree et al. 2002) ; 4) export to cytosol of triose phosphate and its conversion to sucrose (Kromer et al. 1995; Gardestrom et al. 2002). Mitochondria are involved in all these processes. Another important aspect of mitochondrial dissipation of redox equivalent originated in chloroplast is the alternative oxidase (AOX) pathway (McDonald et al. 2002; Vanlerberghe and Ordog 2002). Most of the ATP production of mitochondria is dependent on the cytochrome oxidase (COX) pathway whereas ATP is dissipated by means of the AOX pathway. Experiments with inhibitors suggested that both the COX and AOX pathways are essential for photosynthesis (Padmasree and Raghavendra

1999, 2001). The involvement of AOX pathway increases in the light; when the cytosol and mitochondria are over-reduced (Bykova and Moller 2001): about 60% of mitochondrial respiration in light occurs through the AOX pathway (Ribas-Carbo et al. 2000).

Optimization of photosynthetic carbon assimilation. To optimize the photosynthetic carbon flux the coordination of many biochemical pathways is required. The components needed are: the generation and use of assimilatory power (ATP and NADPH); the induction of photosynthesis; the activation and maintenance of metabolite levels. The oxidative metabolism through COX and AOX pathway are essential for the maintenance of carbon assimilation during photosynthesis (Padmasree et al. 2002; Padmasree and Raghavendra 2001). Moreover, mitochondria and chloroplasts share another important component of cell metabolism, the nitrogen metabolism that has to stay tuned during illumination. The coordinated action of the two organelles is required to sustain the reduction of nitrite and the reductive assimilation of oxoglutarate that allows the proper recycling of ammonia and amino acids (reviewed in Ragahavendra and Padmasree 2003; Dutilleul et al. 2003a; Padmasree and Raghavendra 1998).

Protection against photoinhibition. Photoinhibition occurs under supra-optimal condition of light or suboptimal CO_2 both generating reactive oxygen species (ROS), that damages the photosystem II and occasionally the photosystem I (Andersson and Aro 2001; Ohad et al. 2000). It has been reported that mitochondrial respiration protects photosynthesis against photoinhibition in pea mesophyll (Saradadevi and Raghavendra 1992) and could sustain, through the generation of ATP, the protein biosynthesis needed to repair the damaged photosystems (**Figure 14**; Padmasree et al. 2002; Shyam et al. 1993).

Photorespiratory pathway. Another important aspect of the coordination between mitochondria and chloroplast metabolism in illuminated leaves is the photorespiratory pathway (reviewed in Noguchi and Yoshida 2008). At normal CO_2 concentration mitochondria help to sustain the biosynthesis of sucrose (Kromer 1995; Padmasree et al. 2002) and while in limiting CO_2 conditions the mitochondrial glycine oxidation pathway ensures, not only the dissipation of excess redox equivalent from chloroplast, but also the generation of significant amount of glycerate for the replenishing of the Calvin cycle (Gardestrom et al. 2002).

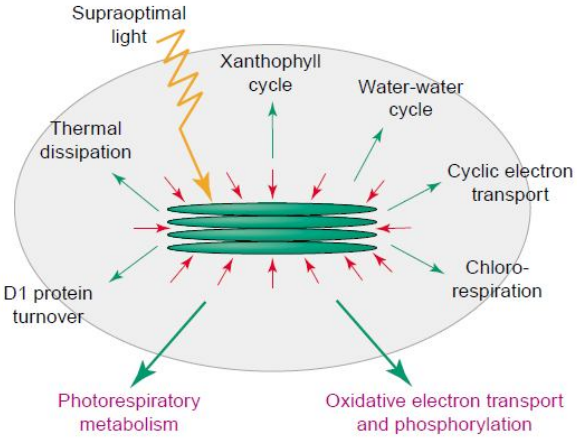


Figure 14 Protection of chloroplast thylakoid membranes against photoinhibition by different process. Red arrows indicate the stress at chloroplast membrane. Green arrows indicate the mechanisms that protect chloroplasts from overexitation. Marked protection against photoihibition is provided by photorespiration and oxidative electron transport, both of which are mediated by mitochondria (from Raghavendra and Padmasree 2003).

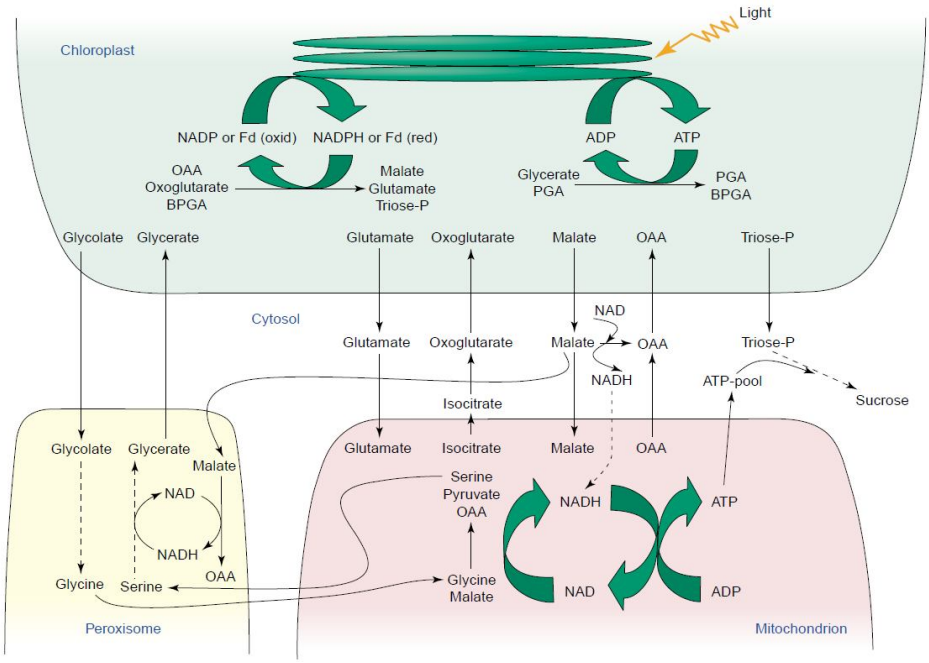


Figure 15 Use of reduced equivalents (NADPH or ferredoxin -Fd-) from chloroplasts. Mitochondria, cytosol and peroxisomesare common sinks for reduced equivalents. The dissipation of excess redox equivalents from chloroplasts is ensured by the export of glycolate, malate, glutamate, and triose phosphate (triose-P). The recycle of these four compounds involves the mitochondria. The arrows indicate the metabolite movement. BPGA: 1,3-bisphosphoglycerate; Fd(oxid): oxidized Fd; Fd(red): reduced Fd; OAA: oxalacetate; PGA: 3-phosphoglycerate (from Raghavendra and Padmasree 2003).

In conclusion, synergies between mitochondria and chloroplast compartments modulate the energy producing pathway and to properly maintain the whole metabolism of the cell redox homeostasis are needed (**Figure 15**; Raghavendra and Padmasree 2003; Dutilleul et al. 2003b).

2.7 THE SENESCENCE PATHWAY IN PLANT TISSUES

Senescence is the developmental program that guides the cell through an ordered schedule of events leading to death of cell/organ and is needed, to allow a variety of functions such as development of new cell types and the remobilization of nutrient substances. Despite that senescence is a degeneration process the cell dismantle is strictly organized and possesses an optimized efficiency to avoid wastes (Granell 1999). A degenerative program is still a program, implies order and order requires energy. Senescence is an active process that requires energy consumption, and some components of the process are designed to sustain process itself (Solomos 1988; Buchanan-Wollaston 1997). Order also implies a genetic control of the information needed on how to dismantle and recently it has emerged that more than one program or variation of the program may exist (**Figure 16**; reviewed in Orzaez and Granell 2003). While the nature of early inducing events that triggers senescence remains elusive, the increase of catabolic activities and the decrease in photosynthetic rate have been clearly indicated as the first symptoms of senescence (Hensel et al. 1993; Quirino et al. 2000; Keskitalo et al. 2005). The two most obvious ultrastructural changes in the morphology of mesophyll cells undergoing senescence are the degeneration of chloroplasts and the vacuolarization of cytosol. While in other tissues the first symptoms may vary, as reported in flower petals were the increment of the autophagic activity of vacuoles was also demonstrated (Noodén 1998). Chloroplast degeneration follows a precise pattern of sequential changes that include in the following order: (1) dilation and breakage of thylakoids, (2) decrease in pigment content and formation of plastoglobules (Barton et al. 1966; Nii et al. 1988; Simeonova et al. 2000; Lam 2004; Keskitalo et al. 2005, van Doorn 2005; van Doorn and Woltering 2005). In addition, in rice coleoptiles the degeneration of the chloroplast is anticipated by the degradation of chloroplastic DNA (Inada et al. 1998a, b) which could be reversible in the early stage of senescence but when the process of

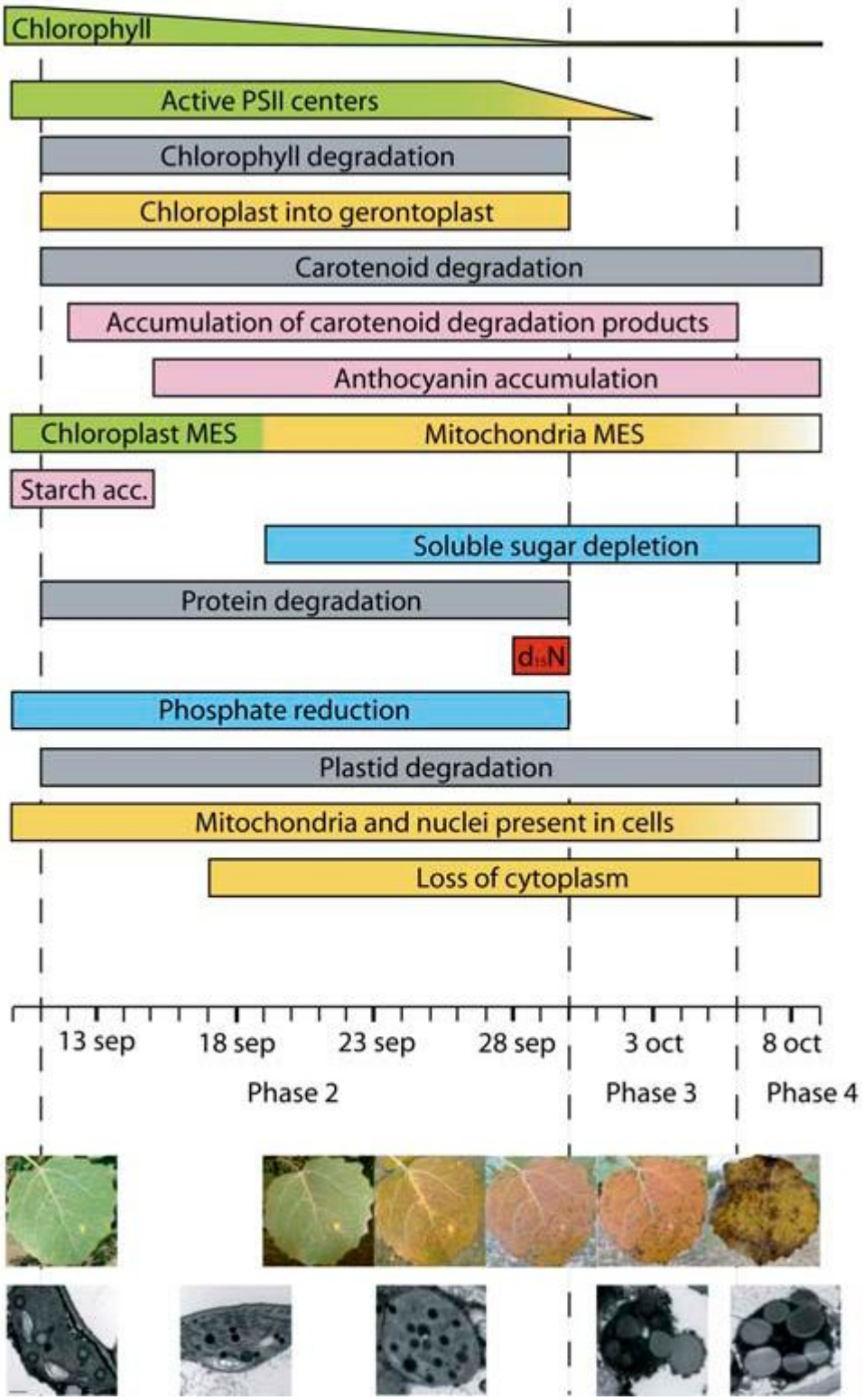


Figure 16 Cellular timetable of *Populus tremula* autumn senescence, compiled from the data presented in this paper. ''Loss of cytoplasm'' denotes the combination of two events, cytoplasm degradation and vacuolar burst. MES, Main energy source.

autophagy takes place the organelles are lost (Zavaleta-Mancera et al. 1999; Orzaez and Granell 2003). In contrast, mitochondria seem to maintain their integrity even in the latest stages of senescence with little or no degradation observed in mitochondrial DNA (Inada et al. 1998a, b). Generally mitochondria are degraded via autophagy near the end of the senescence pathway (Toyooka et al. 2001). The nuclei of senescent cells are also subjected to modifications. Chromatin condensation is reported in relatively early stages during senescense of rice coleoptiles (Inada et al. 1998a, b), petal senescence in carnation (Smith et al. 1992) and carpel degeneration in pea (Orzaez and Granell 1997a, b). Condensation of chromatin is often associated with degradation of DNA, as highlighted by the TUNEL analysis of senescent tissue, however, the stage in which the DNA degradation occurs could be highly variable (Gietl and Schmid 2001). In very early stages of the senescence process the vacuolar compartment increases its size, subsequently the number of vacuolar vesicles increase and only in the later stages of senescence the central vacuole consume nucleus and cytoplasm.

Invaginations of the tonoplast, occasionally containing cytoplasmic material and organelles, are widely reported in different stages of senescence (Matilde and Winkenbach 1971; Inada et al. 1998a, b). In many cases, cell compartimentalization (integrity of the membranes) in maintained until very late in the senescence program. Acidification, probably due to a change in permeability or rupture of the tonoplast, causes activation and exposure to the cytosol of endopeptidases. Once the compartimentalization is lost and the content of lytic organelles is free to digest the remnants of cell corpses. This represents the latest stage of senescence where homeostasis is irreversibly lost (reviewed in Orzaez and Granell 2003 and Lam 2004).

3. *EMPTY PERICARP4* AFFECTS SHOOT AND ROOT DEVELOPMENT AND SENESCENCE IN MAIZE

3.1 INTRODUCTION

The PentratricoPeptide Repeats proteins (PPRs) are putative RNA-binding proteins present in all eukaryots (Rivals et al. 2006; Andres et al. 2006) forming a superfamily in land plants. Few PPR genes have been functionally characterized, some of them are involved in developmental processes (Cushing et al. 2005; Prasad et al. 2005; Gutierrez-Marcos et al. 2007) and others are restorers of fertility (Bentolila et al. 2002; Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003; Akagi et al. 2004; Oguchi et al. 2004; Klein et al. 2004; Shmitz-Linneweber et al. 2005). Moreover, many PPRs play essential roles in mitochondria and chloroplasts (Meieroff et al. 2003; Nakamura et al. 2003; Williams and Barkan 2003; Lurin et al. 2004; Yamazaki et al. 2004; Gothandam et al. 2005; Shmitz-Linneweber et al. 2005).

Despite the fact that PPRs are abundant in plant genome (Aubourg et al. 2000; Andres et al. 2006) their individual role is apparently not redundant as indicated by studies on mutants in single PPR gene in *Arabidopsis* (Meieroff et al. 2003; Kotera et al. 2005), maize (Fisk et al. 1999; Williams and Barkan 2003, Shmitz-Linneweber et al. 2005; Gutierrez-Marcos et al. 2007), rice (Akagi et al. 2004; Gothandam et al. 2005), radish (Ikeda and Gray 1999; Koizuka et al. 2003), sorghum (Klein et al. 2005) and petunia (Bentolila et al. 2002).

Bioinformatic analysis not only revealed that PPRs are mainly targeted to mitochondria and chloroplast but also highlighted that mitochondria is the organelle that receives, from nucleus, the largest part of PPR proteins produced (Lurin et al. 2004). Mitochondria are a semi-autonomous organelle whose proteome can be estimate in 2000-3000 proteins (Millar et al. 2005), the vast majority of proteins located in the mitochondrion are nuclearly encoded and in particular most of those components needed for proper transcription and translation (reviewed in Binder and Brennicke 2003). Mitochondria have a central role in the biochemical reactions that take place in the cell, they are also implicated in cell signaling (Logan and Knight 2003), programmed cell death (Maxwell et al. 2002; Lam 2004) and a complex metabolic interdependencies with chloroplasts (Raghavendra and Padmasree 2003; Noguchi and Yoshida 2008; Woodson and Chory 2008). Many works suggest that the link

between mitochondria and chloroplast is tight and involves many aspects of the energetical reactions of the cell. During illumination, photosynthesis is supported by mitochondrial action in many ways (reviewed in Kromer et al. 1995; Gardestrom et al. 2002); during carbon assimilation (Raghavendra et al. 1994; Padmasree and Raghavendra 2000); in dissipation of the excess of redox equivalent (Moller 2001; Vanlerberghe and McIntosh 1997); in protecting photosynthesis from photoinibition (Saradadevi and Raghavendra 1992; Shiam et al. 1993; Singh et al. 1996) and in the photorespiratory pathway (Douce and Neuburger 1989; Oliver et al. 1990). Thus, it is not surprising that malfunctions of the mitochondrial compartment directly affects the functionality of chloroplasts (Dutilleul et al. 2003; Sweetlove et al. 2006).

EMP4 is a P type-PPR protein that is specifically targeted to mitochondria. Mutant seeds are clearly altered in the Basal Embryo Transfer Layer (BETL) cells both morphologically and functionally. In fact the expression of BETL specific markers is altered in *emp4* seeds. In addition, the accumulation of starch is reduced in the central starchy endosperm of homozygous kernels (Gutierrez-Marcos et al. 2007). Therefore, it has been concluded that *emp4* is essential for the development of maize kernel linking the mitochondrial functionality to the proper development of the seed (Gutierrez-Marcos et al. 2007).

In this section we analyzed the role of *emp4* gene in relation to the development and senescence of the seedling leaves and roots derived from light-exposed and dark-grown seedlings obtained through embryo.

3.2 RESULTS

3.3 MUTANT EMBRYO RESCUE IN DARK AND LIGHT CONDITION

Homozygous emp4 mutant seeds are unable to germinate at physiological maturity, therefore to gain insight into the role exerted by EMP4 during seedling development we have adopted the embryo rescue technique that consists of the excision of embryo from immature kernels and their cultivation on a synthetic medium. According to previous data (Dolfini et al. 2007) embryos were excided at 20 DAP (Days After Pollination); wild-type and mutant embryos were kept in cultures for 12 and 30 days respectively.

For the first four days of culture embryos were grown in a complete dark condition. Subsequently embryos were split in two groups; the first one was exposed to light while the second was kept in the dark (**Table 3.3**). 100% of the light-exposed wild-type embryos germinated and after 8 to 12 DAC (Days After Culture) developed the second leaf. In contrast, mutant embryos showed a lower percentage of germination (about 44%) and only after 30 DAC few plants reached the second leaf stage. Mutant seedlings were very short in stature and had a weak appearance.

In the dark all wild-type embryos were able to germinate although a slight reduction in the number of seedlings that reached the first and the second leaf stage was observed. A substantial increase in the germination rate of mutant embryos was observed in the darkened conditions, moreover the number of seedlings that reached the first and the second leaf stages in the dark treatment were almost doubled in comparison to those in the light condition.

3.4 MORPHOLOGICAL ANALYSIS OF LIGHT-EXPOSED AND DARK GROWN ROOT

The samplings of primary roots were conducted at 5 and 15 DAC for wild-type and mutant respectively and transversal sections were performed in the region of elongation within 1 cm from the root tip. Light and dark grown root tissue anatomies were compared through light microscopy.

The wild-type root cortex from plants grown in light condition appeared more compact then wild-type root cortex from dark-grown plants that showed more intercellular spaces.
Typical root anatomy with the peculiar root cell layers was observed in both wild-type and in mutant samples, however in mutant epidermis and hypodermis cell layers were less ordered and alterations in the cell shape were evident, particularly in light. Moreover, comparison between mutant and wild-type root morphology, indicated that mutant roots either grown in the dark or exposed to light were less developed then wild-types and the cell layers epidermis and hypodermis were not properly organized. (**Figure 3.4**).

					% of plants in different stages of development		
Phenotype	**Treatment**	**DAC**	**No. of cultured embryos**	**Germination (%)**	**Coleoptile[1]**	**First leaf**	**Second leaf**
WT	Light	12	753	100	0	2,4	97,6
WT	Dark	12	153	100	5,4	2,4	92,2
emp4	Light	30	2677	44,6	41,3	2,1	1,2
emp4	Dark	30	249	83,6	76,8	4,7	2,1

Table 3.3 Germination and developmental profile of seedlings obtained trough embryo rescue at 12 (WT) and 30 (emp4) DAC (Days After Culture). Light: embryos grown for four days in the dark condition and subsequently exposed to light. Dark: embryos entirely cultivated in the dark. (1) Coleoptile stage includes the plants with open or closed coleoptile. WT: wild-type

3.5 MORPHOLOGICAL ANALYSIS OF LIGHT-EXPOSED AND DARK GROWN MESOPHYLL

First leaf samples were collected at 5 DAC and 15 DAC, for wild-type and mutant seedlings respectively, and histological analysis was performed on transversal sections (**Figure 3.5/6**).

The leaf tissues of both mutant and wild-type dark grown plants were yellowish in color and wild-type leaves have a straight and longer leaf blade in comparison with light-grown siblings.

All the mesophyll compartments (epidermis, vessels and parenchyma) were clearly detectable in both mutant and wild-type tissues in both conditions.

In the dark, mutant tissues were composed by smaller cells with abnormal shapes. Moreover in both genotypes, chloroplasts, that were distinguishable in wild-type leaf grown in light treatment, could not be observed.

In the light, mutant leaves were smaller in comparison with wild-type and displayed a pale green color. Abnormalities were founded in sections of homozygous mutant plants, like alteration in cell shape and size, a smaller population of chloroplasts and lack of nuclei. (**Figure 3.5/6**).

3.6 ANALYSIS OF EMP4 ACTION IN ADULT LEAVES

Double *Ds* elements are well known for their ability to break the chromosome when simultaneously excided by the action of the *Ac* transposon. This event happens in somatic cell lines generating, ongoing with development, clones of cells with modified karyotype.

The DS-1L3 genetic stock heterozygous for a double *Ds* element located proximal to the *bz2* locus and to the emp4 locus on the long arm of chromosome one and carrying an active *Ac* element was adopted to perform clonal mosaic analysis. These plants that were heterozygous for the *Ds* and carried one dosage of *Ac* were crossed with +/emp4 heterozygous plants. Progeny plants were grown in the field and their emp4 genotype determined through PCR analysis. We expected that one fourth of the 64 +/*emp4* genotyped individuals would have carried both *Ds* and *Ac* elements. In these plants, breakage of the

long arm of chromosome, caused by the *Ds* element would have originated hemizygous *emp4* clonal sectors. They were therefore visually scored for the presence of somatic sectors exhibiting some developmental abnormalities and on 18 plants at the 9-10 leaf stage yellow sectors were detected on leaves (**Figure 3.6.1**). PCR analysis indicated that 15 sectors, each one belonging to a single plant, had a hemizygous genotype in which the *emp4* mutant allele was retained and the *Emp4* allele was lost, while the surrounding green tissue still had a heterozygous constitution. These data indicated that absence of *Emp4* was correlated with the phenotypic change, and also suggested that the *emp4* gene action is cell autonomous. Hemizygous sectors as well as heterozygous sectors were collected for phenotypic analysis.

Hemizygous *emp4/-* yellow sectors appeared on 9-10th leaves of heterozygous plant carrying the *Ds/Ac* elements and heterozygous *emp4/Emp4* tissue samples were collected for the histological analysis (**Figure 3.5/6**). Transversal sections visualized at optic microscopy revealed the presence of an irregular epidermis and compression of vessels as well as parenchimatic areas; the alterations could be ascribed to the age of the leaf sampled and to the open field growing condition. Significant differences were observed between hemizygous and heterozygous sectors since hemizygous sectors showed a strong alteration in the shape of cells and a reduced population of clearly distinguishable chloroplasts and nuclei.

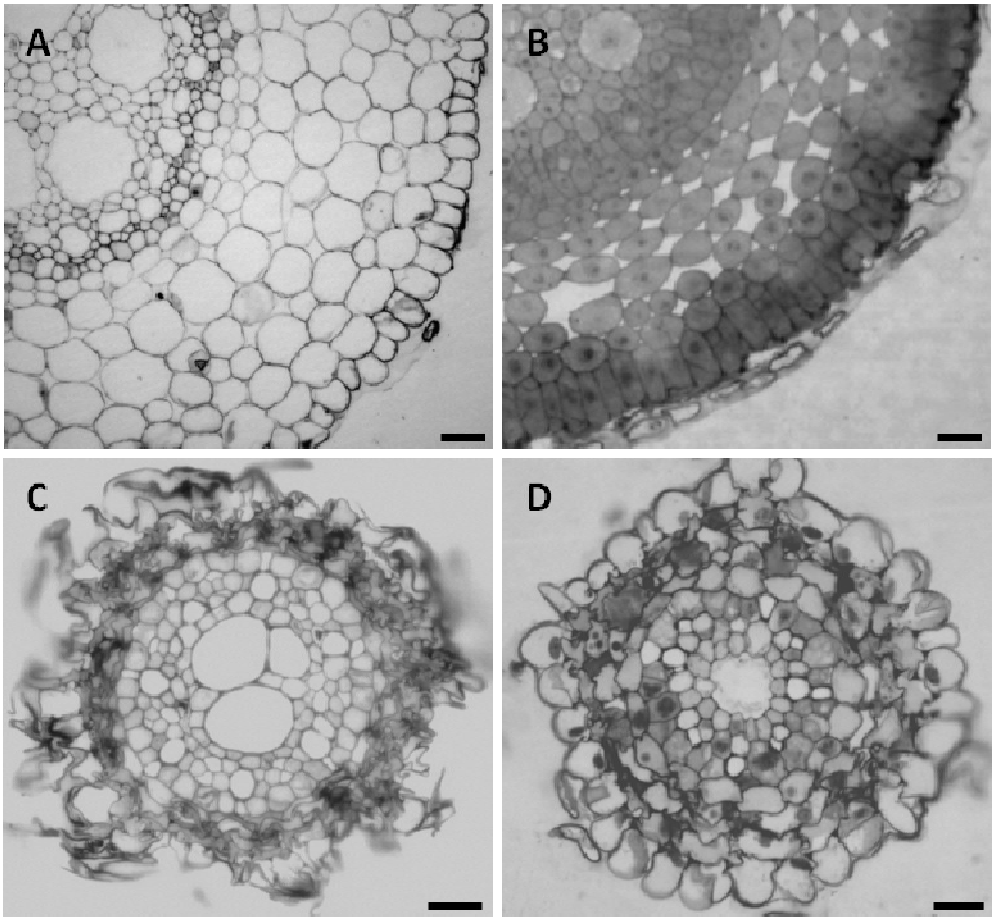


Figure 3.4 Transversal sections of primary root tissues derived from embryo rescued plants. A, B) wild-type; C, D) emp4 mutant. A, C) light-exposed roots; B, D) dark-grown roots. Bars = 20 µm

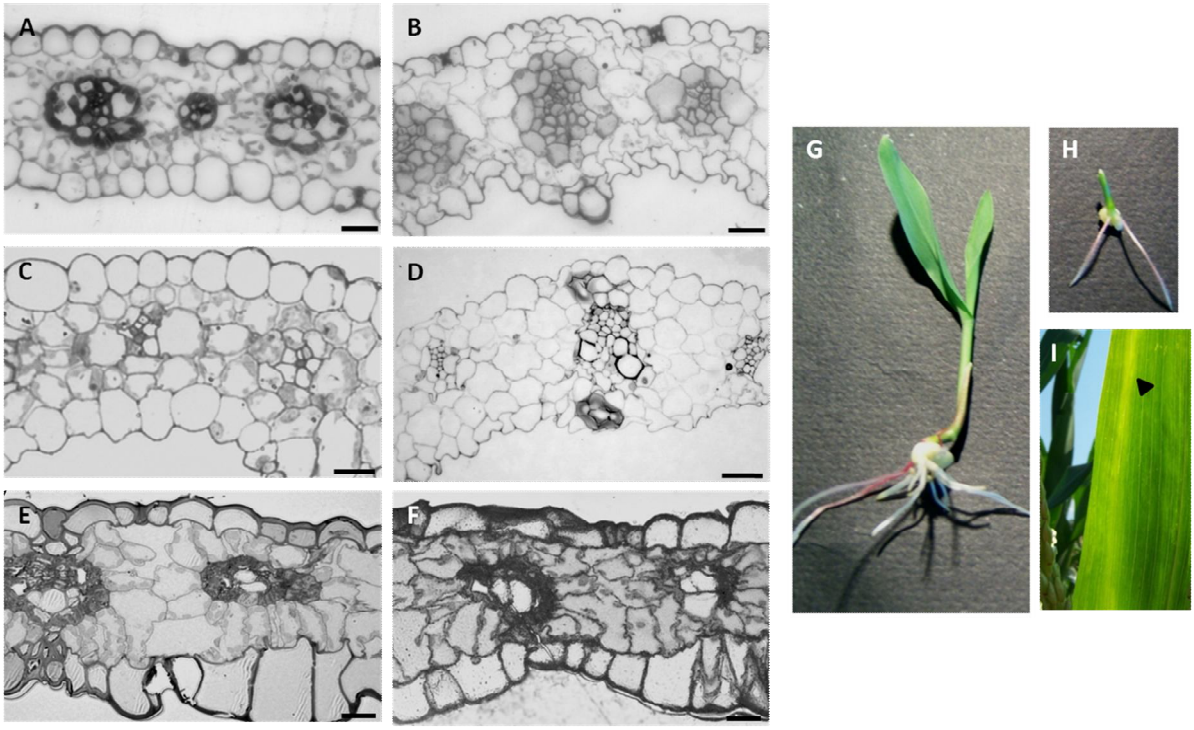


Figure 3.5/6 Transversal sections of a first leaf tissues derived from an embryo rescued seedling (A, B, C, D) and from adult leaf sectors (E, F). A, B) light-exposed leaf tissues; C, D) dark-grown leaf tissues E, F) chimeric sectors. A, C) wild-type; B, D) emp4 mutant; E) heterozygous (+/*emp4*) sector; F) hemizygous (-/*emp4*) sector. Wild-type (G) and emp4 mutant (H) plants at 8 and 30 DAC (Days After Culture) respectively. I) Adult leaf with a clonal sector; the arrow indicates the hemizygous sector. Bars = 20 µm

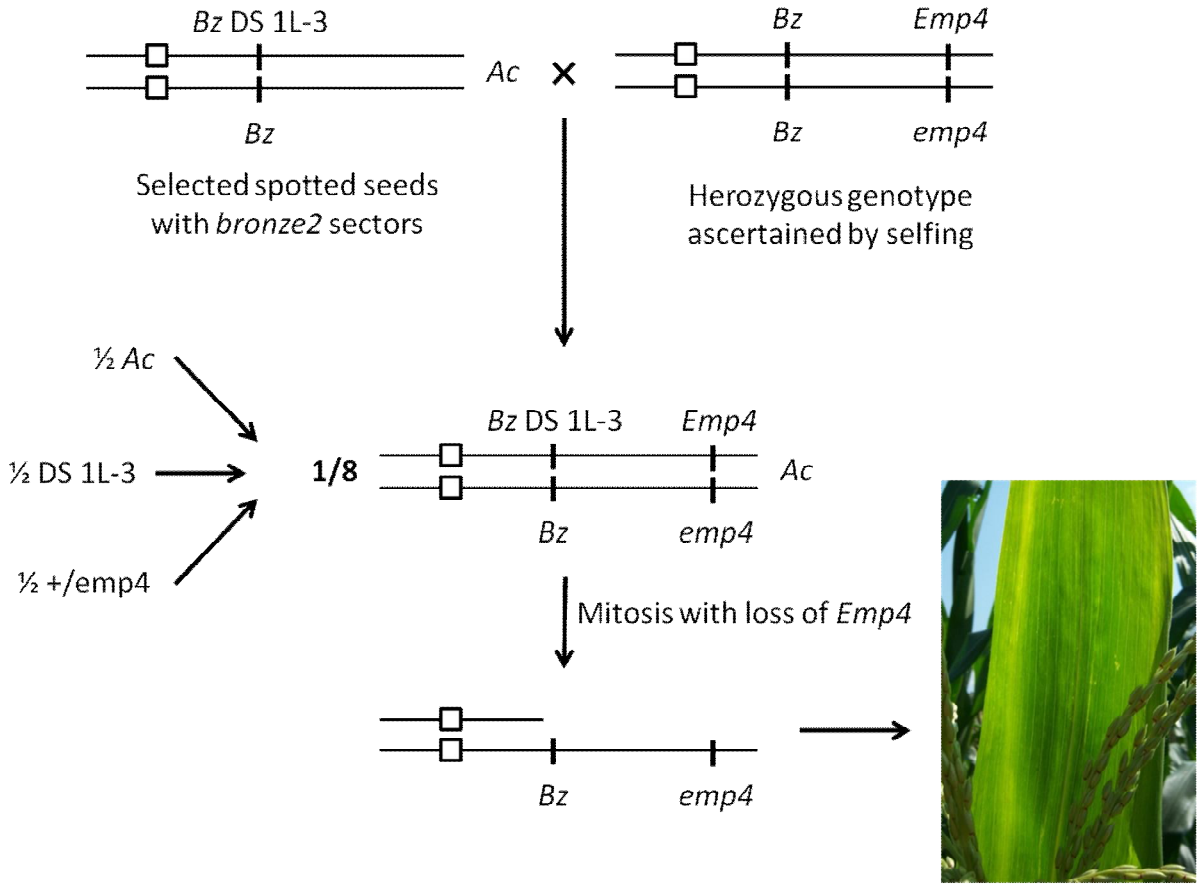


Figure 3.6.1 Cross mode used for the production of chimeric plants.

3.7 ULTRA-STRUCTURE ANALYSIS OF DARK GROWN AND LIGHT-EXPOSED CORTEX CELLS

The primary root cortex cells derived from light-exposed and dark grown roots were analyzed using transmission electron microscopy (TEM). Representative electron micrographs from wild-type and mutants are reported in **Figure 3.7** and **3.7.1** In wild-type samples all the cellular components was clearly distinguishable and with proper morphology. Tissue analyzed showed well defined organells, membranes and cell walls. The plastids population was mainly composed by amyloplast, in which starch deposit was evident. Mitochondria was in an orthodox conformation and showed a well developed inner membrane system. Little vacuoles were present in the cytosol. Mutant roots presented some cells completely empty but in the vast majority of the cells all the cellular components were observed. Around the empty cells a thickened cell wall could be observed. Nucleus, amyloplast and mitochondria were morphologically similar to those observed in wild-type tissues. In mutant cortex cells vacuoles were less evident and not detected in all the cells. In some mutant cells an electron dense condensed matter surrounded by an incomplete membrane was distinguished, this matter was very similar to a tight condensed chromatin and could be interpretable as nucleus remnants (**Figure 3.7**).

In the dark grown mutant cortex the sub-cellular structure was similar to wild-type. The main observed alteration was the presence of few void cells that did not show the thickened cell wall observed in light-exposed mutant cortex. In dark grown primary root cells the plastid population was mainly composed by amyloplast and pro-plastid, mitochondria were abundant and were present in an orthodox conformation. Some cells presented the same tight condensed matter surrounded by incomplete membrane as observed in light-exposed mutant cortex (**Figure 3.7.1**).

In conclusion the ultrastructure analysis revealed that the morphology of cortex cells did not present variations in relation to the different light conditions in which mutant and wild-type plants were grown.

3.8 ULTRA-STRUCTURE ANALYSIS OF DARK GROWN AND LIGHT-EXPOSED MESOPHYLL CELLS

TEM analysis of light-exposed mesophyll tissues from mutant seedlings obtained through embryo rescue revealed many alterations. All cells exhibit an irregular shape and presented a thinner cell wall. Most of the cells appeared empty and only few cells containing organelles were detected, although they appeared not well structured.

When detected nuclei were deeply disorganized, the nuclear envelop was irregular and the nucleus content was principally constituted of clumps of chromatin. Very rarely a less regular tonoplast was detected, that was not adherent to organelles (**Figure 3.8**). Mitochondria were less electron-dense, smaller, with a poorly organized system of inner membranes and their number per cell was smaller than that in the wild-type tissues. In contrast with wild-type chloroplasts that were well developed and showed the peculiar morphology of tylacoid stacked in tight granas, mutant chloroplasts were collapsed, and showed incomplete or deeply irregular outer membrane. In mutant chloroplasts, tylacoids did not show their peculiar structure and organization and small electron-dense bodies were observed that could be referred to as plastoglobules (Barton et al. 1966; Nii et al. 1988; Simeonova et al. 2000; Lam 2004; Keskitalo et al. 2005, van Doorn 2005; van Doorn and Woltering 2005). Rough endoplasmic reticulum (RER) was not observed in mutant mesophyll cells while it was clearly detectable in wild-type tissues (**Figure 3.8**).

The main differences between dark grown wild-type and mutant mesophylls were the cell shape that was irregular in the mutant, and the presence of empty cells, (although the number of empty cells was lower than in the light grown tissues).

In wild-type cells the nucleus was morphologically complete (presence of nucleolus and dispersed chromatin), all membranes were clearly distinguishable even if the inner mitochondrial membranes were poorly defined. The plastids population was abundant and mainly composed by etioplast and amyloplast. The poor conformation of tylacoids and the presence of amyloplast (with massive starch grains) are typical of plants grown in a continuous dark condition (**Figure 3.8.1**).

Mutant nuclei, clearly distinguishable in most cells, were slightly less electron-dense in comparison with wild-type; in addition nucleolus was not observed. In mutant leaves plastid

were detected both as amyloplast and as etioplast; mitochondria had a well defined morphology. In many mutant cells the presence of RER, plasmodesmata junctions and tonoplast were observed, and in few cells a structure similar to a small vacuole was observed.

In conclusion, in the dark grown mutant tissues cellular morphology was very similar to wild-type cells, therefore dark grown mutant tissues appeared less damaged then irradiated tissues (**Figure 3.8.1**).

3.9 ULTRA-STRUCTURE ANALYSIS OF HEMIZYGOUS CLONAL SECTORS AND HETEROZYGOUS LEAF TISSUES

In heterozygous *+/emp4* leaf sectors cells were well structured with all organelles, membranes and the cell wall clearly distinguishable and properly organized. Nuclear compartment was defined and presented dispersed and aggregated chromatin. The central vacuole was clearly developed and the tonoplast was adherent to all the organelles within the cytosol. The chloroplasts were abundant within the mesophyll cells and in every organelle the tylacoidal membranes were packaged in tight grana. Mitochondria did not present any alterations in their membrane system. In contrast, hemizygous cells exhibited many alterations in almost all cellular compartments. First of all the cell shapes were highly irregular and in many cells a thickened cell wall was observed. Some cells lacked nucleus and generally the nuclei was less homogenous; vacuoles could not be distinguished in some cells, while in other cells numerous vacuoles of small size were detected. Mitochondria retained their morphological characteristic while chloroplasts appeared to be the organelles with the most severe alterations. Tylacoids were disorganized, they did not form grana and in general were less packaged; inside chloroplasts some spherical-elliptical electron-dense bodies very similar to plastoglobules could be distinguished (**Figure 3.9**).

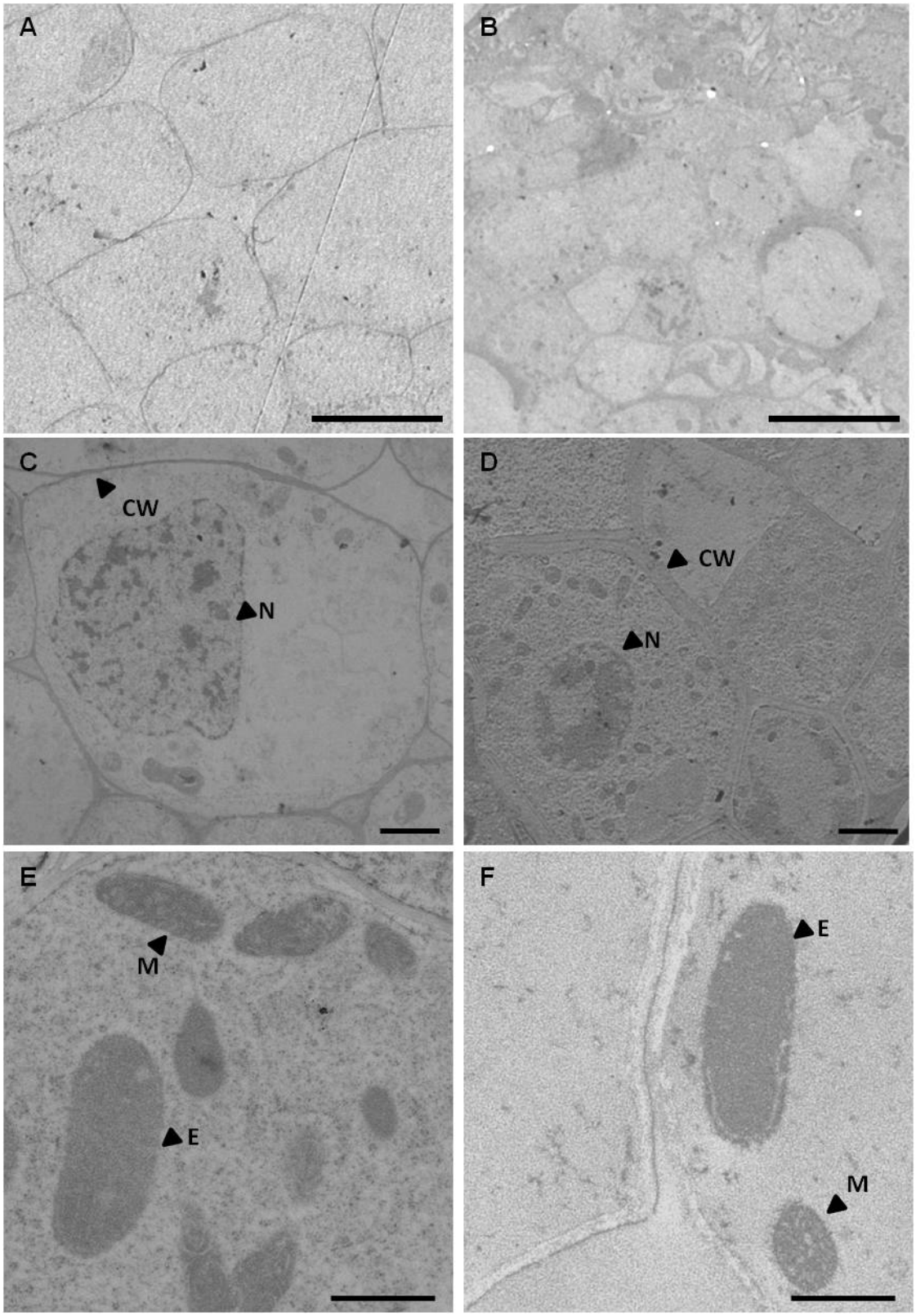


Figure 3.7 Electron microscopy analysis of wild-type cortex primary root tissues (transversal section), from derived from light-exposed seedlings obtained through embryo rescue. A, C, E) wild-type; B, D, F) *emp4* mutant. Abbreviations: CW = cell wall; N = nucleus; M = mitochondria; E = etioplast. A, B) Bars = 10 µm; C, D, E, F) Bars = 2 µm.

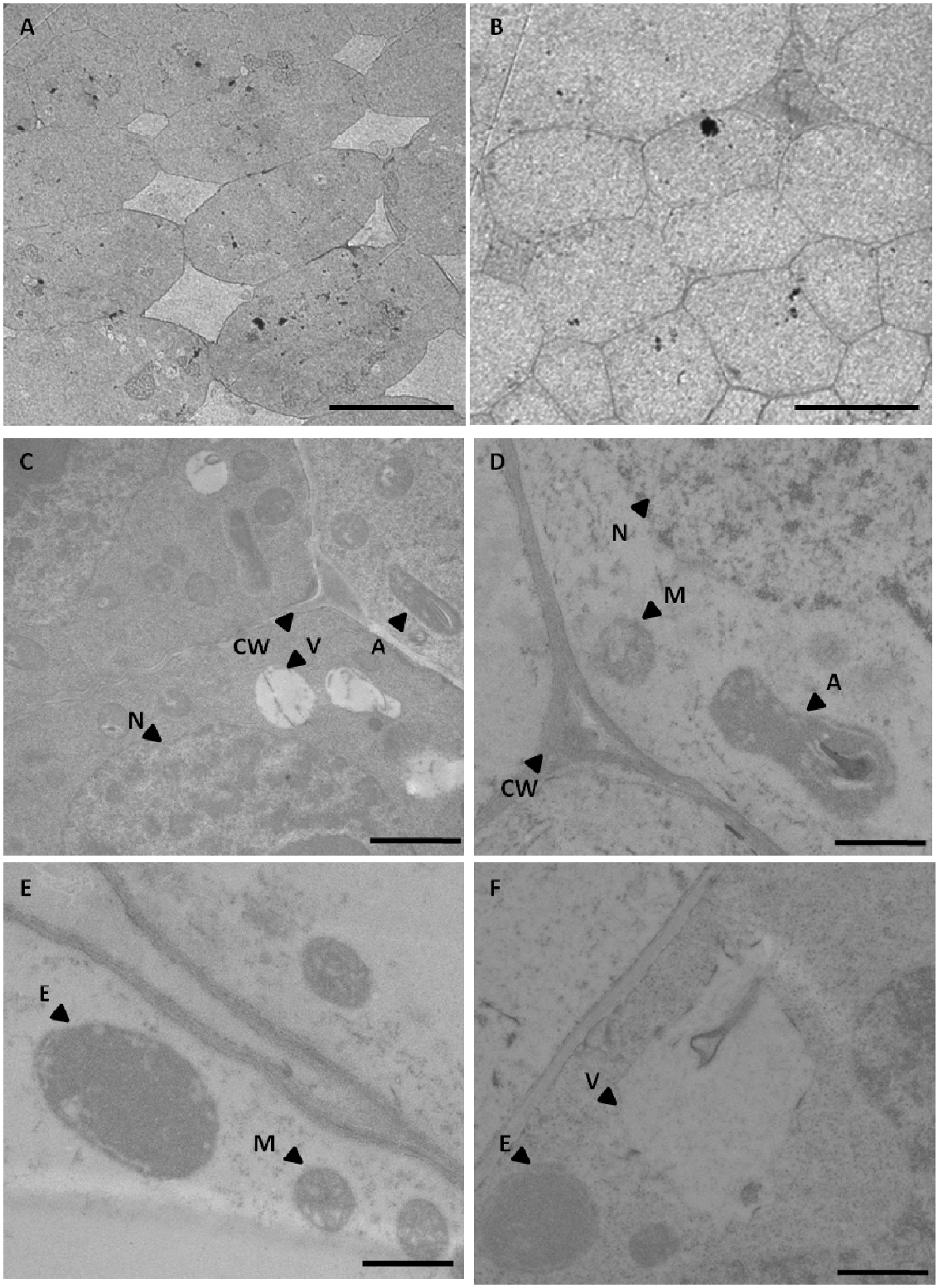


Figure 3.7.1 Electron microscopy analysis of wild-type cortex primary root tissues (transversal section), derived from dark-grown seedlings obtained through embryo rescue. A, C, E) wild-type; B, D, F) *emp4* mutant. Abbreviations: CW = cell wall; N = nucleus; V = vacuole; M = mitochondria; A = amyloplast; E = etioplast. A, B) Bars = 10 µm; C, D) Bars = 2 µm; E, F) Bars = 1 µm.

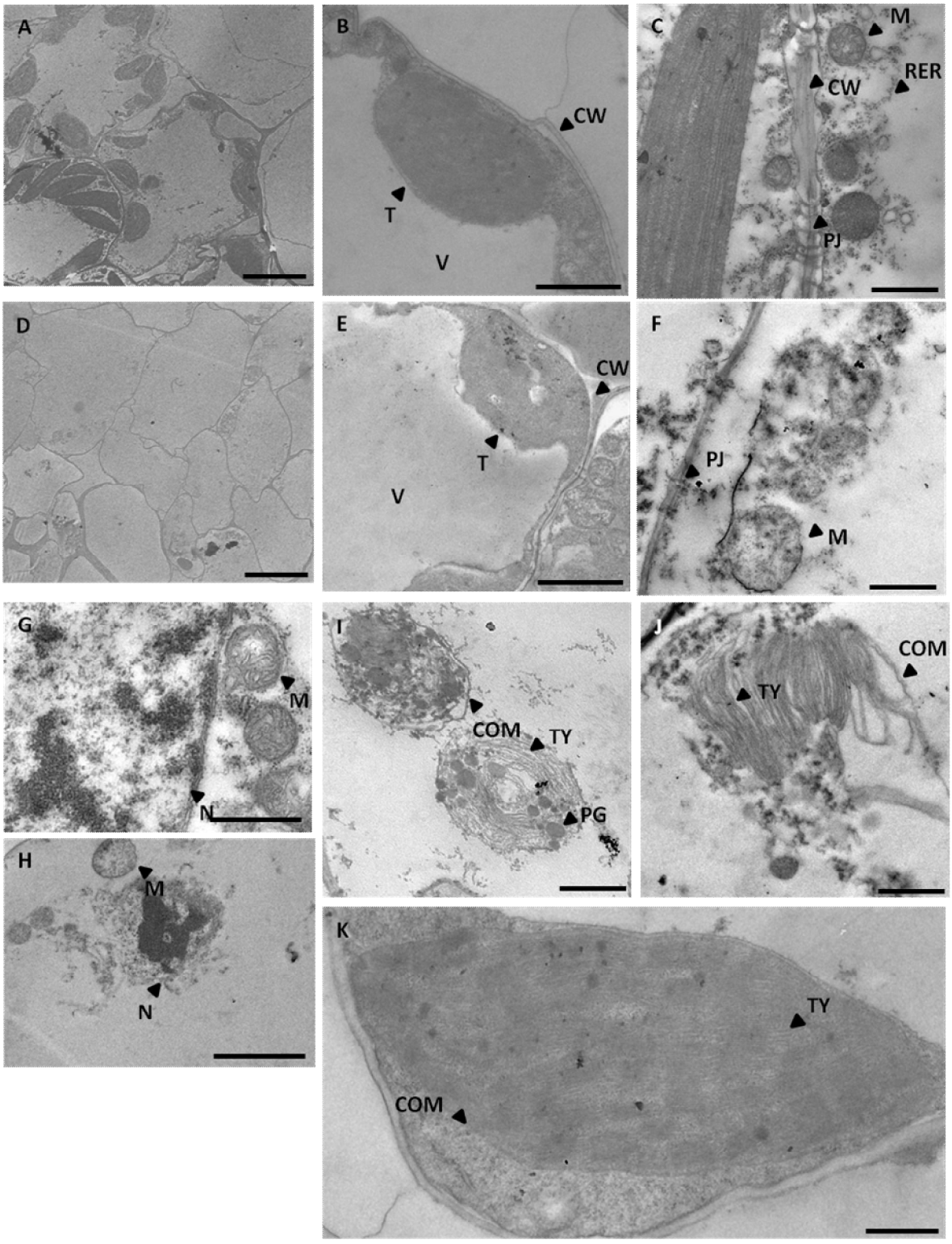


Figure 3.8 Electron microscopy analysis of wild-type mesophyll tissues (transversal section), derived from light-exposed seedlings obtained through embryo rescue. A, B, C, G, K) wild-type; D, E, F, H, I, J) *emp4* mutant. Abbreviations: CW = cell wall; N = nucleus; T = tonoplast; PJ = plasmodesmata junction; V = vacuole; RER = rough endoplasmic reticulum; M = mitochondria; COM = chloroplast outer membrane; PG = plastoglobules; TY = thylakoid. A, D) Bars = 10 µm; B, C, E, F, G, H, I, J, K) Bars = 1 µm.

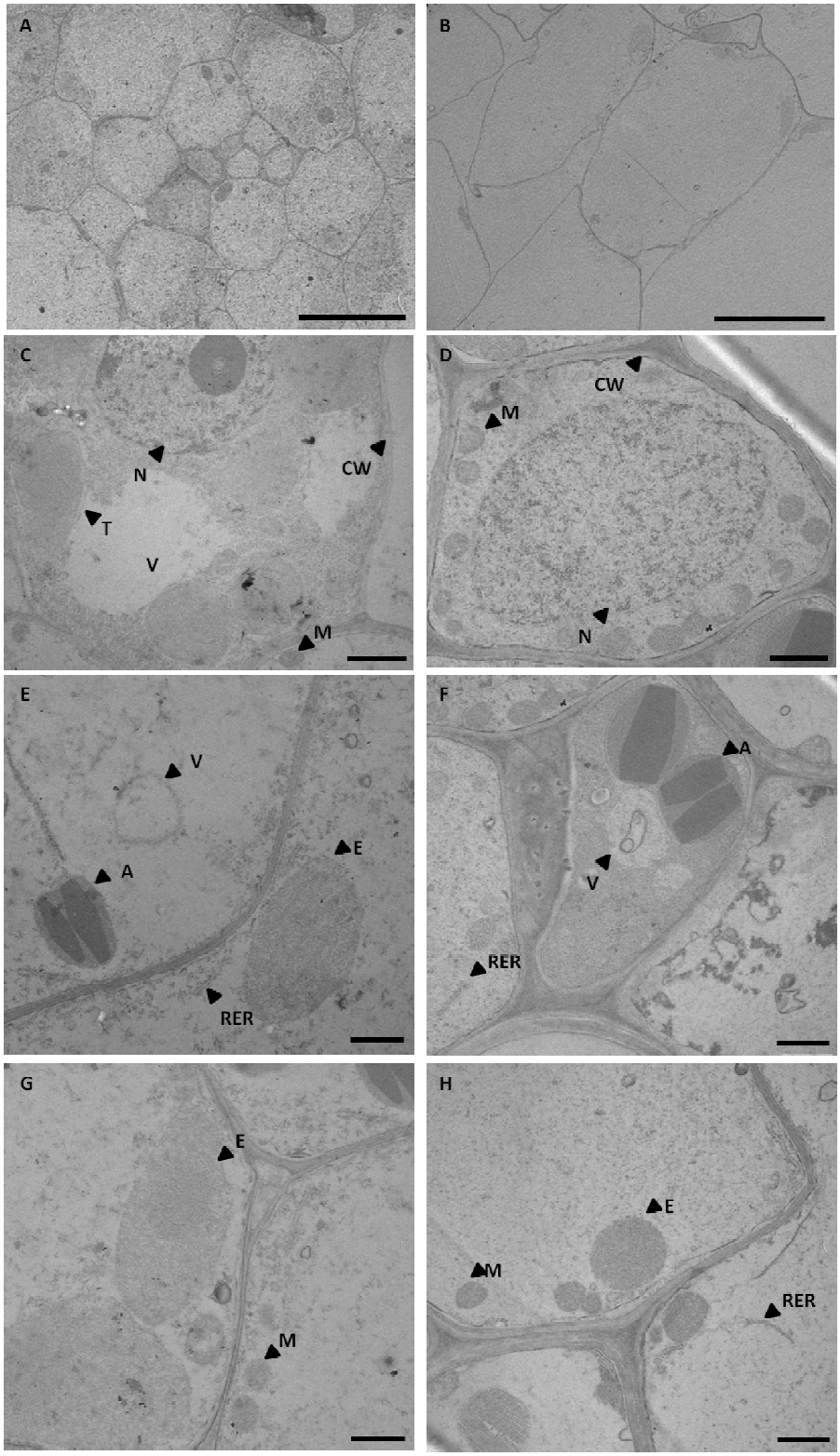


Figure 3.8.1 Electron microscopy analysis of wild-type mesophyll tissues (transversal section), derived from dark-grown seedlings obtained through embryo rescue. A, C, E, G) wild-type; B, D, F, H) *emp4* mutant. Abbreviations: CW = cell wall; N = nucleus; T = tonoplast; RER = rough endoplasmic reticulum; V = vacuole; M = mitochondria; A = amyloplast; E = etioplast. A, B) Bars = 10 μm; C, D, E, F, G, H) Bars = 1 μm.

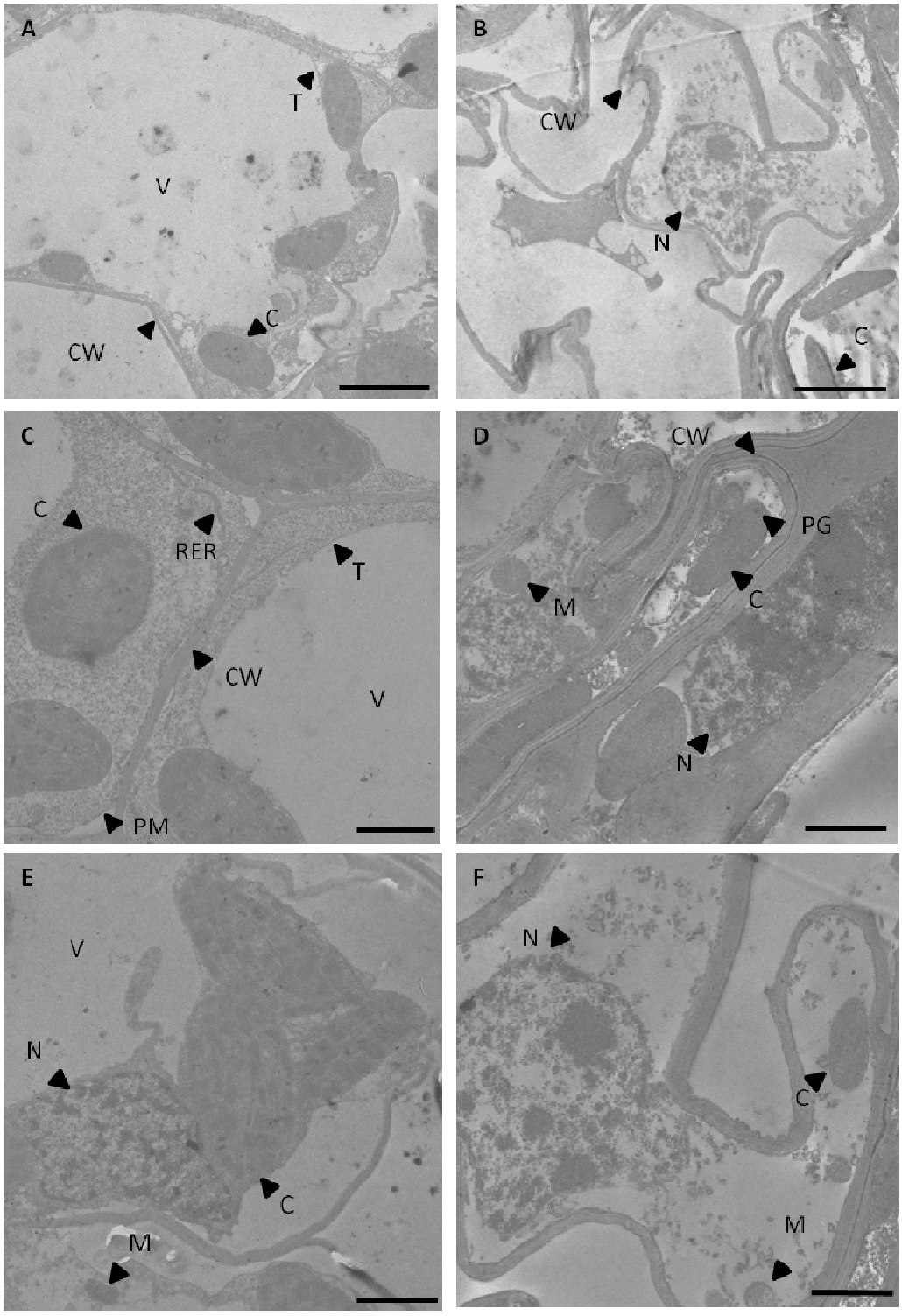


Figure 3.9 Electron microscopy analysis of wild-type mesophyll adult leaf tissues (transversal section). A, C, E) heterozygous (*+/emp4*) sector. B, D, F) hemizygous (*-/emp4*) sector. Abbreviations: M = mitochondria; T = tonoplast; CW = cell wall; N = nucleus; V = vacuole; PG = plastoglobule; C = chloroplast; PM = plasma membrane; RER = rough endoplasmic reticulum. A, B) Bars = 5 μm; C, D, E, F) Bars = 2 μm.

3.10 EXPRESSION ANALYSIS OF MITOCHONDRIAL TRANSCRIPTS

An RT-PCR based approach was conducted to evaluate the transcripts levels in embryo rescued wild-type and mutant leaves. Due to paucity of mutant material expression analysis were conducted on total RNA preparations on mitochondrial genes that were identify as the putative target of EMP4 (Gutierrez-Marcos et al. 2007). A mitochondrial housekeeping gene (*nad6*) and an intronic sequence of *rps3* gene were analyzed as controls. The analysis of *rps3* in the intronic regions revealed a contamination of mtDNA in RNA samples. Despite the number of protocols developed to eliminate the contamination we were only able to reduce the amount of mtDNA in our samples (**Figure 3.10**, 25 cycles). *mttB*, *rps3exon* and *rpl16* transcripts seem to have different regulation in tissues analyzed. In fact they are higher in the mutant leaf in comparison to the wild-type but the situation is inverted in the seed tissue. Generally speaking the amount of mitochondrial transcripts is very low; this fact together with the contamination of mtDNA do not allows us to draw a general conclusion for this analysis.

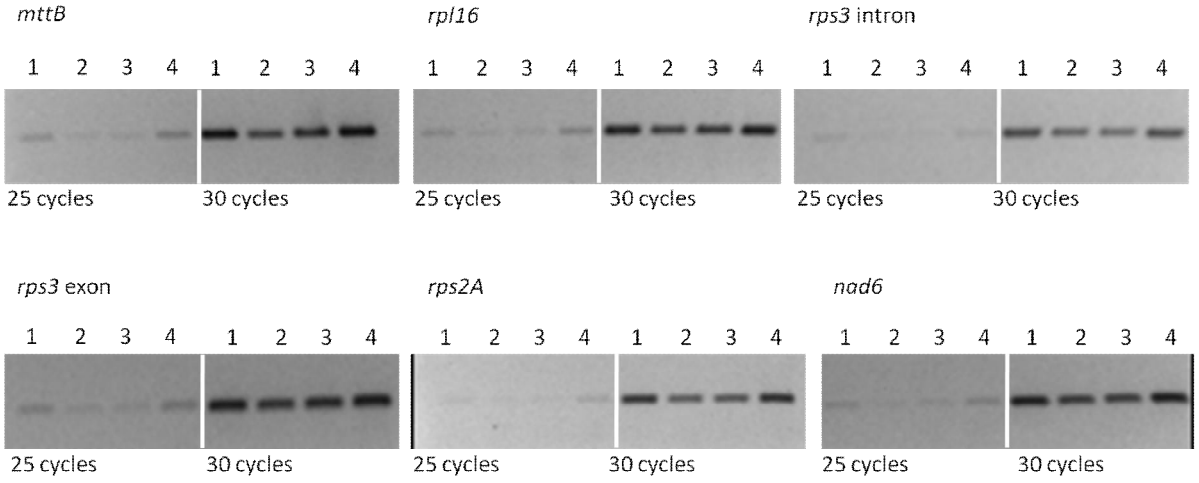


Figure 3.10 Semiquantitative RT-PCR on mitochondrial transcripts. The cDNA used was obtaind from RNA treated with DNase (for 30 minutes) and restriction enzymes (for 30 minutes). 1) emp4 mutant leaf; 2) wild-type leaf; 3) emp4 mutant seed; 4) wild-type seed.

3.11 DISCUSSION

3.12 LACK OF EMP4 AFFECTS GERMINATION, SEEDLING GROWTH AND LEADS TO PRECOCIOUS SENESCENCE IN LEAF TISSUES

The germination of homozygous emp4/emp4 mutant embryos is strongly reduced and germinated seedlings had a very weak appearance and were blocked in the very early stages of development. In emp4 homozygous light-exposed mesophyll tissue, chloroplasts are the organelles with the most severe damage; in addition some cells lack chloroplasts but retain some cellular debris while most of the cells are completely empty. It has been previously shown that the *emp4* products are required for mitochondrial gene activity (Gutierrez-Marcos et al. 2007). In the literature the existence of a strong link between mitochondria and chloroplast functions is well documented especially in illuminated leaf tissues (Kromer 1995; Gardestrom 1996; Raghavendra and Padmasree 2003; Scheibe 2004; Dutilleul et al. 2003 a and b). Studies on mutants that compromise mitochondrial functionality revealed that chloroplasts are the first organelle of the cell that is damaged (Barton et al. 1966; Nii et al. 1988; Simeonova et al. 2000; Lam 2004; Keskitalo et al. 2005, van Doorn 2005; van Doorn and Woltering 2005; Inada et al. 1998 a and b). We propose that the lack of mitochondrial functionality, due to the mutation in the emp4 gene, destabilize the biochemical buffering between mitochondria and chloroplasts that is required for the photosynthesis.

The profound alterations observed at the level of nuclei, chloroplasts, vacuole and mitochondria could resemble those observed in the senescence process in leaf tissue. The process of cellular senescence in the mesophyll has been morphologically characterized by electron microscopy analysis in different species (Keshitalo et al. 2005; van Doorn 2005; van Doorn and Woltering 2005). According to van Doorn (2005) in a cell with chloroplasts the point of no return for cellular death coincides with the degradation of the last chloroplast. The alterations observed in chloroplasts are the same reported in the literature for senescence: irregular or interrupted outer membrane, tylacoids not organized, and presence of plastoglobules in the stroma and loss of starch granules (Keshitalo et al. 2005, van Doorn 2005; van Doorn and Woltering 2005; Lam 2004). These alterations resemble (for some

aspects) in part the macro-autophagy model proposed by van Doorn (2005) and in part the model for senescence reported in (Orzaez and Granell 2003) and in Keshitalo et al. 2005.

3.13 IN EMP4 MESOPHYLL TISSUES THE SENESCENCE PROCESS IS ENHANCED BY LIGHT

Germination percentage was double in the dark versus light condition and the number of mutant seedlings that reach the first and second leaf stages was higher. The cellular morphology and ultra-structure is more deeply affected in emp4 mutant leaf exposed to light. Differences between light grown and dark grown plants has been observed. Mutant plants grown in dark conditions were not phenotipically distinguishable from mutant plants exposed to light a part from the yellow pigmentation of their leaves. Mutant tissues from plants grown in dark conditions were less damaged. Some empty and damaged cells were present, but the vast majority of dark-grown mutant mesophyll cells displayed minor alterations. Therefore, the morphological status of dark-grown emp4 cells could be interpreted as the first steps of the senescence process. Light exposure had a deep effect on cell morphology of emp4 cells, as if a magnification in the senescence process was triggered by this signal, particularly visible in the deterioration of the whole cellular components within the mutant cells.

3.14 CLONAL SECTORS LACKING THE *EMP4* GENE DISPLAY A PRE-SENESCENCE ULTRA-STRUCTURAL ALTERATIONS

Hemizygous (*emp4/-*) sectors were distinguished in adult heterozygous (*emp4/+*) leaves for their yellow color and this phenotypic change was correlated with the lost Emp4 wild-type allele, due to the excision of the double *Ds* element linked to the *emp4* gene and consequent chromosome breakage. The ultra-structural analysis of hemizygous clonal sectors revealed many alterations in mesophyll cells morphology. Cells in clonal sectors varied from highly

damaged to completely empty. Although less severe, the alterations found in the clonal sectors are very similar to those found in emp4 homozygous tissues derived from light-exposed embryo rescued seedlings. In both tissues, the vast majority of the cells showed irregular shapes and most cells lack vacuoles and are devoid of organelles. It is remarkable that the most similar defects could be distinguished at the level of chloroplast inner membrane system, in which tylacoids are poorly organized and the grana are not stacked as in the heterozygous sectors; moreover plastoglobules could be distinguished in the stroma compartment and starch grains appeared smaller than in the green sectors. This morphological situation resembles the alterations founded in homozygous tissues but with less severe effects. This can be interpreted as if the cells in the hemizygous clonal sectors were in an initial step of the senescence process.

3.15 *EMP4* GENE IS REQUIRED FOR PROPER DEVELOPMENT OF THE PRIMARY ROOT

The main differences between wild-type and mutant roots were detected at the ultra-structural levels. The development of primary root in mutant samples is irregular, and their growth is reduced in the wild-type; some cells, completely empty, were found in the primary root along with partially developed cells with altered morphology. The senescence hypothesis postulated for the leaves could also explain the morphology of the mutant root tissues. Some features, already reported in the literature for root tissues entering plant cell death and senescence (Lam 2004; van Doorn 2005; van Doorn and Woltering 2005), could be distinguished in our samples. First of all the presence in the root cortex cells of many empty cells or of cells containing cellular debris, presumably remnants of the organelles.

Moreover, in contrast with wild-type root cells showing a clear vacuolarization, mainly consisting of a central vacuole in mutant cortex cells, such vacuole was never detected, however in some cells small non electron-dense vesicles were present. These two observations could be interpreted as the first symptoms of senescence or celldeath.

Therefore, it can be summarized that the *emp4* gene is essential for the development of the primary root.

In conclusion, *emp4* is essential for the proper development of primary root and leaf tissues. The morphological alteration observed in homozygous and hemizygous samples could be described as an acceleration of the senescence process. Moreover, we have demonstrated that light plays a role during the senescence process in the mesophyll tissues accelerating the process that leads to the cellular death. The acceleration of the senescence could be likely due to generation of ROS species scattering from chloroplasts that are unable to substain the photosynthetic process without an active role of mitochondria. The ROS generation probably is not due to mitochondria impairment because their ultra-structure is conserved even in the more damaged tissues.

4. FUNCTIONAL STUDY OF *EMPTY PERICARP4*: RECOMBINAT PROTEIN EXPRESSION ANALYSIS AND PUTATIVE INTERACTION WITH THE *EMP9475* GENE.

4.1 INTRODUCTION

The Pentratrico Peptide repeats (PPRs) motif, first described by Small and Peters (2000), is a degenerate 35 amino acid sequence closely related to 34 aa TetratricoPeptide repeat (TPR) motif. TPR and PPR occur in tandem repeats in a widespread protein family of both prokaryotes and eukaryotes for TPR, while PPR are eukaryotes exclusive (Andres et al. 2006; Lurin et al. 2004). On the basis of the solved 3D structure of TPR domain, as well as modeling approaches, PPR motif was described as two anti-parallel alpha helices that form a superhelix with a central groove with a positively charged ligand-binding surface (Small and Peters 2000). The nature of PPR motifs, as well as the literature, suggests that PPR proteins are able to bind other macromolecules in particular the nucleic acids DNA and RNA (Meieroff et al. 2003; Nakamura et al. 2003; Williams and Barkan 2003; Lurin et al. 2004; Yamazaki et al. 2004; Gothandam et al. 2005; Shmitz-Linneweber et al. 2005). PPR proteins have been found involved in the development (Cushing et al. 2005; Prasad et al. 2005; Gutierrez-Marcos et al. 2007), in the restoring of fertility (Bentolila et al. 2002; Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003; Akagi et al. 2004; Oguchi et al. 2004; Klein et al. 2004; Shmitz-Linneweber et al. 2005), and that many play essential roles in mitochondria and chloroplasts (Meieroff et al. 2003; Nakamura et al. 2003; Williams and Barkan 2003; Lurin et al. 2004; Yamazaki et al. 2004; Gothandam et al. 2005; Shmitz-Linneweber et al. 2005).

The origin of PPR gene family is still unclear and the wide colonization of plant genome, where PPR are one of the most abundant super-families of proteins, remains unclear. Some authors suggest that PPR have evolved with a mechanism like to “Birth and Death” initially described for immunoglobulins (Nei et al. 1997) and adapted for the resistance genes by Michelmore and Meyers (1998). Also divergence and duplication may be possible mechanisms by which the PPR family has expanded in plants (Geddy and Brown 2007). Geddy and Brown (2007) also suggested that PPR are under great selective pressure to alter their sequences, thus creating changes that will diversify the PPR population. This evidence supports the hypothesis that PPR proteins act as sequence specific binding proteins, requiring changes in their own sequence to match the sequence they will bind. The position

of PPR gene in the genome of *Arabidopsis* (Lurin et al. 2004), petunia (Bentolila et al. 2002), radish (Brown et al. 2003) and rice (Kazama et al. 2003; Komori et al. 2004; Akagi et al. 2004) was extensively studied. No clusters of PPR genes have been discovered apart from the chromosome 1 of *Arabidopsis* that bears 19 putative PPR genes (or pseudogenes) in an approximately 1 Mb region (Lurin et al. 2004).

Despite the growing number of studies in PPR field the mechanism of action of PPR proteins is still elusive. In addition it is still not clear if PPR proteins act alone or with some molecular partners. There are few well documented cases in which PPR proteins are confirmed to be associated in protein complexes where PPR could act as adapters, recruit some additional factors on the RNA target (reviewed in Andres et al. 2006) or, in addition, work in a dimeric state like the *Arabidopsis* HCF152 (Nakamura et al. 2003).

This study want to obtain insight into the action mechanism and the molecular relationship of the emp4 mutant, with empty pericarp9475 mutant. A second approach was conducted, in parallel, to elucidate the mechanism of action of EMP4.

4.2 RESULTS

4.3 COMPLEMENTATION TEST ON EMPTY PERICARP MUTANTS

A collection of empty pericarp mutant was crossed to understand their allelic relationship; originally the mutants have been isolated in populations carrying an active *MuDR* or *Spm* and they all behave as single gene mutants. To establish their allelic relationship we made crosses of each mutant with the others. To perform this test, pollen of 10-20 plants of emp9475 plants, whose heterozygous condition was ascertained by selfing, was applied to the silks of plants representing the selfed progeny of *+/emp4* parents. The resulting ears were then scored for visual evidence of mutant segregation. If only wild-type seeds were observed in all ears produced by this cross, the two mutants were considered not allelic, whereas if some of the ears yield mutants in about one-quarter of the seeds this was taken as evidence of allelism. Wild-type seeds were then taken for further test in F2 and F3, the expectation being that ears should be recovered segregating 3 to 1 for the mutant or not segregating, in a 3 to 1 ratio. If the F2 obtained by selfing non-mutant plants of the F1 progeny includes ears segregating an excess of mutants (30-40%), this segregation value, approaching a 9 to 7 ratio, was taken as evidence of heterozygosity for two *emp* mutants in the parental F1 plant, thus defining two genes. The results of this test were generally concordant in their conclusions. However, the results obtained in F1 and in F2/F3 lead to contrasting conclusions*, i.e*. one gene as inferred from the lack of complementation observed in F1 and two genes based on the observation of a segregation close to the 9 to 7 ratio, expected when the heterozygous *emp* F1 plants identify two genes (**Table 4.3**).

F1		F2			F3				
+/+	3:1	+/+	3:1	9:7	+/+	3:1	9:7		
Expected ratios									
		1	2	1	1	4	4	**Inferred number of genes**	
Cross mode	**Observed data (# of ears harvested)**							**F1**	**F2/F3**
emp 9475 x emp4	**31**	**9**	**21**	**48**	**6**	**2**	**23**	**16**	**1**	**2**

Table 4.3 Complementation test between emp9475 and emp4 mutants. +/+) non segregating ears; 3:1) ears segregating 3 to 1 for wild-type and empty pericarp phenotype respectively; 9:7) ears segregating 9 to 7 for wild-type and empty pericarp phenotype respectively and ears segregating for empty pericarp phenotype over the 30%.

4.4 STUDY OF THE EMP9475 MUTANT

According to the data obtained in the F1, one possible explanation of the data obtained from the allelism test might be that emp9475 is an allele of emp4. Alternatively it might be that *emp9475* and *emp4* are two independent genes whose products interact. The interaction between defective proteins in the double heterozygotes may lead to the mutant phenotype segregation in the F1. To gain inside the role exerted by the *emp9475* and to investigate about its possible interaction with *emp4* we have characterized the emp9475 mutant.

Embryo rescue was performed starting from immature, 20 DAP, kernels were excided and grown on synthetic medium for 30 days. The embryo rescue technique was successfully used for other empty pericarp mutants (Dolfini et al. 2007) and also for emp4. For the first four days of culture embryos were grown in a complete dark condition, subsequently embryos were split in two groups, the first one was exposed to light while the second was kept in the dark (**Table 4.4**). 100% of the light-exposed wild-type embryos germinated and after 8 to 12 DAC (Days after culture) developed the second leaf. In contrast, mutant embryos shown a lower percentage of germination (about 30,9%) and after 30 DAC few plants were able to reach the second leaf stage. Mutant seedlings were very short in stature and had a week appearance. In the dark all wild-type embryos were able to germinate although it was observed a slight reduction in the number of seedlings that reached the first and the second leaf stage. A substantial increase in the germination rate of mutant embryos was observed in the dark conditions, moreover the number of seedlings that reached the first and the second leaf stages in the dark treatment were almost doubled in comparison with those in the light condition.

Heterozygous females were crossed with heterozygous or hyperploid B-A translocation males with the aim of establishing the chromosomal arm location of *emp9475*. The F_1 that revealed the mutant was obtained from crosses involving the TB-1L-a male parent, thus indicating that *emp9475* lies on the long arm of chromosome 1. A more refined position for *emp9475* was achieved by analysis of simple sequence repeat (SSR) marker distribution in a segregating population obtained by crossing heterozygous females with B73, Mo17 and LEL (Large Embryo Line) inbred male parents. A polymorphism for the marker bnlg 1347

established a distance of about 1 cM (1 recombinant out of 13) between this marker and *emp9475* on the long arm of chromosome 1 at 1.10 bin (**Figure 4.4**). Because the emp4-1 mutant was isolated from an active *Mu*-population, we performed a cosegregation analysis in an attempt to identify whether insertion of a *Mu* element in the Emp9475 locus was responsible for the mutant phenotype.

Genomic DNA extracted from leaves of *emp9475/+* and *emp9475/emp9475* individuals whose genotype had been ascertained by selfing was compared by different approaches.

In a first approach, PCR was performed with specific *Mutator* primers in conjunction with *emp4* gene specific primers. No amplification were obtained in the emp9475 mutant DNA indicating that no transposons are located in the coding sequence of *emp4* gene. In addition TAIL-PCR approach was undertaken, to verify if a *Mutator* related polymorphism was associated with the mutant phenotype. To this aim we analyzed DNA extracted from heterozygous (*+/emp9475*), homozygous wild-type (+/+) and homozygous mutant (*emp9475/emp9475*) plants. TAIL-PCR (Liu et al. 1995) approach did not allow to identify a polymorphic band associated to the DNA extracted from emp9475 homozygous and heterozygous seeds.

Preliminary data obtained with AIMS technique, that is more sensitive than TAIL-PCR (Frey et al. 1998), failed to identify a polymorphic fragment associated to the *Mutator* element in the homozygous and heterozygous emp9475 plants. AIMS was performed with a primer mix as well as with 4 separate primer species that differs from the last base to increase the specificity, 3 replicas were performed but no polymorphic bands were detected on the electrophoretic profiles.

					% of plants in different stage of development		
Phenotype	**Treatment**	**DAC**	**No. of cultured embryos**	**Germination (%)**	**Coleoptile[1]**	**First leaf**	**Second leaf**
WT	Light	12	175	100	0	3,8	96,2
WT	Dark	12	85	100	6,8	2,4	90,8
emp9475	Light	30	679	30,9	29,5	0,9	0,5
emp9475	Dark	30	116	83	75,1	6,2	1,7

Table 4.4 Germination and developmental profile of seedlings obtained trough embryo rescue at 12 (WT) and 30 (emp9475) DAC (Days After Culture). Light: embryos grown for four days in the dark condition and subsequently exposed to light. Dark: embryos entirely cultivated in the dark. (1) Coleoptile stage includes the plants with open or closed coleoptile.
WT: wild-type

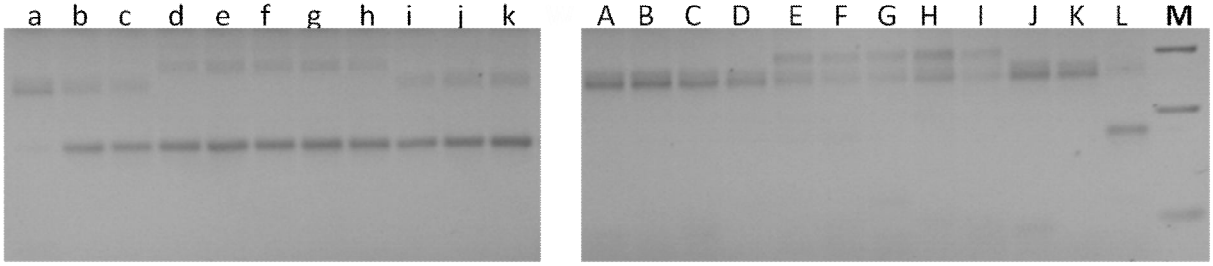


Figure 4.4 SSR analysis, bnlg 1347, on emp9475 plants. Minorcase letters: heterozygous (+/*emp9475*) plants ; Capital letters: homozygous (+/+) plants. a, b, c) Mo17; d, e, f, g, h) LEL (Large embryo line); i, j, k) B73. A, B, C, D) Mo17; E, F, G, H, I) LEL; J, K, L) B73. M = molecular marker.

4.5 EMP4 RECOMBINANT PROTEINS SYNTHESIS IN *E. COLI*.

The expression of recombinant protein in *E. coli* is a wide use technique for the production of proteins in a rapid and cheap fashion. Although the expression of proteins *E. coli* is quite simple for many polypeptides some more attentions are needed in chase of proteins that interfering with *E. coli* life, like toxic proteins or some transcription factor that could interact with bacterial gene expression.

Full EMP4 recombinant protein and two fragments of EMP4 (N-terminal and C-terminal) was produced in *E.coli*. A system with tight control of induction (pBAD-Thio-TOPO® vector Invitrogen and LMG194 strain) was chosen to allow a minimal expression level of un-induced *emp4* gene. The *emp4* sequence, a part from the signal peptide and 15 bases that encodes for highly hydrophobic sequence, was sub-cloned in the expression vector pBAD-Thio-TOPO® (Invitrogen, **Figure 4.5.1**). The recombinant vector was transferred to the LMG194 *E. coli* strain and cells were grown in liquid cultures. Total proteins were extracted and separated in SDS-PAGE electrophoresis. Gel staining revealed the presence of a band whose molecular size (about 94000 Da) is attributable to EMP4-thioredoxin fusion protein only in cell samples transformed and induced by arabinose (**Figure 4.5.2**).

We introduced some variations to protocol suggested from the producer of the expression kit (Invitrogen). The higher expression of EMP4-thioredoxin fusion protein was achieved using LB medium supplied with glucose (0,1%), an induction time of 54 hours at 29 °C with a concentration of inductor of 2%. In particular we noticed that low growth temperature increase the accumulation of the putative EMP4-thioredoxin fusion protein in *E. coli* cells. The 94000 Da protein is more abundant in cells grown at 29 °C in comparison with those grown at 37 °C in the same conditions. The 94000 Da protein could be founded in *E. coli* cells induced a minimum of 4 hours. The amount of the putative EMP4-thioredoxin fusion protein, produced in *E. coli*, tend to increase during time of induction until the 70 hours. Prolonged inductions (over 70 hours) seem to had no effect on further accumulation of the 94000 Da protein.

The conditions used for the production of the full-lenght EMP4 recombinant protein in *E. coli* were used also for the production of the N and C-terminal domains of EMP4 (fragments were predicted to be 23-26000 Da respectively). SDS-PAGE analysis revealed the presence of bands attributable to N/term-thioredoxin and C/term-thioredoxin (**Figure 4.5.2**).

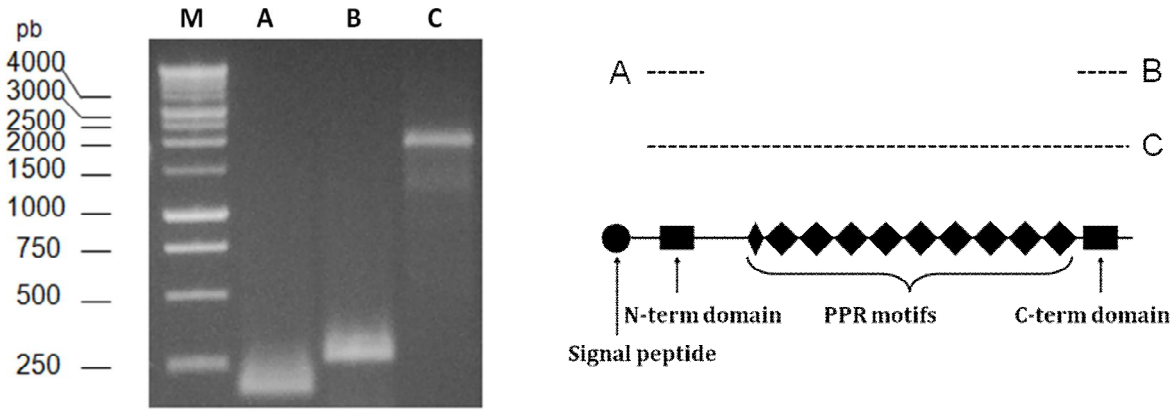


Figure 4.5.1 Agarose gel of purified amplification products used for cloning in pBAD-Thio/TOPO vector. A) N-terminal region; B) C-terminal region; C) full length *emp4* without signal peptide. M = molecular marker.

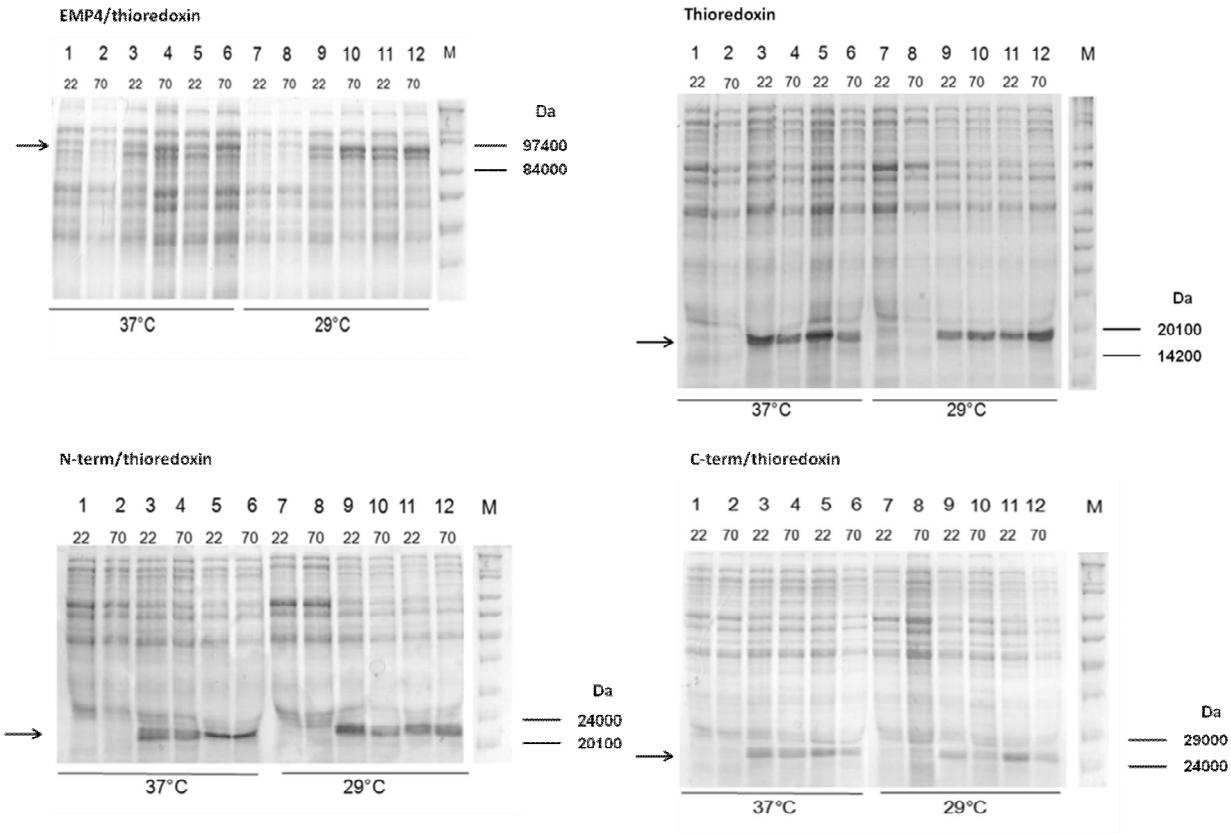


Figure 4.5.2 SDS-PAGE of *E. coli* LMG194 proteins after the transformation with EMP4/thioredoxin, N-term/thioredoxin and C-term/thioredoxin constructs or with the empty vector (thioredoxin) used as control of induction. 1, 2, 7, 8) not induced; 3, 4, 9, 10) induced with 0.2% arabinose; 5, 6, 11, 12) induced with 2% arabinose. 22 and 70 indicate the hours of growth after induction. Cell were grown at 37 and 29 °C. Arrows indicate the bands whose increase during induction. M = molecular marker (6000-200000 Da)

4.6 EMP4 BELONGS TO THE P CLASS OF PPR PROTEINS

Searches conducted with EMP4 sequence revealed high similarity with many PPR proteins. Among those a sequence of *Sorghum bicolour* (XP 002466528.1 protein and XM 002466483.1 nucleotide) and an accession of *Oryza sativa Japonica* sub-specie (NP 001051146.1 protein and NM 001057681.1 nucleotide) were identified as putative orthologues of *emp4* (**Figure 4.6.1**). Other accessions shown significant identities to EMP4 were of dicotyledonous species (*Popolus trichocarpa*, *Vitis vinifera* and *Arabidopsis thaliana*). However, these sequences were similar mainly in the PPR region, so we concluded that was not so strongly related with EMP4. BLAST similarity searches addressed on N and C-terminal region of EMP4 recovered few sequences with high identities rates. Only the sequence of *S. bicolour* and *O. sativa* judged as putative orthologues of EMP4 showed significant identity; we concluded that the domains both at N (**Figure 4.6.2**) and C-terminal (**Figure 4.6.3**) regions are specific for EMP4 protein. The *emp4* gene lies on chromosome 1L, a region sinthenic with chromosome 3S of rice in which the putative orthologue of *emp4* gene is located (**Figure 4.6.4**).

The PPR domains of EMP4 protein were subjected to sequence analysis by mean of global alignment *in silico* tool CLUSTAL W. The results showed that EMP4 PPR domain were highly similar to P-type motif. All EMP4 PPR motifs showed about the same similarity with the consensus sequence of the P-type motif. Alignment with other kinds of PPR motifs given no results therefore, we concluded that EMP4 is a peculiar P-type PPR protein with two uncharacterized motifs on both N and C-terminal regions.

The transcript sequences of the four mitochondrial genes (*mttB*, *rps2a*, *rpl16* and *rps3*), putative targets of EMP4, were retrieved from NCBI data bank and analyzed by *in silico* alignment tool CLUSTAL W. The sequence of the fifth putative target *rps2B*, was not included because its retained a pseudo-gene (Perrotta et al. 2002). Both the coding region and the upstream sequences of these mitochondrial genes were aligned to highlight the presence of common sequence features that could explain the regulation described in a previous work

(Gutierrez-Marcos et al. 2007), however no common sequences could be retrieved from the analysis.

The secondary mRNA structures were predicted using a web resource (**Figure 4.6.5**; www.genebee.msu.su/services/rna2_reduced.html), the superimposition of the structures obtained showed that these secondary structures contain no specific secondary motifs that could explain the co-regoulation of the four putative targets of EMP4. We concluded that no common features both at level of sequence and in secondary structure could be recognized in the four mitochondrial genes analyzed.

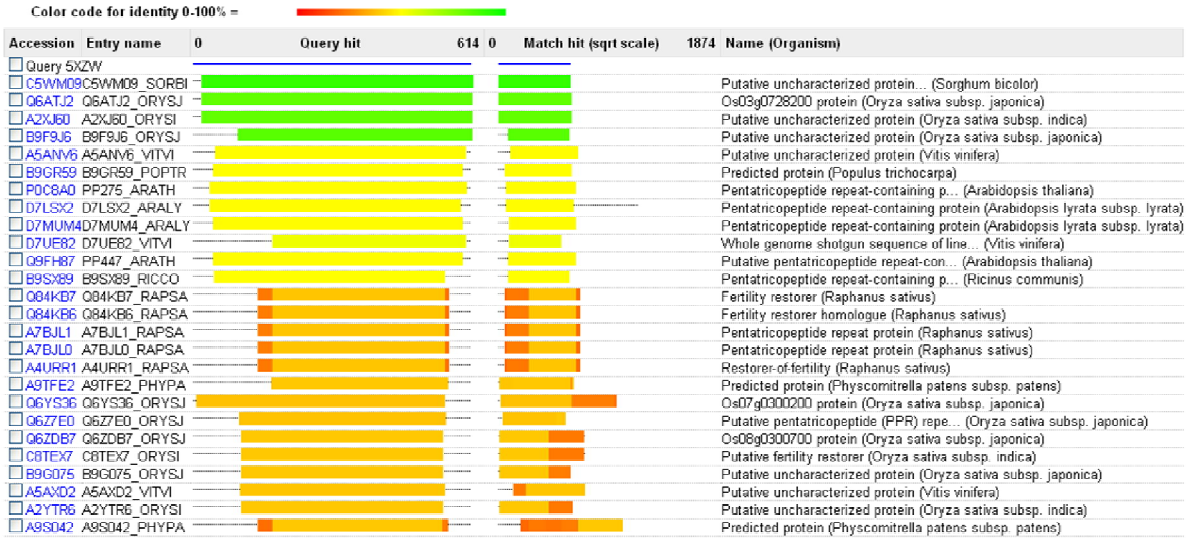
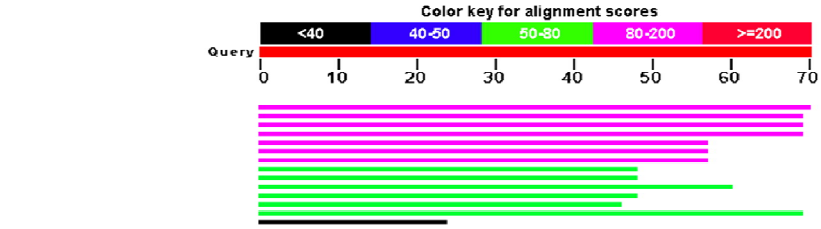


Figure 4.6.1 Alignment with the EMP4 sequence (at PROSITE) showing a nearly perfect match with monocotyledonous species *Sorghum bicolor* and *Oryza sativa* and significant identities with dicotyledonous species *Popolus trichocarpa*, *Vitis vinifera* and *Arabidopsis thaliana*.

Figure 4.6.2 Alignment with the EMP4 N-terminal using BLAST (at NCBI) sequence showing identities with monocotyledonous species *Sorghum bicolor* and *Oryza sativa* and similarities with uncharacterized proteins of other dicotyledonous plants.



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Links
XP_002466528.1	hypothetical protein SORBIDRAFT_01g009420 [Sorghum bicolor] >gb|EER935	119	119	100%	1e-25	U G
EAY90870.1	hypothetical protein OsI_12477 [Oryza sativa Indica Group]	112	112	98%	1e-23	
NP_001051146.1	Os03g0728200 [Oryza sativa Japonica Group] >gb|AAT77067.1| putative PPR	112	112	98%	1e-23	U G
EEE59444.1	hypothetical protein OsJ_11623 [Oryza sativa Japonica Group]	112	112	98%	1e-23	
XP_002302657.1	predicted protein [Populus trichocarpa] >gb|EEE81930.1| predicted protein [P	88.2	88.2	81%	3e-16	U G
NP_201383.1	pentatricopeptide (PPR) repeat-containing protein [Arabidopsis thaliana] >sp|	80.9	80.9	81%	5e-14	U G
CAA16678.1	predicted protein [Arabidopsis thaliana]	80.9	80.9	81%	5e-14	
CAN71515.1	hypothetical protein [Vitis vinifera]	77.8	77.8	68%	3e-13	
CBI41047.3	unnamed protein product [Vitis vinifera]	77.8	77.8	68%	4e-13	
XP_002866691.1	pentatricopeptide repeat-containing protein [Arabidopsis lyrata subsp. lyrata]	77.8	77.8	85%	4e-13	G
XP_002877696.1	pentatricopeptide repeat-containing protein [Arabidopsis lyrata subsp. lyrata]	72.4	72.4	68%	1e-11	G
CAB66911.1	putative protein [Arabidopsis thaliana]	70.1	70.1	65%	7e-11	
NP_190542.4	unknown protein [Arabidopsis thaliana] >sp|P0C8A0.1|PP275_ARATH RecNam	70.1	70.1	98%	9e-11	U G
XP_002530608.1	pentatricopeptide repeat-containing protein, putative [Ricinus communis] >gb	38.9	38.9	34%	0.18	G

Figure 4.6.3 Alignment with the EMP4 C-terminal using BLAST (at NCBI) sequence showing identities with monocotyledonous species *Sorghum bicolor* and *Oryza sativa* and similarities with uncharacterized proteins of other dicotyledonous plants.

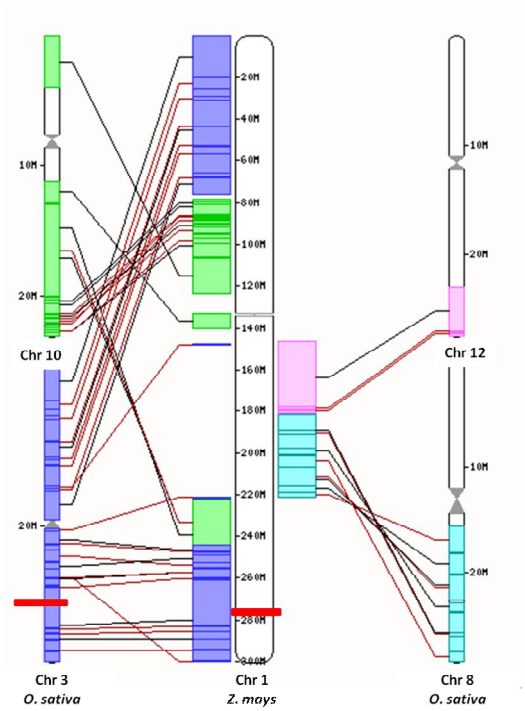


Figure 4.6.4 Sinthenic regions of *Zea mays* chromosome 1 with *Oriza sativa* chromosomes. Red bars indicating the position of *emp4* and its putative orthologue in rice.

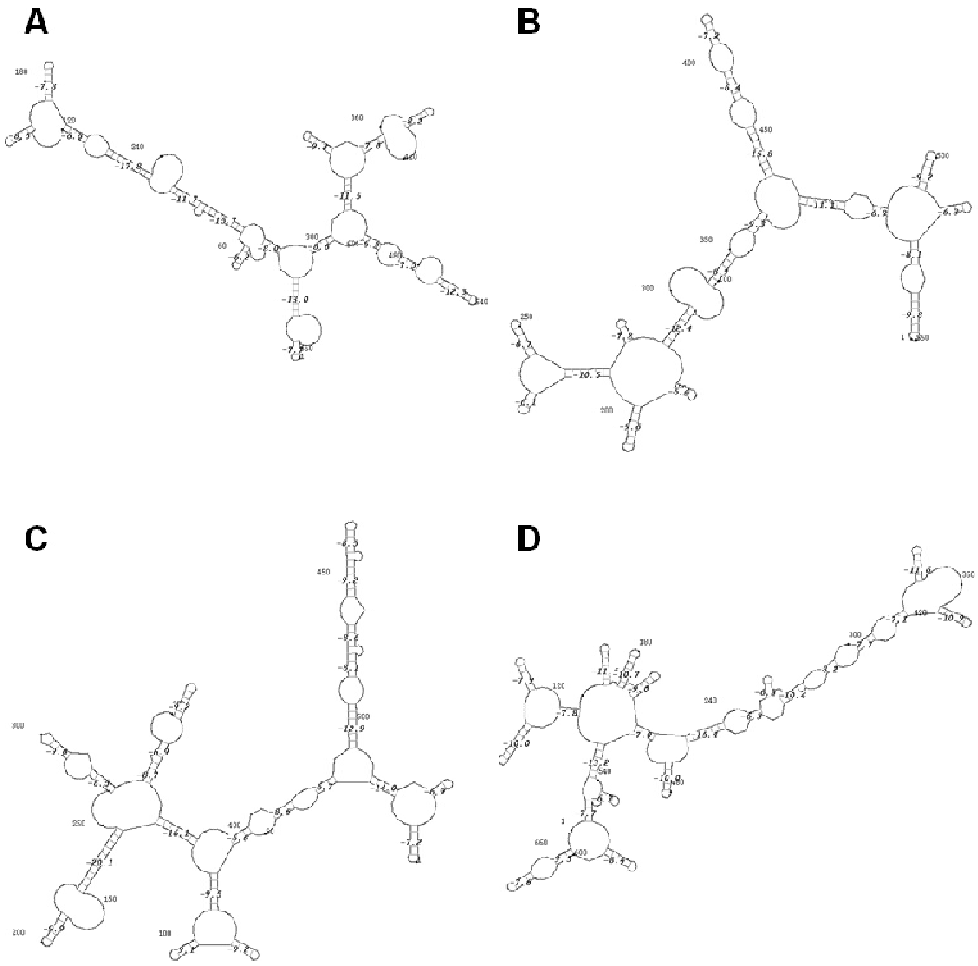


Figure 4.6.5 Second dimension structure of upstream region of mitochondrial genes predicted with the web resource - www.genebee.msu.su/services/rna2_reduced.html A) *mttB*; B) *rpl16*; C) *rps2A*; D) *rps3*.

4.3 DISCUSSION

4.4 *EMP4* AND *EMP9475* PUTATIVE INTERACTION

The origin of PPR gene family is still unclear and the wide colonization of plant genome, where PPR are one of the most abundant super-families of proteins, remains unclear. Geddy and Brown (2007) also suggested that PPR are under great selective pressure to alter their sequences, thus creating changes that will diversify the PPR population. This evidence supports the hypothesis that PPR proteins act as sequence specific binding proteins, requiring changes in their own sequence to match the sequence they will bind. No clusters of PPR genes have been discovered apart from the chromosome 1 of *Arabidopsis* that bears 19 putative PPR genes (or pseudogenes) in an approximately 1 Mb region (Lurin et al. 2004). There are few well documented cases in which PPR proteins are confirmed to be associated in protein complexes where PPR could act as adapters, recruit some additional factors on the RNA target (reviewed in Andres et al. 2006) or, in addition, work in a dimeric state like the *Arabidopsis* HCF152 (Nakamura et al. 2003).

The study of empty pericarp mutants is complicated by the fact their phenotype is almost the same with slight differences in the wrinkles of pericarp and so is quite impossible to classify the phenotype. The behavior of *emp9475* described in embryo rescue resembles the *emp4* comportment and the phenotype of the two mutants is the same thus, the first question to answer is: are *emp4* and *emp9475* allelic or two independent genes? To answer this central question we used the complementation test. This test generally answers in a "yes or no" fashion but, in this case the result is not the answer. In fact, the segregation relationship of emp4 and emp9475 cross are discordant in F1 versus F2/F3; this result is consistent for the great number of ears scored during the years and is not unknown by the literature (Hawley and Gilliland 2006). This intriguing result could be explained in two ways: the two mutants are allelic and a third *emp* gene segregates in the F2/F3 progenies, or the two mutants are ascribable to two linked genes whose product interact functionally as described in the Second Site Non Complementation cases (Hawley and Gilliland 2006).

For the moment we have some indication that could suggest that *emp4* and *emp9475* genes are independent. The map position for *emp9475* was determined by means of B-A translocation and confirmed with an SSR microsatellite: *emp9475* lies on the long arm of chromosome 1 (Bin 1.10). This fact could suggest that in maize a little cluster of embryo-essential genes are located on the 1L chromosome on which *emp4* and also *dek22* genes reside. The *dek22* genes have not been studied intensively so far but it have a strong defective kernel phenotype (very similar with *emp4* phenotype), is lethal and therefore essential for seed development. The characterization of *emp9475* and *dek22* will address their genetic nature and maybe we would face the first cluster of PPR genes discovered in *Z. mays*.

4.5 IDENTIFICATION OF THE EMP9475 GENE

Unfortunately, AIMS and TAIL-PCR approaches, used to identify the nature of the mutation in the *emp9475* gene, gives no results with the *Mutator* specific primers thus, the mutation in the *emp9475* may be due to other genetic causes. The isolation of the *emp9475* gene could be the first step to understand if the SSNC hypothesis is true and, therefore, *emp4* and *emp9475* gene products are functionally associated in a protein complex.

Mutant embryos shown a lower percentage of germination (about 30,9%) and after 30 DAC few plants were able to reach the second leaf stage. Mutant seedlings were very short in stature and had a weak appearance and a pale green color. A substantial increase in the germination rate of mutant embryos was observed in dark conditions, moreover the number of seedlings that reached the first and the second leaf stages in the dark treatment were almost doubled in comparison with those in the light condition.

Likely for the *emp4* mutant, the dark treatment allowed recovery of more seedlings from mutant embryos. This fact would suggest that also in *emp9475* the light exposure could drastically increase the effects of the mutation.

4.6 RECOMBINANT PROTEIN EXPRESSION ANALYSIS

The sequence of the *emp4* gene was cloned in the pBAD-Thio/TOPO Vector that allowed the expression of proteins that are putatively toxic or interfering with *E. coli* life. The generally accepted endosimbiotic evolutionary theory proposes that mitochondria was derived from an ancient alpha-proteobacteria (Margulis 1981), thus, their genetic background as well as their transcription/translation mechanism are very similar. EMP4 is a PPR protein that putatively plays a role in maize mitochondria transcription, and therefore, could also interact with the gene expression in *E. coli*. For this reason the vector we chose allowed a tight control of un-induced proteins limiting their basal expression. The strategy chosen allowed the isolation of a band with a molecular mass ascribable to EMP4-Thioredoxin protein fusion. The recombinant protein, after the purification and the immunological test needed to confirm its nature, will be used for a technique useful for the isolation of the putative partner of EMP4.

5. MATERIALS AND METHODS

5.1 EMBRYO RESCUE

20 DAP segregating ears was surface sterilized with bleach (4% of NaClO) for 20 minutes and then rinsed with sterilized water. Embryos were excised from kernels with a scalpel and disposed on sterile solid (agar 0,8%) MS medium (Murashige and Skoog 1962) supplied with sucrose (20%) and adjusted to 5,6-5,8 with NaOH. Embryos were grown in a on growth chamber at 25 °C. All the embryos were grown for the first four days in dark conditions then some embryos were exposed to light (photoperiod: 16 hours of light and 8 hours of dark) and some were grown without light for 30 days. Sampling was performed at 12 DAC (Days After Culture) for wild-type plants and 30 DAC for emp4 plants.

5.2 DNA PREPARATION, GENOTYPING, SSR ANALYSIS

DNA was extracted with the method proposed by Arthur et al. (2003). Two PCR reactions with primers specific to the wild-type (RT5rev/Oest11 primers) and mutant allele (RT5rev/Mu58) were used to address the genotype of plant material analyzed (**Table 5**). PCR were performed with Taq polymerase (Promega) adding betaine (Sigma, final concentration 0.5M) to each reaction according to the manufacturer's instructions. SSR microsatellite was detected, using the conditions as indicated on Maize GDB (www.maizegdb.org), and separated on high-resolution agarose gel (Invitrogen).

5.3 RNA PREPARATION AND RETROTRANSCRIPTION

RNA was extracted with a phenol chloroform method as reported previously (Bateman et al., 2004), with the single exception of doubling the LiCl step. RNA was subsequently treated with restriction enzymes (ECORI and PSTI at 37 °C for 30 minutes; New England BioLab) and DNase I (at 25 °C for 30 minutes, Invirogen) then purified with another phenol-chloroform extraction. cDNA was produced with polyT oligo-nucleotides as primers using the commercial kit SuperScript III (Invitrogen) according to the manufacturer's guide.

5.4 LIGHT AND TRANSMISSION ELECTRON MICROSCOPY

Samples were cut from the first leaf and immediately fixed in 3 % glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 2 h under vacuum, washed in buffer and placed in 0,5% OsO4 at 4 °C in 0,5% cacodylate buffer 0.1M pH 7.4 for 1.5 hours in agitation in dark conditions. The specimens, dehydrated in a graded ethanol series, were embedded in a low viscosity epoxy resin (Spurr 1969) and cut on a ultramicrotome. 2µ thick sections, stained with toluidine blue, were examined with Leica DM RA2 microscope. 75 nm thick sections, stained with uranyl acetate (Reynolds 1963), were examined with an Energy Filtered TEM LEO 912ab electron microscope. All experiments were repeated three times. The genotype of all the samples analyzed was addressed with two PCR reactions.

5.5 TRANSFORMATION OF *E. COLI* AND SDS-PAGE

To clone the full emp4 sequence in the appropriate vector proofreading Taq polymerase (Promega) was used (**Table 5**). Fragments were purified by means of a commercial kit (Wizard SV Gel and PCR Clean UP System Promega) and directly cloned in pBAD-Thio/TOPO vector (Invitrogen) according to the manufacturer's protocol. To optimize the expression of our protein in the LMG194 E. coli strain the transformed cells were grown in LB medium supplied with glucose (0,1 and 0,2%) grown in controlled temperature conditions (29 or 37 °C)and induced with arabinose (0,002%, 0.02%, 0,2%, 2%, and 20%) for many hours (from 4 to 70 hours). Afterwards the induction cells were collected, centrifuged for 5 minutes, diluted in water and treated with sonic waves for 30 min at 60 °C. Samples were centrifuged for 10 minutes, then were treated for their preparation and for the SDS-PAGE, as reported in (Shagger and von Jagow 1987).

5.6 B-A TRANSLOCATION AND COMPLEMENTATION TEST

The mutants under test were crossed *inter se* in all pair wise combinations to assay their complementation pattern and progeny ears were analyzed as previously described (Consonni et al. 1998; Consonni et al., 2003). Their chromosomal arm location was

established by crossing the progeny of outcrossed heterozygous +/emp plants with male parents including the entire set of B–A translocations covering about 90% of the genome. The source of these stocks is the Maize Stock Center, Urbana, Ill. USA. Ears (2-3) from these crosses were then scored for the presence of emp seeds with a frequency of 15–20% as evidence that the translocation is uncovering the mutant under test.

5.7 TAIL PCR AND AIMS ANALYSIS

TAIL-PCR was conducted as reported in Liu et al. (1995) with slight modifications to optimize the reaction with Mutator specific primers (TIR6/OMuA; **Table 5**).

AIMS analysis was conducted according to Frey et al. (1998) on DNA derived from heterozygous and homozygous emp9475 plants. Some modifications were developed to perform the AIMS with Mutator specific primers (Mu12/Mu9242 for the linear PCR and AIMS1-Mu2/MseI zero/BfaI zero/TaqI zero for the exponential PCR; **Table 5**).

5.8 SEQUENCE ANALYSIS AND BIOINFORMATIC TOOLS

Sequencing was provided by BMR Genomics (Padova, Italy). Reference sequence for the mitochondrial genes was derived from the NCBI data base (http://www.ncbi.nlm.nih.gov). Bioinformatic analysis was conducted with BLAST at NCBI and with tools provided by the SDSC Biological Workbench (http://workbench.sdsc.edu). Analysis of mitochondrial transcripts was conducted with the web resource at www.genebee.msu.su/services/rna2_reduced.html

Primer	Sequence
TAIL PCR	
TIR6	AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC
OMuA	CTTCGTCCATAATGGCAATTATCTC
AIMS linear PCR	
Mu12	GAATCCCTTCCGCTCTTCGTCTA
Mu9242	AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC
AIMS exponential PCR	
AIMS2-Mu2	GCBCTCTTCGTCYATAATGGCAATTATCTC
MseI zero	GATGAGTCCTGAGTAA
BfaI zero	GATGAGTCCTGAGTAT
TaqI zero	ATGAGTCCTGAACGA
Adapters used for AIMS	
MseAd1	GACGATGAGTCCTGAG
MseAd2	TACTCAGGACTCAT
TaqAd1	ATGAGTCCTGAA
TaqAd2	TACTCAGGACTTGC
Genotyping	
RT5rev	GGGAGCACCAGTGGAAGAAGTGC
Mu58	CCAACGCCTCCATTTCGT
Oest11	ATCGCCCGCATGTGCATCTC
SSR analysis	
bnlg 1347 lp (262)	TTGCAATCACACAGGTGGTT
Bnlg 1347 up (219)	GTGGTCACGACGAAATCCTT
C-terminal (protein expression)	
EXPR3_FNEW	GACACATTTGCAAAGTTGATGA
EMP4EXPR1R	GCCGGAAAATTCTACTTCATCA
N-terminal (protein expression)	
EXPR2_FNEW	CCGTCGCTGCCCTCGCCGCT
EMP4EXPR2R	GGGCTGGTCGTGGTCGGGGC
EMP4 full (protein expression)	
EXPR1_FNEW	TGTCTCTCCTCCACCGGCGC
EMP4EXPR1R	GCCGGAAAATTCTACTTCATCA

Table 5 Primers used and their respectively sequence

6. LITERATURE CITED

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