Genetic Dissection of Organellar-Translation-Dependent Retrograde Signalling in *Arabidopsis*

Dissertation

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“Do you know the difference between an error and a mistake? […]
Anyone can make an error.
But that error does not become a mistake until you refuse to correct it”

(Timothy Zahn)

Summary

Retrograde mechanisms have evolved to communicate the functional and developmental state of the organelles to the nucleus, which in turn modulates anterograde control and cellular metabolism accordingly. Previous works showed that simultaneous impairment in the organelle translational machinery, due to a mutation in the prolyl-tRNA synthetase PRORS1, targeted to both chloroplasts and mitochondria, induces a specific downregulation of nuclear photosynthetic genes. However, this downregulation of nuclear photosynthetic genes was not observed in *mrpl11-1* and *prpl11-1* mutants, impaired in the mitochondrial and plastid ribosome function, respectively, but only in the *mrpl11-1prpl11-1* double mutant, indicating that the translation rate in both chloroplasts and mitochondria contributes synergistically to the regulation of nuclear gene expression. Despite these findings, primary nuclear target genes of retrograde signalling, are still not characterised. In this thesis it was shown that an inducible expression system, used in combination with transcriptomics, might represent a powerful tool to investigate and to identify possible candidate regulatory proteins, involved in the translation-dependent-retrograde signalling. *MRPL11*, *PRPL11* and *PRORS1* were cloned under the control of an ethanol inducible promoter, pAlcA, and *mrpl11-1*, *prpl11-1* and *prors1-2* mutants were transformed, generating *Arabidopsis* overexpressing transgenic lines. The ethanol induction system in both *in vitro* and on soil conditions was established, and it was shown that the system did not influence *per se* photosynthetic performances and did not interfere with the expression of photosynthetic marker genes. Upon induction, p*Alc::MRPL11 mrpl11* and p*Alc::PRPL11 prpl11* lines showed the complete or partial recovery from the mutant phenotype and this seemed to be directly correlated to the amount of the transcript level of the transgenes. By contrast, although phenotype complementation also occurred in p*Alc::PRORS1 prors1.2* plants exposed to ethanol vapour, the detected transcript level of *PRORS1* did not directly correlate with the complementation dose, and therefore the employment of this system seemed to be critical for this genotype. Because of its phenotypic characteristics, *prpl11-1* was chosen to perform expression profiling with Affymetrix ATH1 microarrays: 30% of the products of the significantly differentially expressed genes were located in the chloroplast and, among them, the expression of 18 genes, whose products are related to photosynthesis or chloroplast activities, was upregulated. On the basis of their localisation and function, the expression of five upregulated genes was tested in the other translation impaired mutants and in the ethanol inducible overexpressing lines, to verify their possible involvement in nucleus-organelle signalling. Among the tested genes, just two, *PFKB1* and *HD2A*, displayed an increase in the transcript level in all loss-of-function or knock down mutants. In p*Alc::MRPL11 mrpl11* lines, the transcript level of these genes did not show direct correlation with *MRPL11* expression. Moreover, plastome and chondriome analyses were performed in the corresponding knockout lines: expression patterns of the plastid-encoded genes displayed a transcript increase of genes encoding for ribosomal proteins. Furthermore, the observed transcript increase of plastid-encoded genes in the *mrpl11-1* mutant confirmed the tight interdependency between plastid and mitochondrion.

Zusammenfassung

Um den Funktions- und Entwicklungsstatus von Organellen dem Zellkern zu kommunizieren, entwickelten sich im Laufe der Evolution retrograde Signalwege, die wiederum Veränderungen in der anterograden Kontrolle des Zellkerns über die Organellen zur Folge haben können. Frühere Experimente zeigen, dass eine zeitgleiche Beeinträchtigung der Translation in den Mitochondrien und Plastiden eine spezifische Verringerung der Expression im Kern kodierter photosynthetischer Gene induziert. Die zeitgleiche Beeinflussung der Translationsraten ist durch eine Mutation im Gen der Prolyl-tRNA-Synthetase PRORS1 möglich, welche in beiden Organellen lokalisiert ist. Im Gegensatz hierzu konnte diese Veränderung der Expression nicht in den jeweiligen Einzelmutanten *mrpl11-1* und *prpl11-1*, die jeweils nur in der mitochondrialen bzw. der chloroplastidären Ribosomenfunktion beeinträchtigt sind, festgestellt werden. Jedoch tritt dieser Effekt ebenso in der Doppelmutante *mrpl11-1 prpl11-1* auf, was darauf hinweist, dass die Translationsraten in beiden Organellen synergistisch dazu beitragen, die Expression kernkodierter Gene zu regulieren. Trotz dieser Beobachtungen wurden primäre Zielgene des retrograden Signalweges noch nicht charakterisiert. In dieser Dissertation wird gezeigt, dass Systeme basierend auf induzierbarer Expression kombiniert mit Transkriptomanalysen ein kraftvolles Instrument darstellen können, mögliche Kandidaten für regulatorische Proteine, die im translationsabhängigen Signalweg involviert sind, zu isolieren. *MRPL11*, *PRPL11* und *PRORS1* wurden unter die Kontrolle eines mit Ethanol induzierbaren Promotors pAlcA kloniert und mit diesen Konstrukten die jeweiligen *Arabidopsis thaliana* Mutanten *mrpl11-1*, *prpl11-1* und *prors1-2* transformiert, um Linien mit einer induzierbaren Überexpression der Gene zu erhalten. Die Anwendung eines auf Ethanol basierenden induzierbaren Systems auf in Erde und *in vitro* angezogenen Pflanzen wurde etabliert, wobei ein Einfluss des induktionsauslösenden Ethanols auf die photosynthetische Leistung der Pflanzen und auf die Expression photosynthetischer Markergene überprüft und ausgeschlossen wurde. Nach Induktion zeigten die Linien p*Alc::MRPL11 mrpl11* und p*Alc::PRPL11 prpl11* eine komplette oder teilweise Regeneration des für die Mutanten typischen Phänotyps und diese korreliert direkt mit dem Transkriptlevel des Transgens. Die Komplementation des Phänotyps, die auch in der Linie p*Alc::PRORS1 prors1.2* nach Induktion mit Ethanol zu beobachten war, hingegen zeigte keine direkte Korrelation mit dem gemessenen Transkriptlevel von *PRORS1*. Aus diesem Grund ist die Anwendung des Systems auf diesen Genotypen in Frage zu stellen. Aufgrund ihrer phänotypischen Eigenschaften wurde die Linie *prpl11-1* ausgewählt, um ein Expressionsprofil mithilfe von Affymetrix ATH1 Microarrays zu erstellen. 30% der Proteine der signifikant unterschiedlich regulierten Gene sind im Chloroplasten lokalisiert und davon waren 18 Gene hochreguliert, die die Photosynthese oder die Chloroplastenaktivität betrafen. Basierend auf ihrer möglichen Funktion in Signalwegen wurden hieraus fünf Gene ausgewählt und deren Expression im Hintergrund der anderen in der Translation beeinträchtigten Mutanten und in den Ethanol induzierbaren Überexpressoren untersucht. Unter den getesteten Genen zeigten nur *PFKB1* und *HD2A* einen Anstieg des Transkriptlevels in allen Knock-out- oder Knock-down-Mutanten. Dies deutet auf eine mögliche Funktion dieser beiden Gene in einem Signalweg zwischen Zellkern und Organellen hin.

In den Linien p*Alc::MRPL11 mrpl11*, zeigte die Expression dieser Gene dennoch keine direkte Korrelation zur Transkriptmenge von *MRPL11*. Des Weiteren wurden Plastom und Chondriom-Analysen mittels Macroarrays in den jeweiligen Mutanten durchgeführt. Das Transkriptionsmuster der plastidär kodierten Gene zeigte einen generellen Anstieg des Transkriptlevels der Gene ribosomaler Proteine, was auf ein Bestreben hindeutet, Defekte durch die T-DNA-Insertion auszugleichen. Dazu deutet der beobachtete Anstieg plastidärer Transkripte in der Mutante *mrpl11-1* darauf hin, dass es eine starke gegenseitige Kommunikation zwischen Chloroplasten und Mitochondrien gibt.

Index

Abbreviations

°C	Degree Celsius
CaMV 35S	35S promoter of the Cauliflower Mosaic virus
Alc	Ethanol inducible overexpression system
Alc lines	Overexpressing transgenic lines
ATP	Adenosine triphosphate
bp	base pair
cDNA	Complementary deoxyribonucleic acid
Chl	Chlorophyll
Col-0	Columbia 0
C. reinhardtii	*Chlamydomonas reinhardtii*
cyt b_6/f	Cytochrome b_6/f complex
DNA	Deoxyribonucleic acid
EtOH	Ethanol
F_V	Variable fluorescence
g	Gram
GST	Genomic Sequence Tags
h	Hour
LB	Left border
LHC	Light-harvesting complex
m	Meter
min	Minute
M	Molarity
mol	Mole
MRPL11 ribosome	Protein 11 of the 50S subunit of the mitochondrion
$NADP^+/H$	Nicotinamide adenine dinucleotide phosphate
NDH	NADPH deydrogenase complex or NAD(P)H-plastoquinone-oxidoreductase complex
NF	Norflurazon
NPQ	Non-photochemical quenching
PAM	Pulse amplitude modulation

PCR	Polymerase chain reaction
PFD	Photon flux density
PGE	Plastid gene expression
PRORS1	Prolyl-tRNA-synthetase
PRPL11	Protein 11 of the 50S subunit of the chloroplast ribosome
PSI	Photosystem I
PSII	Photosystem II
qPCR	Quantitative polymerase chain reaction
RB	Right border
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
s	Second
SD	Standard deviation
T-DNA	Transfer-DNA
UBI	Ubiquitin
$^{V}/_{V}$	Volume per volume
$^{W}/_{V}$	Weight per volume
WT	Wild-type
XVE	Estradiol inducible chimeric transcription activator
Φ_{II}	Effective quantum yield of photosystem II

1. INTRODUCTION

1.1 Endosymbiotic origin of mitochondrion and chloroplast

Chloroplasts and mitochondria descended from free-living bacteria ancestors (Dyall et al, 2004). Phylogenetic, biochemical and structural analyses suggest that mitochondria descended from an α-proteobacterium-like ancestor, that invaded or was engulfed by an archaeal-like host 1.5 billion years ago or earlier. This transformation was accompanied by a massive translocation of bacterial genetic information into the nuclear genome, as testified by present-day mitochondrial genomes, which contain between 12 and 20 protein-coding genes (Andersson et al, 2003).

In a second endosymbiosis event, between 1.5 and 1.2 billion years ago, a mitochondrion-possessing eukaryote engulfed or was invaded by a cyanobacterium, that was subsequently converted into a plastid (chloroplast) (Dyall et al, 2004). Also this endosymbiosis was followed by a large-scale information transfer out of the endosymbiont genome into the nuclear genome of the host cell. At the same time, gene transfer from the mitochondrion to the nucleus continued on a small scale and, in addition, some nuclear and plastid nucleic acid sequences invaded the mitochondrial genome (Unsel et al, 1997; Bock et al, 2008) (**Fig.1.1**).

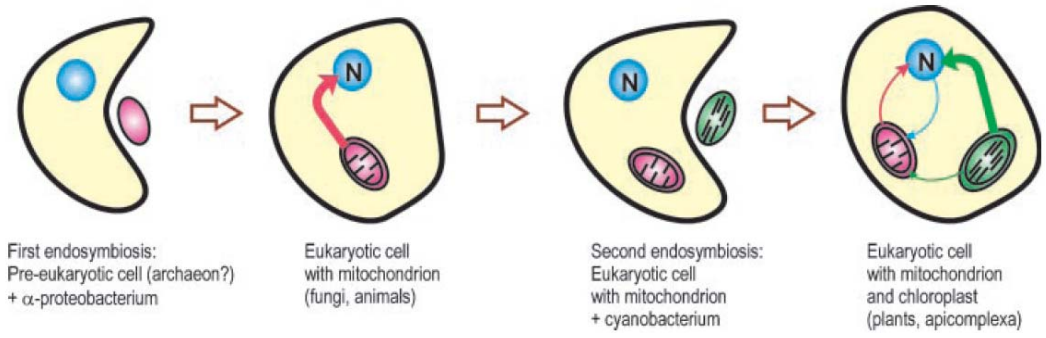


Figure 1.1. Intracellular gene transfer between genomes in the evolution of eukaryotic cells (from Boch and Timmis, 2008).

Genome comparisons between current plastid and contemporary cyanobacteria reveal a severe genome reduction process during the course of endosymbiosis (Martin and Herrmann, 1998), because, while around 80 proteins are encoded by the plastome, the contemporary genome of cyanobacteria encodes several thousand proteins (Kaneko et al, 1996).

Most of the protein-encoding genes in the chloroplast genome encode components of the four thylakoid photosynthetic complexes, or proteins necessary for their assembly and also encode parts of the organellar genetic machinery (Lopez-Juez and Pyke, 2005). Since the plastid genome encodes less than 80 proteins, it is obvious that a much greater number is required for the variety of plastid functions. Proteomics and genomes analyses of protein localisation sequences estimate that organelles might contain up to several thousand different proteins (Dyall et al, 2004; Richly and Leister, 2004). Therefore, most of proteins (93–99%) found in organelles have been individually identified as being encoded in the cell's nuclear genome, translated in the cytoplasm and imported into the plastids.

The existence of three different and compartmentalised genomes, encoding organelle proteins, requires a tight coordinated gene expression to ensure appropriate and energy-saving assembly of the photosynthetic machinery (Woodson et al, 2008; Kleine et al, 2009). These genome- coordinating mechanisms include both anterograde (nucleus to organelle) and retrograde (organelle to nucleus) signals. Anterograde mechanisms coordinate gene expression in organelles in response to endogenous and environmental stimuli that are perceived by the nucleus. Retrograde mechanisms originate in the organelles to communicate its functional and developmental state to the nucleus, which can then modulate anterograde control and cellular metabolism accordingly (Woodson et al, 2008).

Additionally, in plant cells chloroplasts and mitochondria have complex metabolic interdependencies (Raghavendra and Padmasree, 2003). Photosynthesis provides substrates for mitochondrial respiration, but depends itself on a range of compounds synthesized by mitochondria, including ATP in the dark. Moreover,

mitochondrial respiration protects photosynthesis against photoinhibition by dissipating redox equivalents exported from chloroplasts (Leister et al, 2005).

1.2. Retrograde signalling

1.2.1. Historical overview

About 30 years ago, studies on *albostrians* and *Saskatoon* mutants of barley revealed the first evidence that signals from chloroplast regulate the nuclear gene expression (Bradbeer et al, 1979). These mutants display white or white-stripes leaves, caused by a recessive nuclear allele (Hagemann and Scholz, 1962), which prevents the accumulation of carotenoids and leads to undifferentiated photosynthetically inactive plastids, with traces amounts of chlorophyll and lack of ribosomes (Hess et al, 1994a,b). These plastid defects are associated with a reduction in the expression and activity of nucleus-encoded plastid proteins, including the *LHC* gene family (Light-harvesting chlorophyll a/b binding proteins), the small subunit of RubisCO (RbcS), certain other enzymes of the Calvin cycle, and the ferredoxin:NADP+ oxidoreductase (FNR) that catalyses the final step in the electron transport chain in the light-dependent reactions of photosynthesis (Bradbeer et al, 1979).

Subsequent studies using mutant plants impaired in carotenoid biosynthesis, which causes photobleaching in plastids, have demonstrated that the expression of several nuclear-encoded photosynthetic genes is dramatically reduced in the absence of functional chloroplasts (Mayfield and Taylor, 1984; Oelmueller et al, 1986a,b).

Furthermore, studies on arrested chloroplast development, after employing specific inhibitors, showed inhibition in plastid gene expression (PGE), helping to further define plastid-to nucleus signalling (Oelmueller et al, 1986; Adamska, 1995; Yoshida et al, 1998; Sullivan et al, 1999). For example, treatment of mustard seedlings with norflurazon (NF), a herbicide that bleaches and disrupts chloroplasts by inhibiting carotenoid biosynthesis and releases ROS upon illumination, resulted in photooxidation of chloroplasts (Frosch et al, 1979; Reiss

et al, 1983). Norflurazon-treated mustard seedlings showed a decrease in transcripts of *LHCB* and *RbcS* genes (Oelmüller et al, 1986). Similar effects on *Lhcb* and *RbcS* transcripts were observed if chloramphenicol (an inhibitor of plastid translation) was applied to mustard seedlings during early development (Oelmüller et al, 1986).

Although no chloroplast retrograde pathway is well-understood mechanistically, several signals have been reported to trigger retrograde signalling from chloroplasts, including accumulation of Mg–protoporphyrin IX (Mg–proto, the first unique intermediate of chlorophyll biosynthesis), redox state of the organelle, and signals that are generated by inhibiting plastid gene expression or by accumulating various ROS (Woodson et al, 2008).

1.2.2. Tetrapyrroles

The first evidence for the involvement of chlorophyll biosynthesis precursors in retrograde signalling appeared during studies with an unicellular green alga, *Chlamydomonas reinhardtii*. In light-dark synchronized cultures of *C. reinhardtii*, *Lhcb* mRNA begins to accumulate about two hours after the transition to light, primarily due to transcriptional activation (Jasper et al, 1991). *Lhcb* mRNA accumulated normally when chlorophyll synthesis was blocked by inhibitors such as hemin and levulinic acid which interfere with early steps in the chlorophyll biosynthesis pathway prior to the formation of magnesium protoporphyrin methyl ester (Johanningmeier et al, 1984). By contrast, the accumulation of *Lhcb* mRNA was prevented by the chlorophyll-synthesis inhibitor α,α-dipyridyl (DP) which blocks late steps in the chlorophyll biosynthetic pathway and leads to the accumulation of the porphyrin intermediate magnesium protoporphyrin methyl ester (Mg-ProtoIXme) (Johanningmeier et al, 1984). These results strongly suggest that accumulation of porphyrin intermediates are necessary for *Lhcb* repression. Furthermore *C. reinhardtii* mutant *brs-1*, defective in the H-subunit of Mg-chelatase, is impaired in light induced *Lhcb* expression (Johanningmeier et al, 1984), suggesting that ProtoIX accumulation can also repress *Lhcb* transcription.

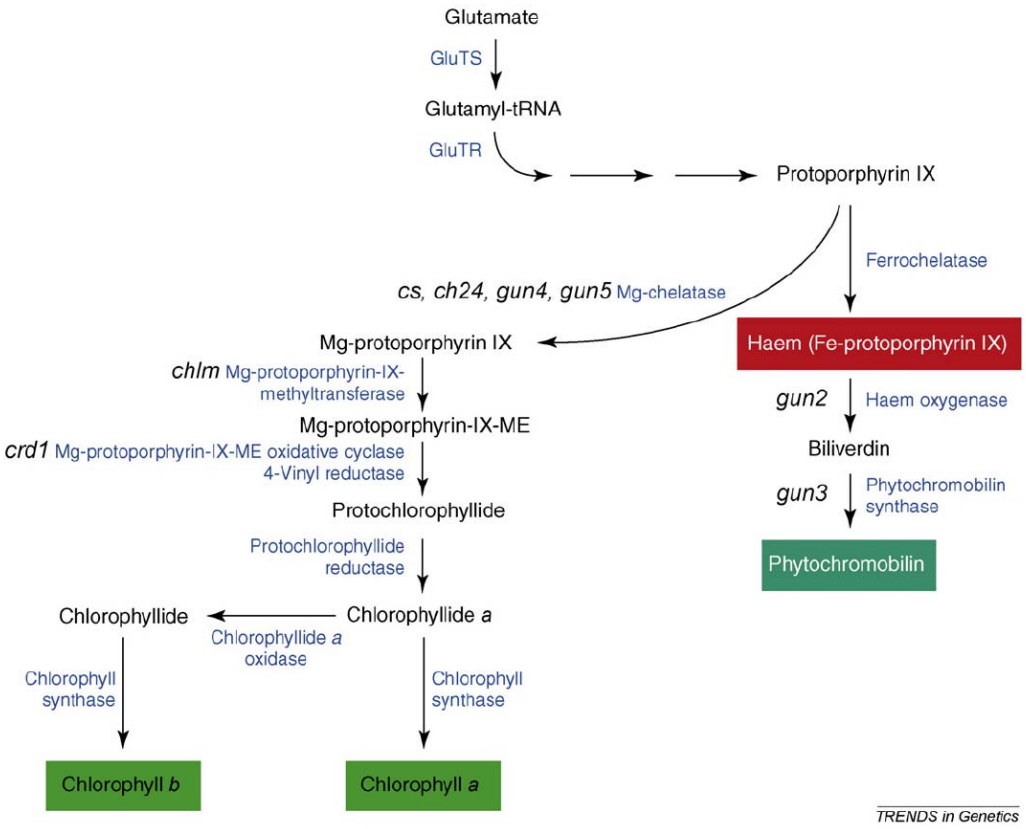


Figure 1.2. The tetrapyrrole biosynthesis pathway. From Kleine et al, 2009.

Although *C. reinhardtii* represents the first organism in which intermediates of tetrapyrrole synthesis in the chloroplast were suggested to influence nuclear gene expression, the first mutant screen specifically designed to identify components of plastid signalling was performed in the model flowering plant *Arabidopsis thaliana*. Using a genetic approach, Susek et al (1993) identified several nuclear-encoded genes required for plastid-to-nucleus signaling in *Arabidopsis*. An *Lhcb* promoter fused to both a selectable and screenable marker was integrated into the nuclear genome and seeds mutagenized with ethyl methanesulfonate (EMS). These lines were then used to isolate mutants in which *Lhcb* expression is uncoupled from the functional state of the chloroplast (Nott et al, 2006). Wild-type plants grown on NF, under continuous light, have low expression of reporters driven by the *Lhcb* promoter, owing to photobleaching of the chloroplast. In contrast, mutants have high levels of *Lhcb* expression on NF-containing medium, even though the chloroplasts are photobleached. From this

initial screen, five nonallelic loci impaired in retrograde signaling were identified and named *genomes uncoupled* or *gun* mutants (Susek et al, 1993; Mochizuki et al, 2001). With the exception of GUN1, which encodes a chloroplast-localised PPR protein (Koussevitzky et al, 2007), all GUN proteins were found to be involved in tetrapyrrole biosynthesis (Mokizuki et al, 2001; Larkin et al, 2003).
Although *gun* mutants were extensively characterised, their role in the plastid signalling remains obscure and, sometimes, contradictory. In fact, if ChlH, a subunit of the Mg-chelatase, was originally suggested to act as a sensor (Mochizuki et al, 2001), later, the tetrapyrrole pathway intermediate Mg-protoporphyrin IX (Mg-proto IX) was proposed to act directly as a signalling molecule (Strand et al, 2003) and to traverse the cytosol (Ankele et al, 2007). These studies were contradicted by precise and reproducible tests, that have re-evaluated this hypothesis by quantifying the steady-state levels of protoporphyrin IX (Proto IX), Mg-proto IX and the methylester of Mg-Proto IX (Mg-proto-me) in *Arabidopsis* plants with altered plastid signalling responses: all analysis employed did not show any correlation between the steady state levels of Mg-Proto (Mg-Proto-Me) and *Lhcb1* expression or with any of the other genes tested, leading to the conclusion that Mg-proto IX does not act as a direct signalling molecule (Mochizuki et al, 2008; Moulin et al, 2008).

1.2.3. Reactive oxygen species (ROS)

Exposure to both biotic and abiotic stresses can leads to the increased accumulation of ROS, which causes irreversible oxidative damage to cells. ROS that are generated in chloroplasts act as retrograde signals, to inform the nucleus to increase antioxidant enzyme production and to adjust the photosynthetic machinery for more efficient light harvesting (Vandenabeele et al, 2004; Lee et al, 2007). Among ROS, H_2O_2 and singlet oxygen ($^1O_2$) generated in chloroplasts by high light could act as chloroplast redox signals. In *Arabidopsis*, the *fluorescent in blue light* (*flu*) mutant, which overaccumulates the photo-excitable chlorophyll precursor, photochlorophyllide in the dark, is known to generate $^1O_2$ in the chloroplast, when shifted to light (Meskauskiene et al, 2001). The transcriptional response is distinct from that induced by H_2O_2 and/or O_2^-, suggesting that

separated retrograde signalling pathways respond to H_2O_2 and $^1O_2$ (op den Camp et al, 2003).

Recently, the *Arabidopsis* EXECUTER 1 (EX1) and EXECUTER 2 (EX2) proteins were identified as components in the $^1O_2$- dependent stress response pathway (Wagner et al, 2004; Lee et al, 2007), but the details of the signal transduction mechanisms initiated by H_2O_2 and $^1O_2$ still need to be determined.

1.2.4. Redox signals

As light intensity fluctuates, the chloroplast modulates the expression of photosynthesis-related genes to optimise photosynthesis and decrease ROS production (Woodson et al, 2008). The redox state of the photosynthetic electron transport chain (PET), which is the link between the transmitted light energy and metabolism, fluctuates owing to varying light intensity and quality, and has been shown by microarray analyses to exert retrograde control of nuclear gene expression of photosynthetic genes in *A. thaliana* (Fey et al, 2005). Relevant redox-active components are the pool of plastoquinone (PQ) and the PSI acceptor site (e.g. NADPH, thioredoxin, glutathione and glutaredoxin) (Kleine et al, 2009). Mechanistically, little is known about the retrograde redox signalling, but genetic analyses have recently identified STN7 as the first component of a possible long-term modulation of the nuclear gene expression by the redox state of PQ (Bellafiore et al, 2005; Bonardi et al, 2005; Pesaresi et al, 2009). STN7 is a dual-function thylakoid protein kinase, that is required for state transitions, a post-translational mechanism that underlies the short-term, PQ redox-state-dependent acclimation of photosynthesis to altered light conditions) (Bonardi et al, 2005). Furthermore, studies on *rimb* mutants (*redox imbalanced*), which have uncoupled transcriptional control of the nuclear-encoded chloroplast antioxidant enzyme 2-cys peroxiredoxin (2CPA) from the redox state of the PSI acceptor site, seem to confirm the salient role of the redox state of PSI acceptor site for the short-term regulation of nuclear gene expression (Heiber et al, 2007).

1.2.5. Organellar gene expression-dependent signalling

Treatment with inhibitors of organellar protein synthesis, like chloramphenicol or lincomycin, decreases the expression of nuclear photosynthesis genes in a light-independent manner during early stages of plant development (Pesaresi et al, 2007). This latter observation was first made using two constitutively photomorphogenic mutants, the pea *lip1* (*light independent photomorphogenensis*) and the *Arabidopsis cop1-4* (*constitutively photomorphogenic mutant*) (Frances et al, 1992; Deng et al, 1991; Nott et al, 2006). These mutants accumulate significant levels of the light-induced *Lhcb1.2* transcript if grown in the dark, but its level is reduced if seedlings are exposed to lincomycin (Sullivan and Gray, 1999). This implies that decreases in the general rate of protein synthesis or in the synthesis of particular proteins in the plastid generate a signal only during early plastid development. An alternative explanation is that the treated plastids do not reach the stage at which they could send the appropriate signal (Gray et al, 2003).

Recently, Pesaresi et al (2006) characterised mutant alleles of *PRORS1*, an *Arabidopsis* nuclear gene encoding a prolyl-tRNA-synthetase. This protein is located in both mitochondria and chloroplasts.The reduced gene expression, due to the T-DNA insertion in the promoter region, causes damage to the organellar translational machinery. Interestingly, leaky mutations in the gene, *prors1-1* and *prors1-2*, display altered photosynthetic performances, with reduced effective quantum yield (ΦII) and a significant reduction of the maximum quantum yield (F_v/F_m), implying a defect in energy transfer within PSII. These alterations are more pronounced in *prors1-2*, resulting in yellow leaves and strong reduction in growth rate (Fig.1.3.C). Similar to organellar gene expression inhibitors, the mutation in *PRORS1* induces a specific downregulation of nuclear photosynthetic genes: in particular transcriptional profiling of light-adapted mutant plants shows a predominant downregulation of genes coding for proteins involved in the light reactions of photosynthesis, including antenna and photosystem core proteins (Pesaresi et al, 2006).

Figure 1.3. Growth phenotypes of (A) Wild-type Col-0; (B) *prors1-1*; (C) *prors1-2*; (D) *mrpl11-1*; (E) *prpl11-1*; (F) *prpl11mrpl11*. From Pesaresi et al, 2006.

To investigate the specific role of protein synthesis in mitochondria and chloroplasts in the regulation of nuclear photosynthetic gene expression, Pesaresi et al (2006) employed furthermore two mutants, *mrpl11-1* and *prpl11-1*, specifically affected in the mitochondrial or chloroplast translational activity. *prpl11-1*, described by Pesaresi et al (2001), is impaired in the nuclear encoded gene *PRPL11*, that codes for the protein 11 of the 50S subunit of the chloroplast ribosome. Mutant plants have pale green leaves and are drastically reduced in size (Fig.1.3.E), as result of the marked reduction of (F_v/F_m), implying a defect in energy transfer within PSII (Pesaresi et al, 2006). Although, *prpl11-1* showed a drastic reduction in the level of plastome encoded thylacoids proteins (Pesaresi et al, 2001), analyses of the polypeptide composition of the mitochondrial electron transport chain did not show any difference to WT plants, indicating that the mitochondrial activity remained unaffected (Pesaresi et al, 2006). The *mrpl11-1* mutation is affected in the mitochondrial counterpart of *prpl11-1*. Due to the marked reduction of *MRPL11* gene expression, mutant plants are reduced in size and show a dark green coloration compared to WT, (Fig.1.3.D) (Pesaresi et al, 2006). *mrpl11-1* displays a reduction in mitochondrial protein abundance, but it is not affected in plastid translation rate or thylacoids protein composition, resulting in normal photosynthetic performance.

The *mrpl11prpl11* double mutant, generated by crossing *mrpl11-1* and *prpl11-1*, displayed pale-green leaves and drastic reduction in size (Fig.1.3.F), like *prpl11-1*, as well as the photosynthetic performance (Pesaresi et al, 2006). Interestingly, whereas the single mutants *mrpl11-1* and *prpl11-1* did not show specific downregulation of nuclear photosynthetic genes, Pesaresi et al (2006) observed a marked downregulation in the double mutant, similar to that seen in *prors1* mutants (Fig.1.4). These results led to the conclusion that translation rates in both mitochondrion and chloroplast contribute synergistically to the regulation of nuclear gene expression (Pesaresi et al, 2006; Kleine et al, 2009).

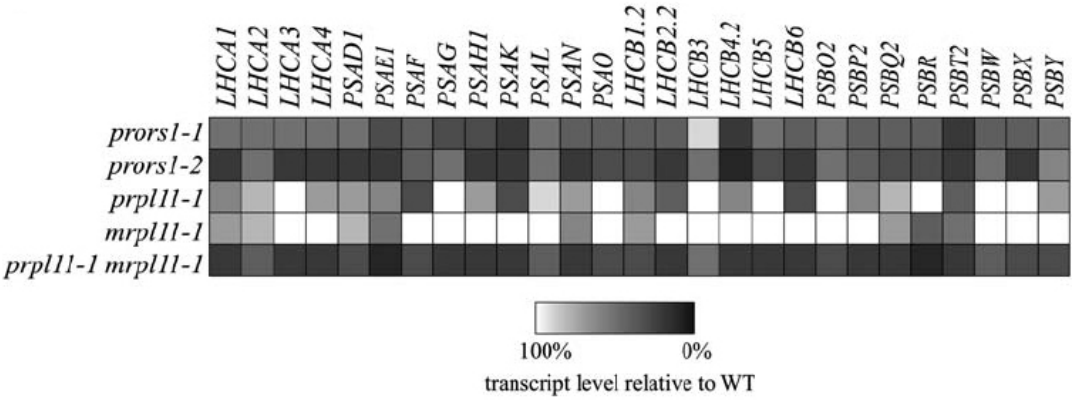


Figure 1.4. Transcript level quantification of nuclear photosynthetic genes in *mrpl11-1*, *prpl11-1*, *mrpl11prpl11* and *prors1-1* mutants (Pesaresi et al, 2006).

Although these experiments prove the existence of an organellar gene expression-dependent signalling, identification of the proteins directly involved in this signalling pathway is difficult: in fact, inhibition of plastid gene expression can be expected to have a plethora of secondary effects (Gray et al, 2003). However, a recent work of Koussevitzky (2007) indicates GUN1 as the first plausible transduction candidate. The *GUN1* gene, that was identified in the *gun* screen performed by Susek et al (1993) and Mochizuki et al (2001) (see chapter 1.2.1), codes for a plastid-localised pentatricopeptide repeat (PPR) protein. Among all the original *gun* mutants, *gun1* is unique because it is the only one where the photosynthesis-related *LHCB1* and *RBCS* genes are expressed in presence of lincomycin, whereas these genes are sensitive to this inhibitors in the *gun2*, *gun4* and *gun5* (Gray et al, 2003; Nott et al, 2006). This indicates that *GUN1* encodes a

component of the signalling pathway affected by inhibition of plastid protein synthesis (Kleine et al, 2009). Double mutant analyses have shown that GUN1 and GUN2–GUN5 define two distinct, but partially redundant signalling pathways that regulate overlapping groups of nuclear genes (Mochizuki et al, 2001; Strand et al, 2003).

1.2.6. Mitochondrion-chloroplast cross-talk

The interaction between chloroplasts and mitochondria is mutual. Photosynthesis provides substrates for mitochondrial respiration but also depends on a range of compounds produced by mitochondria, such CO_2 and ATP. Thus, the two organelles are metabolically interdependent (Hoefnagel et al., 1998; Raghavendra and Padmasree, 2003). In the dark, mitochondria are the main source of ATP for cellular processes, including those in the chloroplasts. Moreover, in the dark, mitochondrial ATP maintains the proton gradient across the thylakoid membrane, thus protecting the chloroplast from photoinhibition upon reillumination (Gilmore and Bjorkman, 1995). In the light, mitochondria provide the chloroplast with carbon skeletons (derived from the tricarboxylic acid cycle) for NH^+_4 assimilation (Kromer, 1995), while ATP supports various biosynthetic reactions, including the repair and recovery of photosystem II (PSII). Furthermore, mitochondrial respiration protects photosynthesis against photoinhibition by processing redox equivalents exported from chloroplasts (Padmasree et al., 2002). The chloroplast provides haem precursors to the mitochondria (Lindemann et al, 2004) and metabolic compounds that are involved in photorespiration (Raghavendra and Padmasree, 2003).

Studies in different model systems suggest that chloroplast–mitochondrion cross-talk involves a retrograde signal from one of the organelles that modulates the anterograde control of the other (Woodson et al, 2008). In particular, the activation of the cytochrome respiratory pathway in mitochondria leads to an increase in expression of photosynthesis-related genes in the nucleus (Matsuo et al, 2006). In the other direction, in mutant barley cells that lack chloroplast ribosomes, the nuclear-encoded mitochondrial RNA polymerase is upregulated, resulting in increased mitochondrial transcription (Emanuel et al, 2004). Furthermore, the redox state of the plastoquinone pool in chloroplasts affects

transcription of the mitochondrial RNA polymerase in the nucleus (Baier et al, 2005). The maize *non-chromosomal stripe* (*ncs*) mutants were used as models to study the requirement of mitochondrial function for chloroplast biogenesis and photosynthesis. Gu et al (1993) suggested that the NCS6 mitochondrial mutation, a cytochrome oxidase subunit 2 (*cox2*) deletion, is associated with a malfunction of PSI in defective chloroplasts of mutant leaf sectors. Jiao et al (2005) quantified the reductions of photosynthetic rate and the activity of PSI, observing that both plastid and nuclear encoding genes were reduced in *ncs6*.

Furthermore, *Nicotiana sylvestris* mitochondrial mutant, *cytoplasmic male sterile II* (*CMSII*) (Li et al, 1988), carries a stable mitochondrial DNA mutation that affects the respiratory electron transport chain: the mitochondrial *nad7* gene encoding the NAD7 subunit of complex I is deleted (Pla et al, 1995), and mitochondria are impaired in complex I structure and function (Gutierres et al, 1997). This mutant exhibits a decrease in the rate of photosynthesis, notably during dark–light transitions or if carbon fixation and photorespiration are simultaneously active (Sabar et al, 2000; Dutilleul et al, 2003a).

1.3. Chemically inducible gene expression systems: a powerful tool for dissecting signalling pathways

In addition to the more traditional forward genetic, reverse genetics has become a powerful tool to understand developmental and physiological processes in plants. Two major strategies employed in reverse genetics are overexpression and knockdown (underexpression) of genes of interest (Zuo et al, 2003). In the past, the use of constitutive promoters or enhancers and of loss-of-function or reduced-expression mutants has proven to be a powerful tool to relate a mutant phenotype to the function of a gene (Chen et al, 2003; Zuo et al, 2003). However, in many cases constitutive overexpression or underexpression of a target gene might cause detrimental effects or even results in lethality of the host plant. Furthermore, the use of knock out or knock down mutants is limited by the untargeted nature of the mutagenesis or of the T-DNA insertion (Chen et al, 2003). To overcome these

potential problems, several inducible gene expression systems have been developed (Moore et al, 2006).

A highly useful and versatile gene control system should have two important features: first, the chemical inducer (i.e. ligand) must be highly specific for the target promoter and must be non-toxic to plants; and second, the target promoter should have low basal (i.e. uninduced, background, leaky) and high induced levels of expression (Padidam, 2003). Systems that have been developed using components from non-plant sources meet these requirements. In general, such systems contain two transcription units. The product of the first transcription unit is a transcription factor that responds to a chemical. The transcription factor may be expressed using a constitutive promoter or a cell/tissue/developmental-stage-specific promoter, providing additional control. The second transcription unit contains a response element through which the activated transcription factor binds a gene of interest. Transgenic plants that contain the chemically inducible system should only express the gene of interest following chemical treatment (Padidam, 2003).

An important criterion in choosing an expression technology is its propensity to silence over generations. Silencing of the chemically responsive transcription factor or target promoter was a problem of the early activation systems (Weinmann et al, 1994; Boehner et al, 1999; Love et al, 2000). Although current inducible gene expression systems are shown to be stable over generations, any transgene may be susceptible to post-transcriptional gene silencing (PTGS), if its transcript accumulate to sufficient levels (Moore et al, 2006). Interestingly, Abranches et al (2005) reported that the probability of silencing of an inducible locus is increased if the locus is induced. This idea is consistent with observations of Zuo and Chua (2003) on the XVE oestrogen-inducible system, which delivers stable and highly inducible expression of many genes of interest, whereas other genes are silence rapidly.

As mentioned above, several inducible gene expression systems have been described and include systems controlled by steroids (Ayoama et al, 1997; Boehner et al, 1999; Zuo et al, 2000), ecdysone (Martinez et al, 1999a and b),

estrogen (Zuo et al, 2000), tetracycline (Gatz et al, 1992) and ethanol (Caddick et al, 1998).

1.3.1. The Alc switch

Amongst the chemical-dependent gene expression systems, the ethanol inducible gene expression (*Alc switch*) has been considered as one of the most promising systems for both laboratory and field use (Li et al, 2005). This system is derived from the filamentous fungus *Aspergillus nidulans* (Felenbock et al, 1988; Felenbock, 1991).

The ethanol-inducible gene expression system consists of two transcription units. The first one is an alcR expression cassette (*p35S:alcR*), which is constructed by cloning a constitutive CaMV 35S promoter with an *A. nidulans alcR* gene placed downstream. CaMV 35S promoter can be replaced by cell/tissue/developmental-stage-specific promoter, providing additional control. The other component is a target gene expression cassette (*palcA + mini-p35S:target gene*), which is composed of a minimal 35S promoter with the upstream activator region of the *alcA* promoter (*palcA*) and a target gene. When ethanol is absent, ALCR protein expressed from the first unit has no activity and can not bind to the *palcA* promoter located in the second unit to trigger gene expression. Upon ethanol adding, ALCR interacts with ethanol, leading to a conformational change and becoming active. The activated ALCR then binds to the specific ciselement in *palcA* promoter, and directs the transcription of the downstream target gene. If ethanol is removed, ALCR looses its activity and is released from the *alcA* promoter region, resulting in termination of target gene expression (Caddick et al, 1998).

Compared with the other gene expression systems, the *Alc switch* has the following advantages:

1. The construction of the system is relatively simple.
2. The alcA promoter exhibits high induction (Caddick et al, 1998; Salter et al, 1998; Roslan et al, 2001).
3. The system responds in an approximately dose-dependent manner, although high levels of ethanol are deleterious; both short and long-term expression

could be achieved through adjustment of concentration or duration of contact of ethanol with the plant (Caddick et al, 1998; Salter et al, 1998; Roslan et al, 2001; Sweetman et al, 2002; Deveaux et al, 2003).

4. ALCR is derived from non-plant organisms;
5. Ethanol is inexpensive, biodegradable and environmentally safe.
6. Ethanol can be applied to plants, using different methods (Caddick et al, 1998; Salter et al, 1998; Roslan et al, 2001; Sweetman et al, 2002; Deveaux et al, 2003).
7. The risk of accidental induction under anaerobic conditions appears small although extreme anoxia can induce the *Alc switch* (Caddick et al, 1998; Salter et al, 1998; Roslan et al, 2001; Sweetman et al, 2002; Deveaux et al, 2003).

1.3.2. The XVE system

The XVE system employs the regulatory moiety of the human estrogen receptor (ER or E) to confer hormone inducibility on a chimeric transcription factor, XVE (Zuo et al, 2003).

The peculiarity of the system is represented by the chimeric transcription activator, XVE, containing the DNA-binding domain of the bacterial repressor LexA (X), the acidic transactivating domain of VP16 (V) and the regulatory region of the human estrogen receptor (E) (Zuo et al, 2000). The expression of the chimeric XVE is controlled by the strong constitutive promoter G10-90 and its transactivating activity is regulated by estrogens, in particular by 17-β-estradiol (Zuo et al, 2000).

The XVE system possesses several key characteristics for effective chemical induction of transgene expression:

1. The inability of endogenous *Arabidopsis* steroids to activate XVE renders 17-β-estradiol specific to the target promoter, with undetectable basal transgene expression levels in the absence of inducer (Zuo and Chua, 2000).

2. There is no evidence that the inducer disturbs neither endogenous gene expression nor growth and development in *Arabidopsis* (Zuo and Chua, 2000).
3. The dose-dependent induction of XVE-regulated genes is equally suited to applications that require only low levels of induction as well as those demanding several thousand-fold increases in expression levels.
4. 17-β-estradiol is readily taken up by aerial tissues as well as roots, but is not volatile, preventing inadvertent gene activation.
5. Owing to the difficulty in grafting *Arabidopsis*, the use of the XVE system to produce genetic chimera provides a facile approach to discriminating between local responses and those involving long-distance signalling (Guo et al, 2003).
6. The activity of XVE-regulated promoters routinely returns to non-detectable levels within 5 to 7 d after removal of the inducer. Expression can be reactivated repeatedly without loss of sensitivity to the inducer (Zuo et al, 2002).

1.4. Aim of the thesis

This thesis focuses on the dissection of the organellar-translation-dependent retrograde signalling pathway, using a reverse genetic approach, based on the employ of loss-of-function and chemically inducible gene expressing mutants.

The first aim of the thesis was to generate and characterise transgenic mutant lines, carrying an inducible gene expression construct, to regulate the expression of the nuclear *MRPL11*, *PRPL11* and *PRORS1* genes, which are involved in the organellar translation process.

The second aim was to perform and analyse the transcriptional profiling of the induced transgenic lines, to distinguish between primary target genes, which are directly regulated, and secondary target genes, which are modulated by the product of primary target genes, and to identify candidate genes involved in the translation-dependent signalling pathway.

2. MATERIALS AND METHODS

2.1 Plant material and growth conditions

Two At5g52520 (PRORS1) and one At1g32990 (PRPL11) insertion mutant lines were identified among a collection of Arabidopsis thaliana (ecotype Columbia-0) lines that had been mutagenized with either AC106 or AC161 T-DNA, generated by Bernd Reiss (Max-Planck-Institute for Plant Breeding Research). The At4g35490 (MRPL11) mutant, corresponding to the Salk_090016 line, was identified in the SALK collection (http://signal.salk.edu/; Alonso *et al.*, 2003) which is made up of flank-tagged ROK2 T-DNA lines (ecotype Columbia-0).
Arabidopsis thaliana wild-type (ecotype Columbia 0) and mutant seeds were sown in Petri dishes on water soaked Whatman paper and incubated three days at 4°C in the dark to break dormancy and to synchronise germination. Plants were grown on soil under greenhouse controlled conditions (PFD: 70-90 $\mu Em^{-2}s^{-1}$, 16h light: 8h dark cycles) or under *Arabidopsis* biological incubator conditions (Percival, ETA associates) (PFD: 100 $\mu E\ m^{-2}\ s^{-1}$, 16h light: 8h dark cycles) . Fertilization with "Osmocote Plus" (Scotts Deutschland GmbH, Nordon Germany) was performed according to manufacturer's instructions.

2.2 Inducible overexpressor and RNAi lines generation

2.2.1 At5g52520 (PRORS1) site- direct mutagenesis

In order to remove the *Hind*III cutting site necessary for the *alc* switch cloning, without altering the aminoacidic sequence, the PRORS1 coding sequence was mutagenised. Two separated polymerase chain reactions (PCR) were performed with the Taq Polymerase (Qiagen) at the following cycling conditions: 3 min at 94°C initial denaturation, followed by 30 cycles of 20 sec denaturation at 94°C, 30 sec annealing at 58°C and 30 sec or 1 min 30 sec elongation at 72°C, according with the length of the products. After a final elongation step of 5 min at 72°C, the products were visualised on 1% agarose gel and then purified by the PCR purification kit (Invitrogen). The primers combinations for the two PCR reactions

were PRORS1 fw and PRORS1 T/G rev, PRORS1 T/G fw and PRORS1 rev, whose sequences are listed in Table 2.1.

The purified products were used as templates in a third PCR reaction, using the Platinum® *Pfx* DNA polymerase, a proofreading polymerase, using PRORS1 fw and PRORS1 rev primers combination, at the following cycling conditions: 94°C for 2 min initial denaturation, followed by 30 cycles of 15 sec denaturation at 94°C, 30 sec annealing at 55°C and 1 min and 45 sec elongation at 68°C, 3 min final elongation step at 68°C. After purification and visualisation on 1% agarose gel, the product was sequenced by the sequencing service at the LMU München (http://www.genetik.biologie.uni-muenchen.de/sequencing), to confirm the mutagenesis.

Primer Name	**Sequence (5'-3')**
PRORS1 T/G fw	GAAGCGTCACACGTTGAGGGATTTAGTCCTGAAC
PRORS1 T/G rev	GTTCAGGACTAAATCCCTCAACGTGTGACGCTTC
MRPL11 fw	CGGTCGACATGGCGGCTGCTGCGAAGGA
MRPL11 rev	CGGTCGACATCACTCCAAATCCTGAACG
PRPL11 fw	CGGTCGACATGGCGTCTTCTTCTCTATC
PRPL11 rev	CGGTCGACACTACCAACCAGGCTTTTAC
PRORS1 fw	CGGTCGACATGGTGTCGTCGTCTCTGAGAC
PRORS1 rev	CGGTCGACTTAATATGACTTGGCAAAGATTGC
p13719 fw	ATTTGGAGAGGACGACCTGC
pJH0022 fw	ATCTATGTTACTAGATCGGG
pJH0022 rev	TCTTCGCTATTACGCCAGCT

Table 2.1. List of primers used for Alc switch cloning

2.2.2 Alc switch cloning

To generate MRPL11, PRPL11 and PRORS1 inducible overexpressing lines (*Alc* lines) the coding sequence of the three genes was cloned into the plant expression vector pJH0022 (Syngenta), in a two- step cloning strategy. A bacterial vector, p13719 (Syngenta), containing the ethanol inducible promoter AlcA, and the plant expression vector pJH0022 (Syngenta), containing the AlcR transcriptional regulator, were respectively digested with *Sal*I and *Hind* III restriction enzymes

(NEB). After purification, the linearized plasmids were dephosphorilated by Antarctic Phosphatase (NEB), which catalyzes the removal of 5´ phosphate groups from the DNA, in order to prevent the self-ligation of the vectors.

The coding sequences of genes of interest were amplified using specific primers containing a cutting site for *Sal*I. The obtained amplification products were cut with *Sal*I and then ligated into p13719. After *Escherichia coli* transformation, the colonies selected on Ampicillin plates were tested by PCR to carry the transformed plasmid, using the p13719 forward primer in combination with the gene specific reverse primer, to select only the colonies, carrying the gene in the right orientation. The positive colonies were inoculated overnight at 37°C in LB liquid containing ampicillin and the plasmid was purified by Plasmid Mini kit (Qiagen). To verify that the cloned sequence was correct, the purified plasmid was sequenced. The positive clones were digested with *Hind*III restriction enzyme, to remove the cassette containing the AlcA promoter, introduced gene and the NOS terminator.

The cassette was subsequently ligated with the plant expression vector pJH0022, previously cut and dephosphorilated. After *Escherichia coli* transformation and selection on kanamycin plates, the selected colonies were screened by PCR, using the pJH0022 forward primer in combination with the gene specific forward primer, in order to select only the colonies carrying the gene orientated in the opposite direction to the Cauliflower Mosaic Virus (CaMV) 35S/AlcR/terminator cassette, to prevent interaction of the 35S promoter and the minimal 35S region, as suggested by the guidance notes provided with the vector (Fig. 2.1).

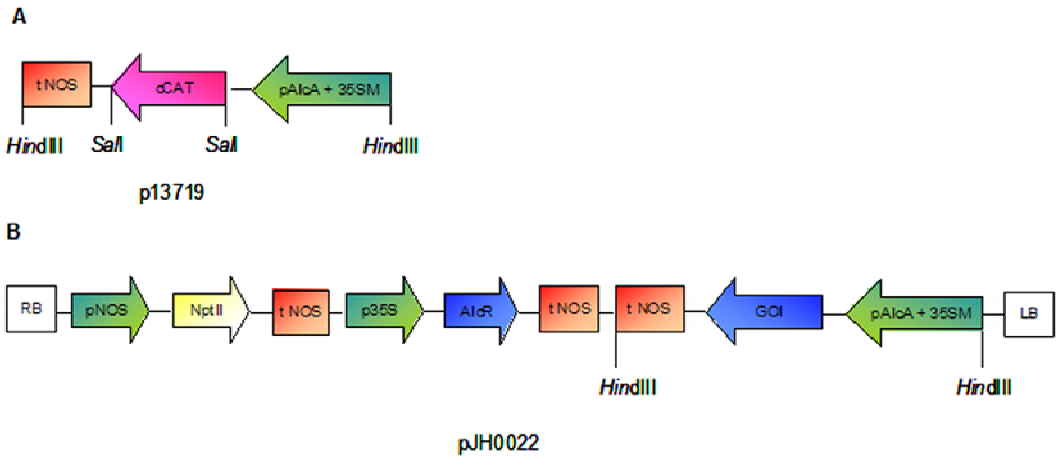


Fig 2.1. Schematic representation of the constructs used to obtain ethanol inducible gene expression. cCAT: chloramphenicol acetyltransferase; 35S M: 35S minimal promoter; tNOS: nopaline synthase terminator; RB: right border; NptII: kanamycin resistance; GOI: gene of interest; LB: left border.

2.2.3 Estradiol inducible RNAi cassette cloning

To generate gene silencing inducible lines (XVE lines) for MRPL11, PRPL11 and PRORS1, Genomic Sequence Tags (GST) of around 200 base pairs (pb) were created, using informations provided in the CATMA webside (http://www.catma.org, Crowe et al. 2003). The obtained GSTs were cloned into the plant expression vector pER8RNAigw (kindly provided by Dr. Vera Bonardi, University of North Carolina at Chapel Hill). The cloning was performed following the strategy based on the GATEWAY Technology (Invitrogen), using the GST specific primers combinations listed in Table 2.2. The GSTs were then cloned into an entry vector (pDONOR201) and subsequently subcloned into the destination vector (pER8RNAigw), in which the GSTs are under the control of an estradiol inducible promoter. To verify the positivity of the cloning, the colonies obtained after the LR reaction were screened by PCR, using vector specific primers (see Table 2.2), designed on the 35S minimal promoter or the 35S terminal region of pER8RNAigw, in combination with a GST specific primer.

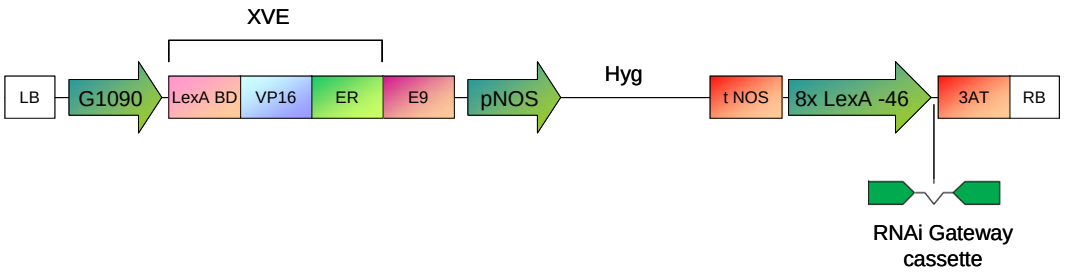


Fig. 2.2. Schematic representation of the constructs used to obtain ethanol inducible gene expression.

2.2.4 Constitutive RNAi cassette cloning

The MRPL11, PRPL11 and PRORS1 GSTs were cloned into the plant expression vector pB7GWIWG2 (Karimi et al. 2002), containing a RNAi cassette, under the control of the CamMV 35S promoter, to generate constitutive gene silencing lines (RNAi lines). The cloning was performed following the GATEWAY Technology (Invitrogen), using the GSTs specific primers listed in Table 2.2.

Primer Name	**Sequence (5'-3')**
MRPL11 GST fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTGACGGTGTC GTGGTACAT
MRPL11 GST rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTACTACTCA AAGCAAAGA
PRPL11 GST fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTACCCGACCT GAACTGCACGA
PRPL11 GST rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATATCGAAC TCTCTTAAG
PRORS1 GST fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTACATTGTGG ATGTAAACTCATACG
PRORS1 GST rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGGAATGTGT GAAAGTGTA
pER8 35 fw	AGGACACGCTGAAGCTAGTC
pER8 3AT rev	CGATGATACGGACGAAAGCT
35S M fw	ACAGTCTCAGAAGACCAGAGG
35S Term fw	ATATGCTCAACACATGAGCGA

Table 2.2. List of primers used for estradiol inducible and the constitutive RNAi cloning

To verify the cloning, the colonies obtained after the LR were screened by PCR using vector specific primers, designed on the CaMV 35S promoter or 35S Terminator region, in combination with the GSTs specific primer.

2.2.5 Agrobacterium-mediated transformation of A. thaliana

All the constructs generated were used to transform Col-0 *Arabidopsis* plants as described by Clough and Bent (1998). Flowering plants were dipped for 15 s in an *Agrobacterium* suspension (strain GV3101, carrying the respective binary vector) containing 5% sucrose and the surfactant Silwet L-77 (0.0005%). After dipping, plants were transferred to the greenhouse and seeds were collected after approximately 3 weeks. *In vitro* selection for resistance to kanamycin (*alc* lines) either to hygromycin (XVE lines), either to BASTA herbicide (RNAi lines) was carried and the transgenic plants were grown on soil under greenhouse controlled conditions (PDF: 70-90 $\mu Em^{-2}s^{-1}$, 16h light: 8h dark cycles). The integration of the transgene in the genome of the resistant plants was confirmed by PCR, using the vector specific primer in combination with the gene specific one (see Table 2.1 and Table 2.2).

The T2 generation of the XVE and the RNAi plants was used for the experiments reported and their inducible and the constitutive silenced expression was confirmed by real-time PCR.

The overexpression of the *alc* plants was confirmed in T2 generation by real-time PCR. Plants showing the highest expression level were chosen to be crossed with the respective mutant plants and the F2 generation of the mutant background transgenic lines was used for the experiments reported.

2.3 Screening for T-DNA insertion

Arabidopsis genomic DNA was isolated grinding fresh material in isolation buffer (200 mM Tris (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS) followed by isopropanol precipitation. Insertion junction sites were identified by sequencing the amplicons of PCRs using a combination of gene and T-DNA insertion specific primers. For the *mrpl11* T-DNA insertion derived from pROK2 was used LBb1 and MRPL11 fw primers combination. *prors1.1* and *prors1.2* T-DNA insertions

derived from AC106 were screened with T-DNA PRORSI rev and PRORSI Scree fw and T-DNA PRORSII fw and PRORSI Scree rev, respectively. For *prpl11* T-DNA insertion derived from AC161 was screened using T4496 fw and PRPL11 int rev. Corresponding gene specific primers combinations in WT were MRPL11 fw and MRPL11 Scree rev, PRORSI Scree fw and PRORSI Scree rev, PRPL11 fw and PRPL11 int rev. The primers sequences are listed in Table 2.3.

PCR runs were performed with Taq polymerase (Qiagen) and the following cycling conditions: 3 min at 94°C initial denaturation, followed by 35 cycles of 20 sec denaturation at 94°C, 30 sec annealing at 55°C and 1 min 30 sec elongation at 72°C. After a final elongation step of 5 min at 72°C, the PCR products were visualised on a 1% agarose gel, containing ethidium bromide.

Primer Name	**Sequence (5'-3')**
LBb1 rev	GCGTGGACCGCTTGCTGCAACTC
T4496 fw	CAGGGTACCCGGGGATCAGATTGTC
T-DNA PRORSII fw	ATATTGACCATCATACTCATTGC
T-DNA PRORSI rev	CTCTTTCTTTTTCTCCATATTGACCAT
MRPL11 fw	CGGTCGACATGGCGGCTGCTGCGAAGGA
MRPL11 Scree rev	CTTTTGGAGCTCAATTATCC
PRPL11 fw	CGGTCGACATGGCGTCTTCTTCTCTATC
PRPL11 int rev	GCAAACACACATACAAACTCC
PRORSI Scree fw	CCAAGCATGAGT TTCTCGAG
PRORSI Scree rev	TCCGGAAAGAGGTCTGTTCC

Table 2.3. List of primers used for T-DNA screening

2.4 mRNA expression profiling

2.4.1 Chondriome and plastome array hybridization and quantification

Chondriome and plastome- encoded genes macroarrays were produced by spotting 165 and 78 gene-specific PCR products (100–300 bp in length, amplified from the 3' end of the cDNA) in duplicate on Hybond N membranes (Amersham Biosciences). Plants were grown in greenhouse conditions as described in 2.1.

Digitoxin- labeled cDNA of wild type, *mrpl11*, *prpl11*, *prors1.1*, *prors1.2* and *mrpl11prpl11* leaves were synthesized from 5 μg of RNA. Each cDNA from 4 independent experiments was hybridized with an array filter of the chondriome macroarrays. Array hybridization and data evaluation were performed as described by Kandlbinder et al (2004).The spot intensities were quantified with the AIDA array vision software (Raytest) and normalized according to whole intensity of all spots on the array. All the data were pooled, and the normalized spot intensities were tested for each genotype against wild type by the use of Student's *t* test analysis ($p < 0.05$). The normalised spot intensities from the different treatments were statistically tested against the specific control by the use of student's *t*-test analysis ($p<0.05$). To quantify differential expression of the different treatments the induction factor (IF) was calculated from the ratio of the averaged and normalised signal intensities of the respective treatment. Chondriome and plastome profiling was performed by Dr. Andrea Kandlbider, Bielefeld, Germany.

2.4.2 Affymetrix ATH1 array hybridization and quantification

10 micrograms of total RNA of two to three biological replicates of the different pools (5 plants each pool) of rosette leaves of 4-week-old Col-0, and *prpl11-1* plants was processed and hybridized to a GeneChip Arabidopsis ATH1 Genome Array using the One-Cycle Target Labeling and Control Reagents according to the manufacturer's instructions (Affymetrix). Reverse transcription was employed to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an in vitro transcription reaction to generate biotinylated cRNA. The fragmented, biotinylated cRNA was used for hybridization. Hybridization, washing, staining, and scanning procedures were performed as described in the Affymetrix technicalmanual. A Hybridization Oven 640, a Fluidics Station, and a GeneChip Scanner 3000 were used. Transcriptome Data Analysis CEL files were imported into FlexArray (http://genomequebec.mcgill.ca/FlexArray/) for further analysis. Raw intensity data were normalized using the robust multiarray average algorithm (Irizarry et al., 2003). The data were log transformed, and a list of differentially expressed genes was generated. The

significance of differential gene expression was estimated by comparing the observed and expected d values. A threshold of 2 for D (the difference between the observed and expected d values) and a threshold of 0.05 for unadjusted P values (rawp < 0.05), together with the twofold change filter, were applied to identify differentially expressed genes.

2.4.3 RT- PCR analysis

To determine the gene expression level of the transgenic lines and the chloroplast and mitochondrion expression profile, total leaf RNA was extracted with TRIzol reagent (Invitrogen, Karlsruhe, Germany) from frozen tissue of WT, *mrpl11*, *prpl11*, *prors1.1*, *prors1.2*, *mrpl11prpl11* mutants and the transgenic *alc*, XVE and RNAi plants. First-strand cDNA synthesis from 1 μg of total RNA was performed either using the iScript cDNA Synthesis Kit (Bio-Rad), to verify overexpression and silencing in the transgenic lines, or using the SuperScript™ III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) for the T-DNA insertion mutants. Both preparations were performed according to manufacturer's instructions.

To quantify and to check the quality of the synthesized cDNA, the reverse-transcriptase (RT)-PCR was performed using specific primer combination for the housekeeping gene Ubiquitin, shown in the Table 2.4. 2 μl of 1:10 cDNA dilution was used in 20 μl of reaction for both RT- PCR and for Real-Time PCR . Thermal cycling consisted of an initial step at 95°C for 3 min, followed by 30 cycles of 10 s at 95°C, 30 s at 55°C and 10 s at 72°C.

For Real-Time PCR analysis, the cDNA and specific primers were added to a solution containing iQ™ SYBR® Green Supermix (Bio-Rad), and the thermal cycling consisted of an initial step at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C, 30 s at 55°C and 10 s at 72°C, after which a melting curve was performed. The Real-Time primers are listened in Table 2.4. Gene expression and standard deviation were calculated by the iQ5™ Optical System Software (Bio-Rad), using the following formula (Pfaffl, 2001):

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct,\ \text{target (calibrator- test)}}}{(E_{\text{ref}})^{\Delta Ct,\ \text{ref (calibrator- test)}}}$$

where E_{target} and E_{ref} are the amplification efficiencies of the target and *UBI*, respectively.

All experiments were performed with a iQ5™ Multi Color Real-Time PCR Detection System (Bio-Rad), using reactions in triplicate with at least two biological replicates.

Primer Name	**Sequence (5'-3')**
MRPL11 RT fw	ATGGCGGCTGCTGCGAAGGA
MRPL11 RT rev	TCCTAGCGTTGAAGTCTTTG
PRPL11 RT fw	CACTGTCTTCGATGATAAGAG
PRPL11 RT rev	GTAGCTGGTCTATTGTTATCAC
PRORS1 RT fw	GTATCTAGTAACAGTGTCGT
PRORS1 RT rev	ATCCACAATGTTACTGTCTC
UBI RT fw	GGAAAAAGGTCTGACCGACA
UBI RT rev	CTGTTCACGGAACCCAATTC

Table 2.4. List of primers used for RT- PCR and Real-Time PCR

2.5 Chlorophyll fluorescence and spectroscopic measurements

Six plants of each genotype were analysed and mean values and standard deviations were calculated.

Chlorophyll fluorescence was measured *in vivo* on single leaves, using the Dual-PAM 100 (Walz, Germany) as described before by Pesaresi *et al.* (2009). The fluorescence of dark adapted leaves was measured (F_0), followed by the application of saturating pulses (800 ms) of red light (5000 μE m^{-2} s^{-1}) to determine the maximum fluorescence in the dark (F_M) and the ratio (Fm-Fo)/Fm= Fv/Fm.

A 15-min illumination with actinic light (80 μE m^{-2} s^{-1}) served to drive electron transport between PSII and PSI before measuring the effective quantum yield of PSII (Φ_{II}), according to the formulas (Maxwell and Johnson, 2000):

$$\Phi_{II} = (F_M'-F_S)/F_M'$$
$$qP = (F_M'-F_S)/(F_M'-F_0).$$

In addition, the NPQ (nonphotochemical fluorescence quenching) was measured at the same experimental conditions described above and it was calculated according to the following equation (Kramer *et al*, 2004):

$$NPQ = 1- Y(II) – 1/(NPQ + 1+ qL(F_M/F_0-1)$$

2.6 Chemical regulated gene expression induction

2.6.1 alc *switch induction*

Induction of the ethanol switch was achieved by ethanol vapour (Salter *et al*,1998; Roslan *et al*, 2001; Deveaux et al, 2003; Maizel and Weigel, 2004; Knowles *et al*, 2008).

a. in vitro

Surface- sterilized seeds were sown onto MS medium (Duchefa), containing 1.5% agarose and 0.2% sucrose. After three days in the dark at 4°C, petri dishes were transferred to a growth chamber at 22 °C/18 °C with a 16 h/8 h light/dark photoperiod. Using cultures plates as convenient vapour chambers, the induction of the 10-days-old seedlings was carried out by wetting paper filter (83 mm diameter; 3MM Whatmann paper) with diluted ethanol and adhering it to the underside of the plate lid. The concentration of ethanol used for standard inductions was 0.1% (v/v).

b. on soil

Induction was performed in a controlled environmental chamber (phytotrone) at 22 °C/18 °C with a 16 h/8 h light/dark photoperiod. 50 ml falcon tubes

(Eppendorf) containing 100% ethanol were placed into 96x trays, containing 4-day-old soil-grown seedlings. The tubes were opened for 8 h per day. In the remaining 16 h they were closed, to minimize the fungal growth. Control samples (not treated plants) were grown at the same conditions, in parallel.

To determine the growth rates of WT, mutant plants and transgenic plants, the leaf areas of 10 plants of each genotype were measured after four weeks of ethanol treatment, using the free software ImageJ (Abramoff et al., 2004).

2.6.2 Estradiol induction

a. in vitro

Hormone treatment was carried out with 10-days-old plants grown in sterile culture (MS containing 1.5% agarose and 0.2%) under long day photoperiod (16 h/8 h light/dark). The plants were sprayed with 1 μM or 5 μM 17-β-estradiol (Roth), prepared in 0.1% dimethyl sulfoxide (DMSO, Roth) and 0.1% Triton-X (Roth), and harvested at a defined time point following the treatment. Due to the 17-β-estradiol light sensitivity and its decrease in the activity, the treatment was repeated every 36 hours.

b. on soil

A solution of 1 μM 17-β-estradiol, 0.1% DMSO and 0.1% Triton-X was sprayed on 4-weeks-old plants, grown in controlled environmental chamber (phytotrone) at 22 °C/18 °C with a 16 h/8 h light/dark photoperiod. Control samples (0 h) were collected before the treatment.

3. RESULTS

To investigate the organellar-translation-dependent retrograde signaling a genetic approach based on chemically inducible systems was chosen. Several works showed how powerful these systems are in dissecting biological mechanisms or unravelling regulatory pathways, especially when the mis- regulation of one component of the system (i.e. a gene) determines lethality (Guo et al, 2003; Padidam, 2003; Battaglia et al, 2006; Rauhut et al, 2009).

3.1 Generation of overexpressing *Alc* transgenic lines

Transgenic lines for *MRPL11*, *PRPL11* and *PRORS1* were generated, cloning the respective coding sequence in the two component alcR/alcA system (Caddick et al., 1998; Salter et al., 1998). The rationale behind this chemically inducible system consists in the activation of *alcR* encoded transcription factor (ALCR) upon exposure to exogenous ethanol. Binding to *alcA* promoter (cloned upstream the heterologous gene), ALCR drives the expression of the target gene in an ethanol-responsive manner (Junker et al, 2003).

As suggested by Deveaux et al (2003), the pAlcA/35SM::*gene*::tNos cassette should be cloned in the opposite direction of the p35S::*AlcR*::tNOS, to prevent interaction between the 35S promoter and the minimal 35S region. While for *PRPL11* (Fig. 3.1.A) the construct was obtained in the suggested direction, for *PRORS1* and *MRPL11* it was possible just to generate the construct in the direct orientation (Fig. 3.1.B).

For each construct several transgenic lines were obtained. To verify the functionality of the system and the efficient overexpression of *MRPL11*, *PRPL11* and *PRORS1* transcripts, 4-week-old transgenic WT plants, carrying the inducible overexpressing cassette, were exposed to 100% ethanol vapours for 16 and 96 hours. Samples of leaves were collected and, after RNA extraction, cDNA was prepared.

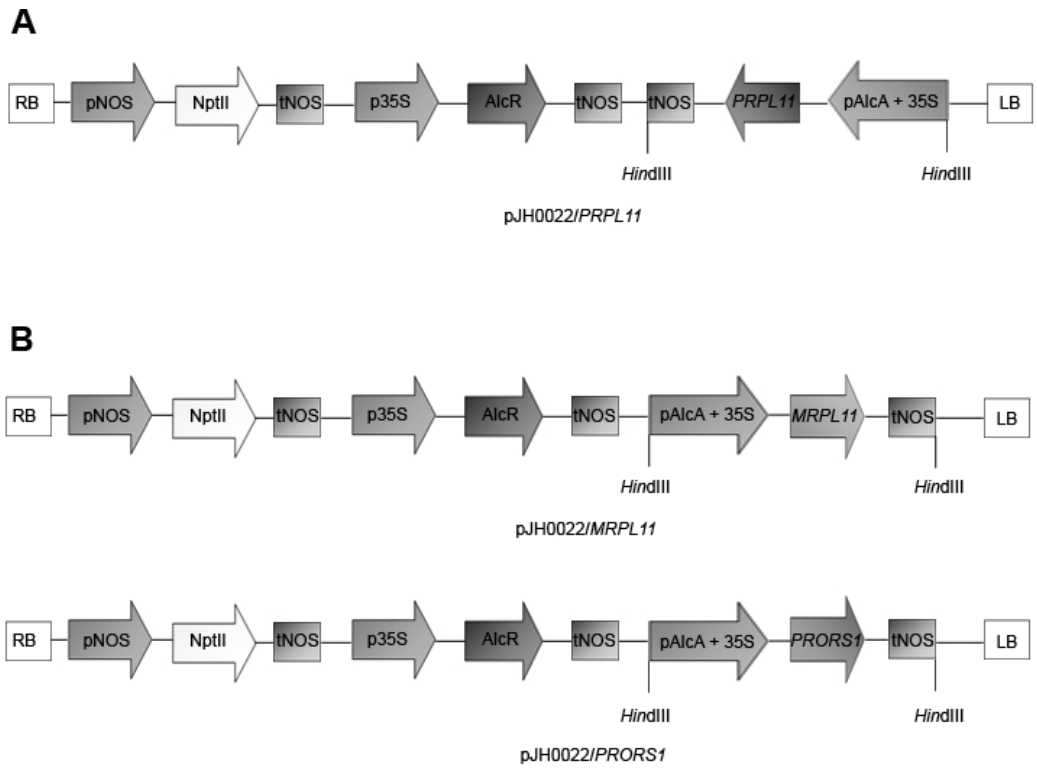


Figure. 3.1. Schematic representation of the constructs obtained to generate transgenic lines.

Real Time PCR (qPCR) was then performed on different transgenic lines. Among the tested p*Alc::mrpl11*-Col0 lines (Col0 plants transformed with the construct shown in Fig.3.1), line 1E and line 12E displayed a strong increase of transcript after 16 hours, which in line 12E decreased after 96 hours, and line 1D showed a moderate induction after 16 hours, which further increased by three times after 96 hours of ethanol exposure (Fig.3.2.A).

Among the collection of p*Alc::prpl11*-Col0 lines tested, line 2 showed a slight change in the expression, lines 6, 8 and 10 displayed an increase of two to four times in the transcript level of *PRPL11*. The most promising line, line 1, showed a 17 folds increase in gene expression, compared to WT, after 16 hours of treatment (Fig.3.2.B).

For *PRORS1* two independent transgenic lines were generated. Line 3 showed just a slight increase in the transcript level, while Line 5 showed enhanced expression (15 fold) in comparison to WT plants, after 16 hours of induction (Fig. 3.2.C).

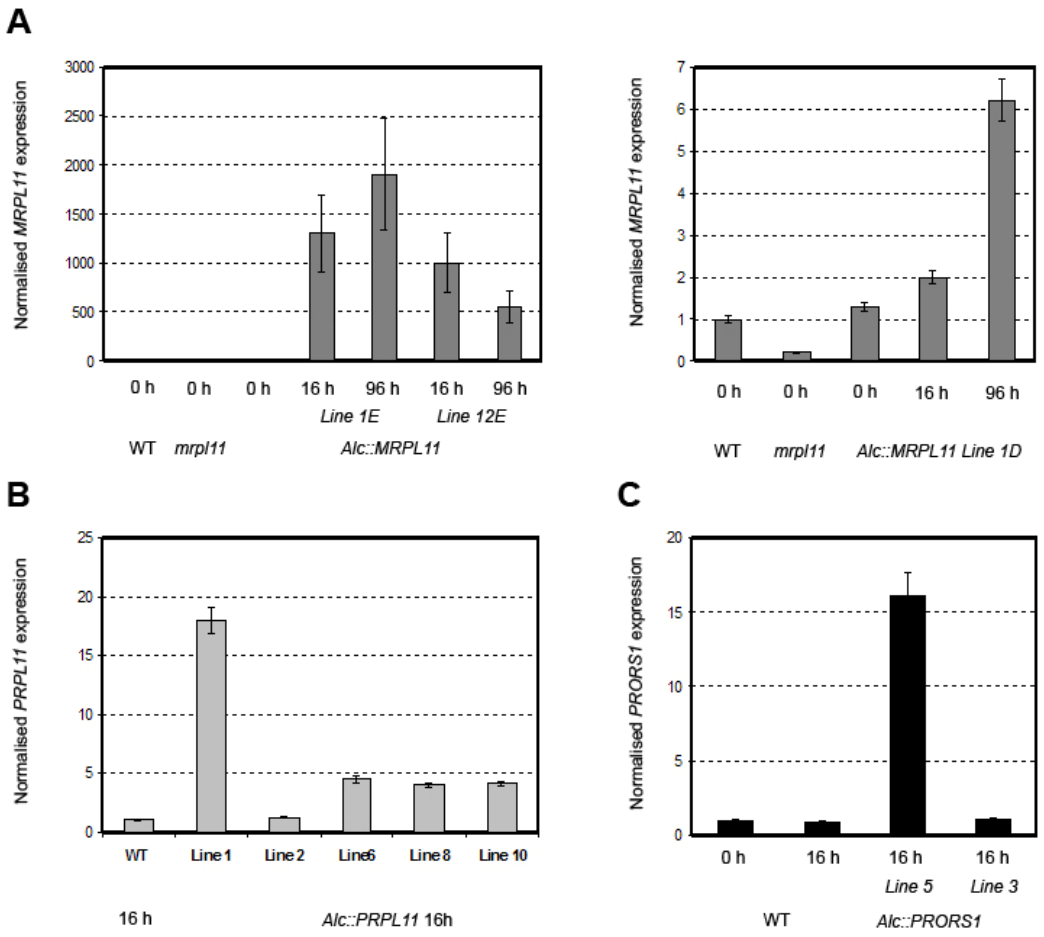


Figure 3.2. Transcript level of **A.** *MRPL11* **B.** *PRPL11* **C.** *PRORS1* in mutant, WT and transgenic lines. The expression levels are shown as arbitrary units, normalized to the expression levels of *UBIQUITIN* which was used as a reference.

To be an effective system for switching transgenes, it is important that the *Alc* system does not result in any secondary effects on i.e. photosynthetic performances and gene expression. WT plants were exposed to ethanol vapours for 24 hours and photosynthetic parameters were measured in order to verify that the inducer has no impact (Table 3.1).

	WT not treated				WT + EtOH			
	0 h	3 h	6 h	24 h	0 h	3 h	6 h	24 h
Fv/Fm	0.815 ±0.002	0.811 ± 0.003	0.812± 0.005	0.811 ± 0.006	0.815 ± 0.002	0.812 ± 0.004	0.81 ± 0.005	0.811 ± 0.003
NPQ	0.133 ± 0.030	0.156 ± 0.010	0.136 ± 0.020	0.145 ± 0.005	0.133 ± 0.030	0.119 ± 0.004	0.128 ± 0.010	0.151 ± 0.020
Yield	0.760 ± 0.001	0.750 ± 0.008	0.750 ± 0.007	0.746 ± 0.001	0.760 ± 0.001	0.755 ± 0.004	0.757 ± 0.007	0.750 ± 0.001

Table 3.1. Spectroscopic data for WT leaves during ethanol vapour exposure. Photosynthetic parameters were measured on WT plants exposed and not exposed to ethanol, in order to check possible secondary effects of the inducer. Mean values ± SD (5 plants each genotype) are shown.

The treatment did not seem to affect performances of photosystem II, whose parameters remained unchanged, compared with the untreated control. The only noticeable difference was a slight decrease in non-photochemical quenching after three and six hours of treatment. Also *LHCB1.2* expression did not seem to be affected when WT plants were exposed to ethanol, as showed in Fig.3.3, confirming that *Alc* system could have been used to dissect the translation-dependent retrograde signalling.

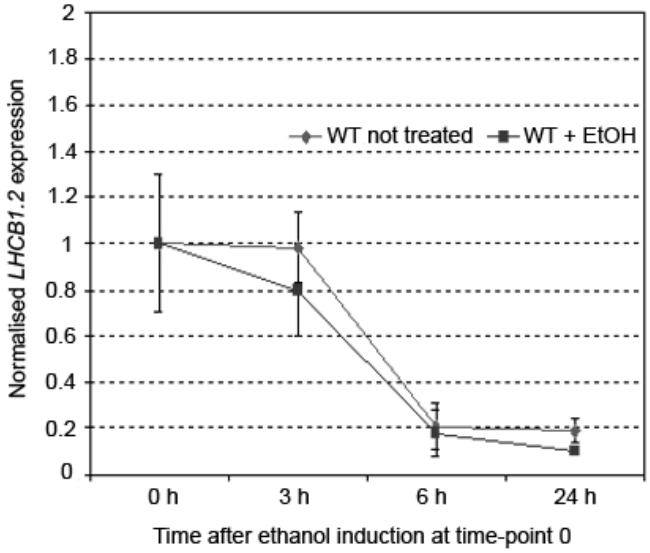


Figure 3.3. *LHCB1.2* expression in WT, exposed for 24 hours to ethanol vapour or in control conditions. For vapour induction open reaction tubes containing 500 µl of 100% ethanol were placed into alternate pots containing 4-week-old soil-grown seedlings. The pots were covered with a lid for the entire experiment. The control plants were treated in the same conditions, but in absence of inducer. Error bars are shown. The experiment was performed using reactions in triplicate with at least two independent biological replicates.

Two highly expressing lines for *MRPL11* and *PRPL11* and one for *PRORS1* were crossed with the respective T-DNA insertion mutant, to obtain inducible overexpressing lines in mutant background. Since here the ethanol inducible overexpressing lines will be mentioned as *Alc* lines.

3.2 Characterisation of *Alc* lines *in vitro*

To characterise the response time of *pAlc::MRPL11 mrpl11* line to ethanol induction, time-course experiments were performed on both seedlings and mature plants. To establish the induction in a highly controlled system, the experiments were carried out *in vitro*, with the culture plate as a vapour chamber.

A paper filter was wetted with 0.1% ethanol and adhered to the underside of the plate lid. 10-day old WT, *mrpl11-1* and *Alc::MRPL11* plants were exposed to ethanol vapours and the gene expression level was monitored during the first 6 hours of treatment (short term time course) and after 3 weeks of continuous induction (long term time course) (Fig.3.4).

After two hours of induction, the expression level of line 21 was still similar to the *mrpl11-1* mutant, but already after six hours, the transcript level increased more than three fold, reflecting the accessibility of the inducer to the tissue. After 3 weeks, the expression of *MRPL11* increased considerably, indicating that the system could be applied also for long term induction.

Moreover, the concentration of ethanol applied in the experiment is sufficient to complement the phenotype of the mutant. In absence of the inducer *Alc::MRPL11* plants displayed the characteristic *mrpl11-1* phenotype (Fig. 3.5.A), whereas the full phenotype complementation was observed after the treatment with ethanol (Fig. 3.5.B). Upon induction, leaf area measurements confirmed an effective increase of more than 1.5 fold in *Alc* line compared to the same line in the control experiment without ethanol (Fig.3.5.C).

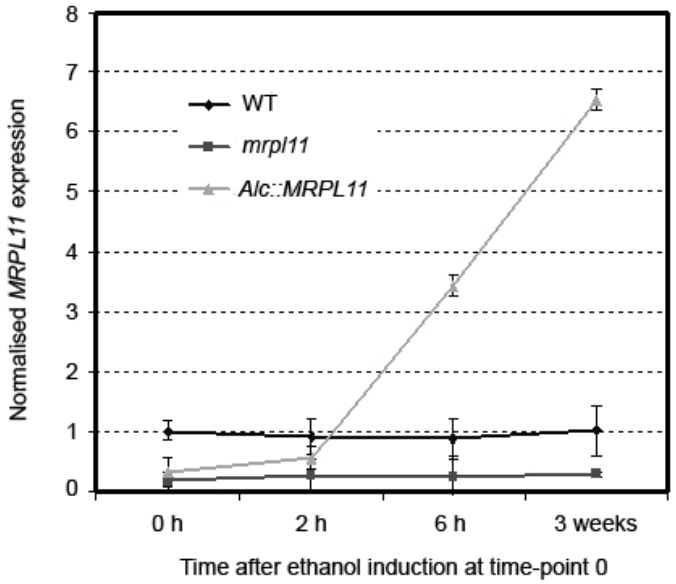
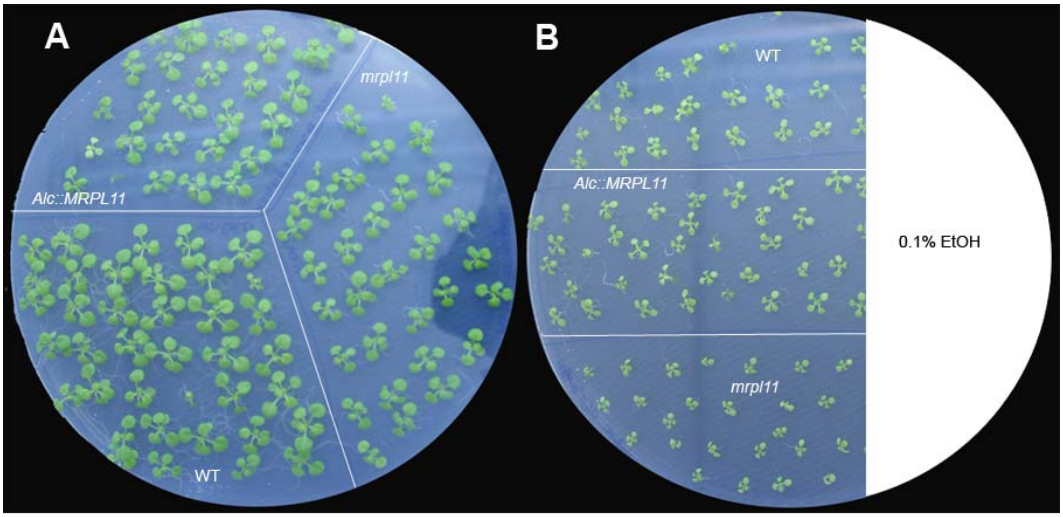


Figure 3.4. Time course of ethanol induction. Plants of WT (blue circle), *mrpl11-1* (pink square) and *pAlc :: MRPL11- mrpl11 line 21* (orange triangle) were induced *in vitro* for 3 weeks with 0.1% ethanol. Bars indicate standard deviation.



C

	WT	*mrpl11*	*Alc::MRPL11*
Control (-0.1% ethanol)	2.4 ± 0.43	1.6 ± 0.39	1.5 ± 0.36
Treated (+0.1% ethanol)	2.0 ± 0.23	1.2 ± 0.13	2.5 ± 0.31

Figure 3.5. The induced overexpression of *MRPL11* is sufficient to fully rescue the *mrpl11-1* phenotype. 10-days-old seedlings were grown *in vitro* for three weeks in absence (**A**) and in presence (**B**) of ethanol vapour. **C.** Growth measurement. Plants area of 10 plants of each genotype were measured. Mean values (in cm^2) ± standard deviations are shown.

The mutation in *MRPL11* gene produces pleiotropic effects on the plant (Pesaresi et al, 2006). To minimize these secondary effects which could negatively influence the analyses, the induction was carried out on 3-day old *in vitro*-grown seedlings. Plants were induced for three weeks with 0.1% ethanol. After this period *Alc* plants showed WT phenotype and a *MRPL11* expression 12 times higher than the WT (Fig. 3.6). At this point, the inducer was removed and leaf samples were collected after 7 and 14 days. While the expression in WT and *mrpl11-1* mutant was stable during the experiment, the *Alc* line displayed a 50% decrease in *MRPL11* transcript after one week without inducer and the achievement of *mrpl11-1* mutant level after 2 weeks (Fig.3.6).

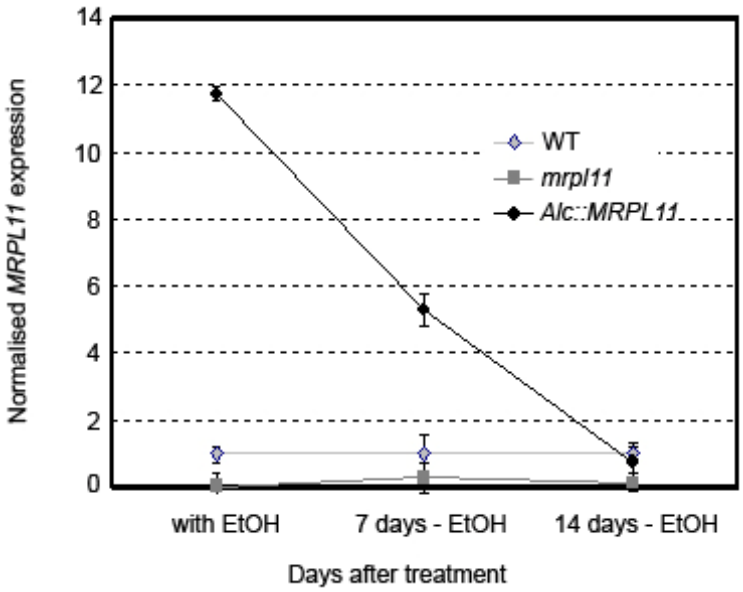


Figure 3.6. Induction of *MRPL11* expression in seedlings. 3-day old plants of WT (blue circle), *mrpl11-1* (pink square) and *pAlc :: MRPL1-mrpl11-1 line 21* (orange triangle) were induced *in vitro* for 3 weeks to ethanol vapour (with ethanol point). At this time the inducer was removed and samples were collected after 7 and 14 days. Bars indicate standard deviation.

Similar experiments were conducted on *PRPL11* and *PRORS1* p*Alc* lines. In absence of the inducer, the p*Alc::PRPL11 prpl11* homozygous line displayed a *PRPL11* transcript level comparable to *prpl11-1* mutant (Fig. 3.7, control), while after three weeks of ethanol exposure the gene expression increased four times in the *Alc* line (Fig.3.7, treated).

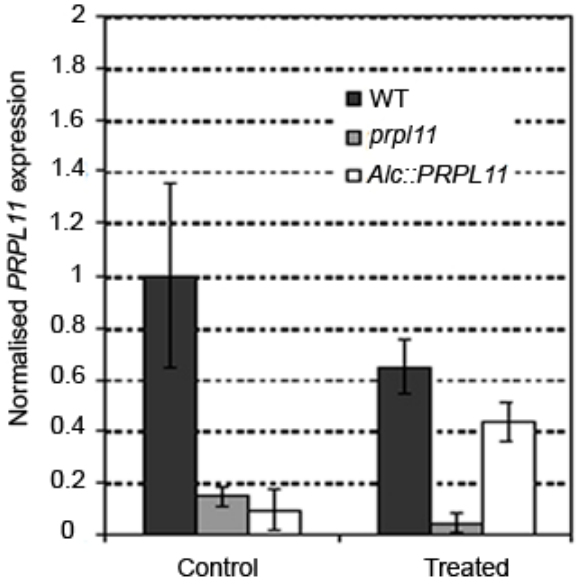
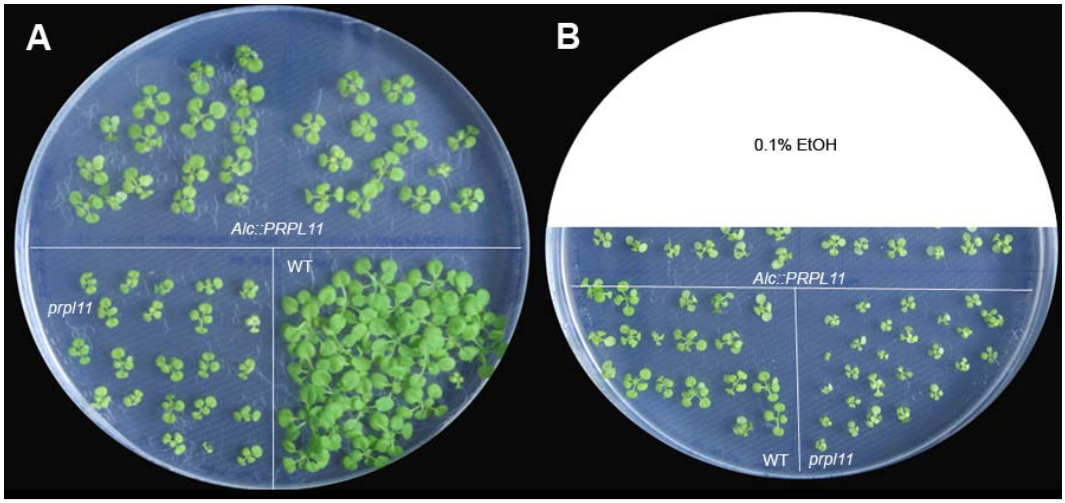


Figure 3.7. Induction of *PRPL11* expression in 3-week old plants. WT, *prpl11-1* and *Alc* plants were exposed for three weeks to 0.1% ethanol vapours *in vitro*. Control plants were grown in absence of the inducer. After this time *PRPL11* expression was determined. Bars indicate standard deviation.



C

	WT	*prpl11*	*Alc::PRPL11*
Control (-0.1% ethanol)	2.24 ± 0.30	0.65 ± 0.08	0.99 ± 0.10
Treated (+ 0.1% ethanol)	1.21 ± 0.20	0.40 ± 0.05	0.73 ± 0.10

Figure 3.8. Phenotype of WT, *prpl11-1* and p*Alc::PRPL11 prpl11* induced by ethanol vapours *in vitro*. **A.** Phenotype of control plants. **B.** Phenotype of treated plants. Three genotypes were grown over 3 weeks in presence of 0.1% ethanol. After this time leaf area of 10 plants were measured (**C**). The table indicates mean values for the plant area (in cm^2) ± standard deviations.

Although the *PRPL11* transcript was raised in treated *Alc* plants, their gene expression was still 30% less than WT. This condition was reflected on the phenotype: after treatment, p*Alc::PRPL11- prpl11-1* plants looked similar to *prpl11-1* mutant, showing that, even if increased, the transcript level was not sufficient to fully complement the phenotype (Fig.3.8.A-B). Detailed leaf area measurements supported this observation: the size of *Alc* plants was in between WT and the knock out mutant, reflecting the transcription profiling (Fig.3.8.C).

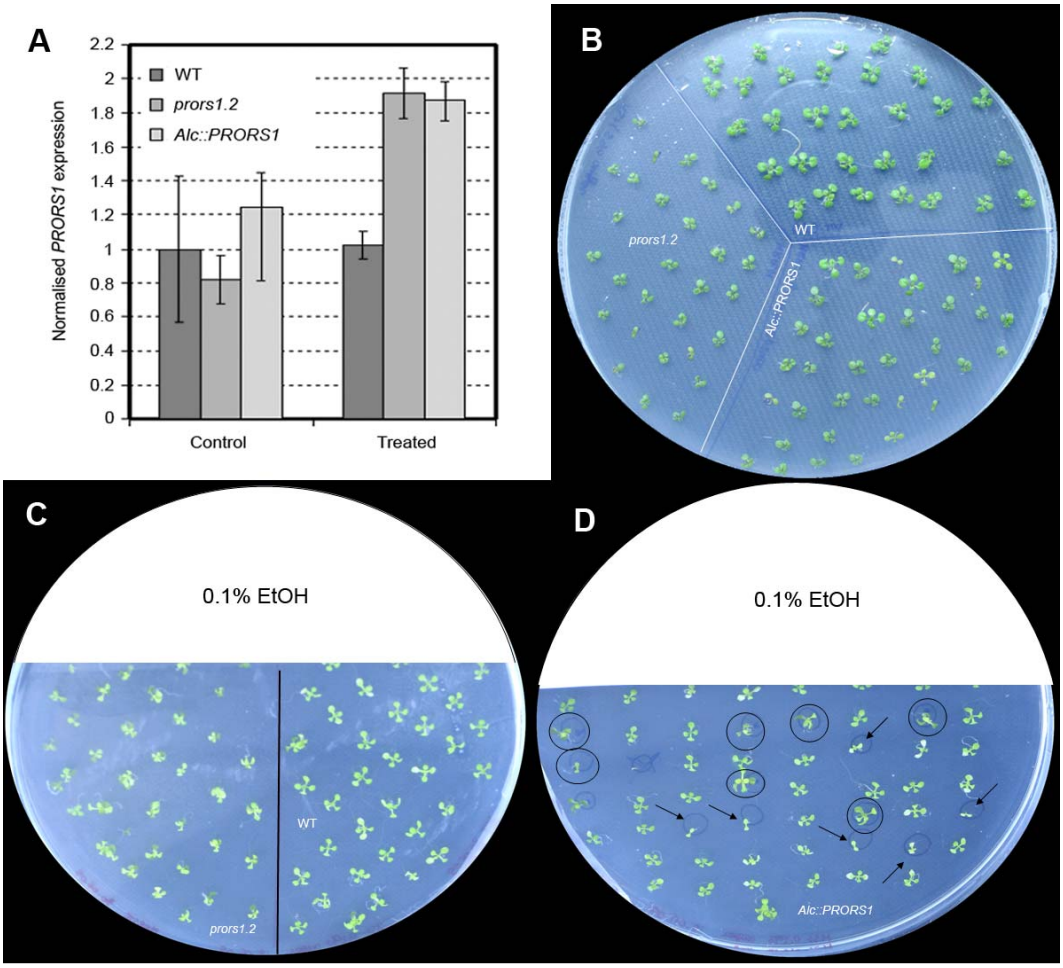


Figure 3.9. Ethanol induction of WT, *prors1.2* and heterozygous *Alc::PRORS1*. WT, *prors1.2* and the *Alc* plants were exposed for three weeks to 0.1% ethanol vapours *in vitro*. Control plants were grown in absence of inducer. After this period *PRORS1* expression was determined (**A**). **B.** Phenotype of control plants. **C and D.** Phenotype of treated plants. Black circles correspond to homozygous *prors1.2* background plants, carrying the Alc :: PRORS1 construct. Black arrows indicate homozygous *prors1.2* plants, not carrying the transgenic construct.

It was observed that *prors1-2* plants carrying the pAlcA/35SM*::PRORS1::*tNOS construct were not able to produce viable seeds. Therefore the induction experiments were carried out using a segregating population, in which p*Alc::PRORS1 prors1-2* homozygous plants were visually distinguished and their position was underlined at the bottom of the culture plate. After three weeks of ethanol induction, a significant increase in size was noted in some of the previously selected plants (Fig. 3.9.D), which showed a phenotype similar to WT (Fig.3.9.C).

Consistently, the increased size was confirmed by leaf area measurements: upon treatment the *Alc* line displayed a 25% increase compared to WT (Table 3.2). The expression level of *PRORS1* was checked in control and treated plants (Fig.3.9.A): in absence of inducer *PRORS1* transcript was substantially comparable in all the genotypes and more abundant in treated *prors1-2* and *Alc* line plants.

	WT	*prors1.2*	*Alc:: PRORS1*
Control (-0.1% ethanol)	1.00 ± 0.12	0.40 ± 0.06	0.50 ± 0.08
Treated (+0.1% ethanol)	0.36 ± 0.03	0.21 ± 0.06	0.45 ± 0.10

Table 3.2. Leaf area measurements of WT, *prors1-2* and p*Alc::PRORS1 prors1.2* plants in control and treated conditions. Leaf areas of 10 plants were measured after three weeks. The table indicates mean values for the plant area (in cm^2) ± standard deviations.

3.3 Characterisation of *Alc* lines on soil

Results obtained *in vitro* were useful to verify the functionality of the ethanol inducible system and to optimise the induction. Nevertheless, the exogenous concentration of sucrose in the media, even in very low concentration, could influence genes expression in plants (Gibson, 2005). To avoid this possible perturbation in the gene expression study, the system was transferred onto soil. Due to previous results obtained *in vitro*, the in soil induction was performed on p*Alc::MRPL11 mrpl11* and p*Alc::PRPL11 prpl11* seedlings, using the induction

model explained in Fig. 3.5. Two 50 ml tubes filled with 100% ethanol were placed in the corners of the 96-plants trays, containing 4-day old soil-grown seedlings. To avoid an excessive inducer evaporation, trays were placed in a *Arabidopsis* biological incubator and the induction was carried out for 8 hours per day. In the remaining 16 h the tubes were closed in order to minimize fungal growth. After three weeks of ethanol vapours exposition, p*Alc::MRPL11 mrpl11* plants displayed WT phenotype, indicating the complete mutation recovery (Fig.3.10.A, C). In agreement with the data obtained from *in vitro* experiment p*Alc::PRPL11 prpl11* plants were not able to completely rescue the phenotype and displayed a slight leaf area increase, compared to *prpl111-1* mutant, but still pale leaf pigmentation (Fig.3.10.B, C).

To quantify the effect of the complementation on the phenotypic changes, photosynthetic performances were measured (Fig.3.10.D). In p*Alc::MRPL11 mrpl11* plants the maximum (F_V/F_M) and the effective (Φ_{II}) quantum yield of PSII remained unaltered, indicating a WT-like efficiency. Also the *mrpl11-1* knock out mutant showed WT behaviour, denoting that the T-DNA insertion did not influence the photosynthetic machinery. Interestingly, an increase in non photochemical quenching (NPQ) was registered in both *mrpl11* (0.2 ± 0.01) and *Alc* line (0.12 ± 0.02), indicating a possible stress situation. *Alc::PRPL11* displayed a *prpl11*- like behaviour (Pesaresi et al, 2001), characterised by a significant reduction in F_V/F_M ratio and Φ_{II}, while the NPQ level was substantially unaltered.

A

B

C

	WT	*mrpl11*	*Alc::MRPL11*	*prpl11*	*Alc::PRPL11*
Plant area (in cm²)	4.60 ± 0.62	1.93 ± 0.19	3.31 ± 0.60	2.13 ± 0.32	2.56 ± 0.42

D

	WT	*mrpl11*	*Alc::MRPL11*	*prpl11*	*Alc::PRPL11*
Fv/Fm	0.81 ± 0.005	0.80 ± 0.010	0.82 ± 0.006	0.73 ± 0.010	0.67 ± 0.030
NPQ	0.06 ± 0. 020	0.20± 0.010	0.12 ± 0.021	0.08 ± 0.050	0.10 ± 0.007
ΦII	0.78 ± 0.006	0.76 ± 0.010	0.78 ± 0.007	0.67 ± 0.010	0.59 ± 0.050

Figure 3.10. Growth characteristics of plants after three weeks of induction in soil. **A.** WT, *mrpl11* and *Alc::MRPL11* plants. **B.** WT, *prpl11* and *Alc::PRPL11* plants. 4-day old plants were induced with ethanol vapours 8 hours per day, over three weeks. After this period *Alc::MRPL11* plants were similar to WT, while *Alc::PRPL11* exhibited a partially rescued phenotype. **C.** Growth measurements. Mean values (in cm^2) ± standard deviation are shown. **D.** Photosynthetic parameters. Six plants for each genotype were measured. Mean values (μmol m^{-2} s^{-1}) ± standard deviation are shown.

To confirm the complete or the partial complementation, the transcript level of the induced genes was determined by qPCR. After three weeks of induction, p*Alc:: MRPL11-mrpl11* plants showed a two fold increase in *MRPL11* gene expression compared to WT (Fig.3.11). In the same experimental conditions p*Alc::PRPL11 prpl11* displayed an increase of two fold if compared with the mutant, but still the transcript level was lower than in WT (Fig.3.11). This transcript condition was set as "Time 0", after which the inducer was removed from the environment and the time course of the gene expression was performed (Fig.3.11). Notably *MRPL11* gene expression remained unaltered for 8 hours after the inducer removal (Fig.3.11). The gene expression decreased dramatically after 19 hours, to be settled at the mutant level until the end of the experiment.

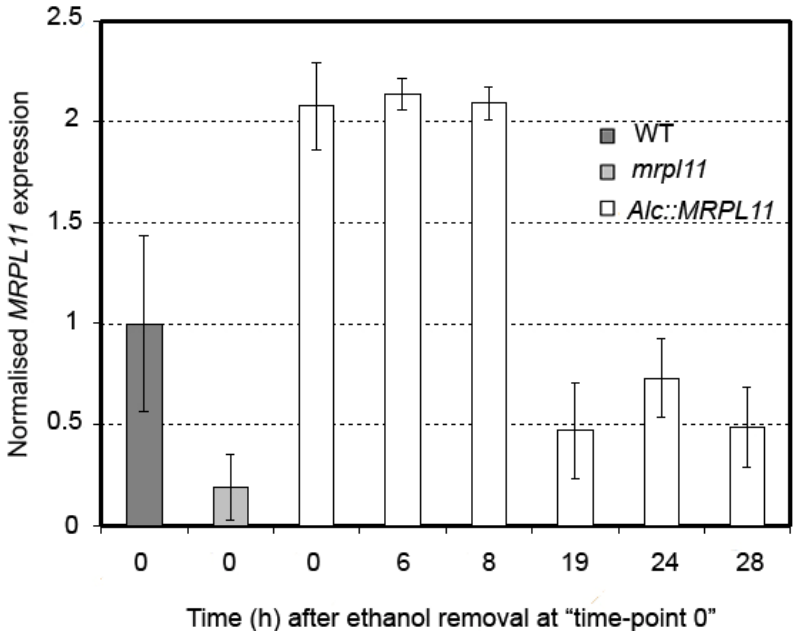


Figure 3.11. Time course of in-soil grown seedlings. 4-day old WT, *mrpl11-1* and p*Alc::MRPL11 mrpl11* plants were induced with ethanol vapours 8 hours per day, over three weeks. After this period the inducer was removed from the environment and *MRPL11* gene expression was detected by qPCR. Error bars are shown. The experiment was performed using reactions in triplicate with at least two independent biological replicates.

Due to the *prpl11-1* foliar anatomy, in particular the thin cuticle, the inducer could have easily reached the target, but as easily the induced effects could have disappeared. Therefore time points in the *PRPL11* expression kinetic were taken every three hours (Fig.3.12). The already slight induction effect disappeared three hours after the removal, settling at the mutant level.

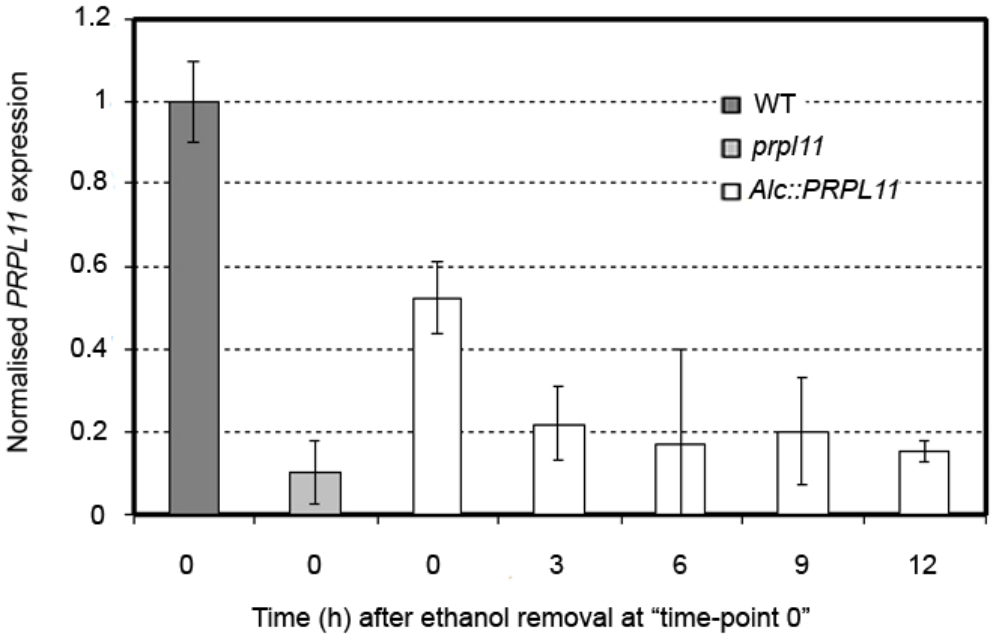


Figure 3.12. Time course of in-soil growth seedlings. 4-day old WT, *prpl11* and *Alc::PRPL11* plants were induced with ethanol vapours 8 hours per day, over three weeks. After this period the inducer was removed from the environment and the *PRPL11* gene expression was detected by qPCR. Error bars are shown.

In parallel, a control experiment was performed, using the same growth and environmental conditions, but in the absence of ethanol. The difference between treated and not treated p*Alc::MRPL11 mrpl11* plants was evident: in control conditions, plants appeared to be small and dark green, displaying the characteristic *mrpl11-1* phenotype, as previously described by Pesaresi et al (2006) (Fig.3.13.A and Table 3.3).

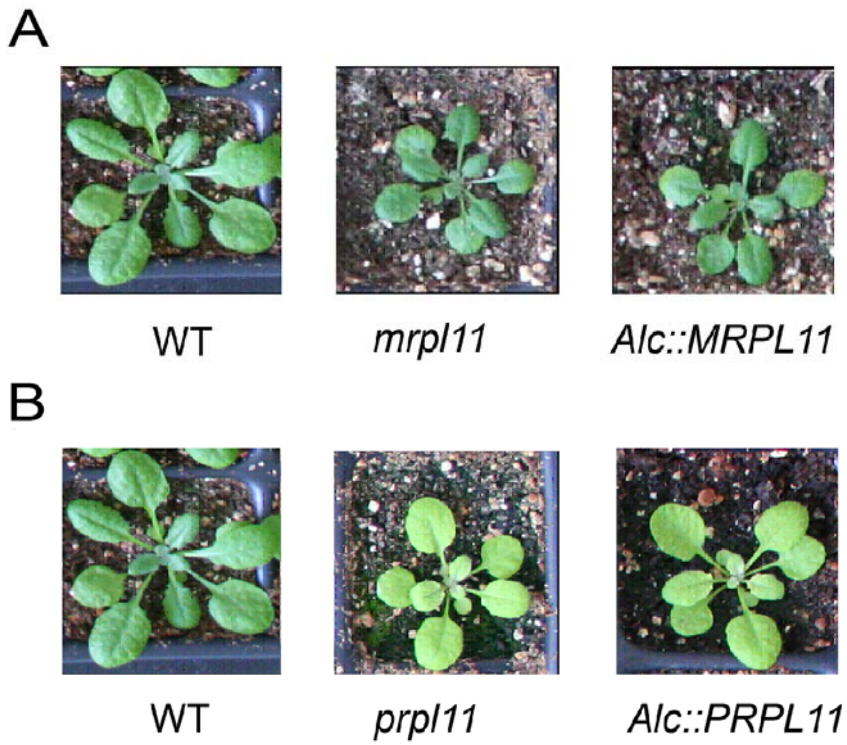


Figure 3.13. Growth characteristics of control plants **A.** WT, *mrpl11* and *Alc::MRPL11* plants. **B.** WT, *prpl11* and *Alc::PRPL11* plants. Plants were grown as explained in Fig.3.9, except for the absence of ethanol.

As showed during ethanol treatment, also under control conditions *pAlc::PRPL11* plants displayed the *prpl11-1* mutant phenotype (Fig.3.13.B and Table 3.3).

	WT	***mrpl11***	***Alc::MRPL11***	***prpl11***	***Alc::PRPL11***
Plant area (cm^2)	5.02 ± 0.53	2.05 ± 0.40	1.72 ± 0.47	1.71 ± 0.28	1.75 ± 0.19

Table 3.3. Leaf area measurements of control plants. Mean values (in cm^2) ± standard deviation are shown.

Moreover, the p*Alc:: PRPL11-prpl11* line showed an alteration in the photosynthetic performances due to a reduced $f\Phi_{II}$, as reported for *prpl11-1* (Table 3.4); also the F_v/F_m appeared significantly reduced, implying a defect in energy transfer within PSII, while the high level of non-photochemical quenching

(NPQ) and the strong decrease in Φ_{II} indicated a severely impaired light utilization and increased photoinhibition. On the contrary, the *Alc* line for *MRPL11* did not show any significant defect, displaying *mrpl11-1* -like photosynthetic performances (Table 3.4).

	WT	*mrpl11*	*Alc::MRPL11*	*prpl11*	*Alc::PRPL11*
Fv/Fm	0.81 ± 0.010	0.81 ± 0.010	0.80 ± 0.001	0.65 ± 0.010	0.66 ± 0.020
NPQ	0.16 ± 0.020	0.17± 0.020	0.12 ± 0.050	0.18 ± 0.050	0.13 ± 0.040
ΦII	0.76 ± 0.010	0.75 ± 0.010	0.75 ± 0.070	0.53 ± 0.010	0.55 ± 0.030

Table 3.4. Photosynthetic parameters of control plants. All the genotypes were grown in the conditions described in Fig.3.9, but in absence of the inducer. Six plants for each genotype were measured. Mean values (μmol m^{-2} s^{-1}) ± standard deviation are shown.

3.4 Transcription studies on *Alc* lines and knock out mutants

Recent report showed that the nuclear mutations in *mrpl11prpl11* and *prors1* negatively affect translation in the chloroplast and also lead to similar light-independent repressive effects on nuclear genes. In particular the repression of nuclear genes observed in these mutant plants, compared to what observed for *mrpl11-1* and *prpl11-1* single mutants, suggested that both organelles send retrograde signals when protein synthesis is impaired, and that their signals are synergistic (Pesaresi et al, 2006; Woodson and Chory, 2008).

To dissect this signalling pathway and discover possible candidates involved in it, transcription studies on chloroplast and mitochondrion genomes were performed on *mrpl11-1*, *prpl11-1*, *prors1-2* knock out mutants and the *mrpl11prpl11* double mutant. The chloroplast transcription profile showed a relatively small number of differentially expressed genes in all the genotypes tested (Fig.3.14).

Interestingly, all genotypes tested showed a strong increase in the expression of chloroplast ribosomal subunits, indicating a possible attempt to compensate the impaired organelle(s) translational machinery. All genotypes showed the same expression pattern for all the genes considered and a general tendency was not detectable in the expression of the two photosystems.

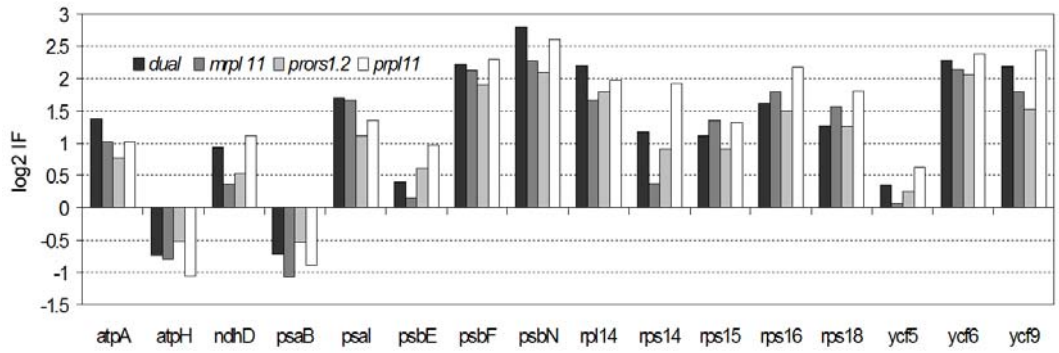


Figure 3.14. Differentially expressed chloroplast genes of *mrpl11*, *prpl11*, *prors1.2* and *mrpl11prpl11* double mutant. Leaf tissue of 3-week old incubator-grown plants was harvested and chloroplast macroarrays were performed for each genotype. The expression profile is displayed as mutant versus WT ratios of transcript levels (logarithmic scale).

This gene profile was also displayed in the mitochondrion arrays, where the mutation in *MRPL11* led to a strongest differentiation in gene expression, compared with the other genotypes (Fig. 3.15).

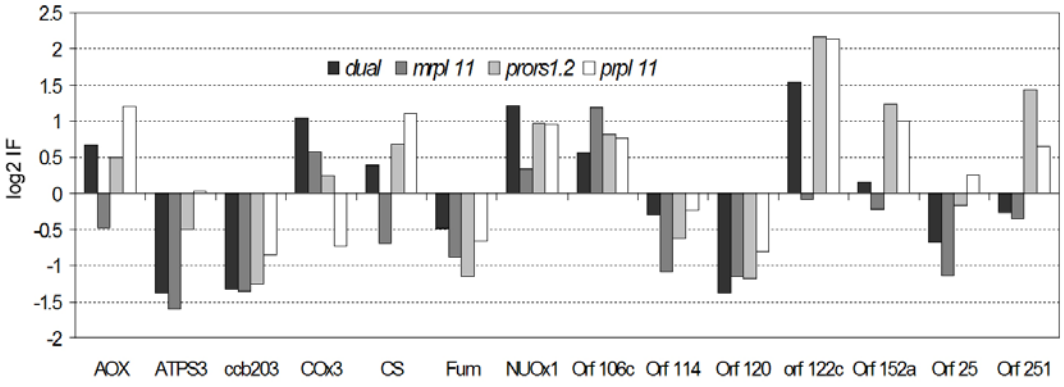


Figure 3.15. Differentially expressed mitochondrion genes of *mrpl11*, *prpl11*, *prors1.2* and *mrpl11prpl11* double mutant. Leaf tissue of 3-week old incubator-grown plants was harvested and chloroplast macroarrays were performed for each genotype. The expression profile is displayed as mutant versus WT ratios of transcript levels (logarithmic scale).

prpl11-1 mutant carries the T-DNA insertion in a nuclear gene encoding for an organelle targeted protein: the displayed phenotype seems stronger than *mrpl11-1* one , with pale leaves and small size, but *prpl11-1* seems to be less impaired than *prors1.2*. Because of this intermediate phenotype, *prpl11* was chosen to perform

an expression profile. The analysis of the microarray data highlighted 68 genes that are shown to be differentially expressed. These genes were categorised according to the predicted or known subcellular location of their gene products. Of 67 proteins, 55 could be localised to a cellular compartment, based on annotation. Approximately 30% of these proteins were localised to the chloroplast, 15% to the nucleus and another 10% to mitochondrion (Fig.3.16.B). When classified according to known or predicted molecular function, 20 of 67 genes displayed unknown function, 11 were involved in protein synthesis and processing, with a further 6 and 4 involved in general stress response and RNA processing and regulation (Fig.3.16.A).

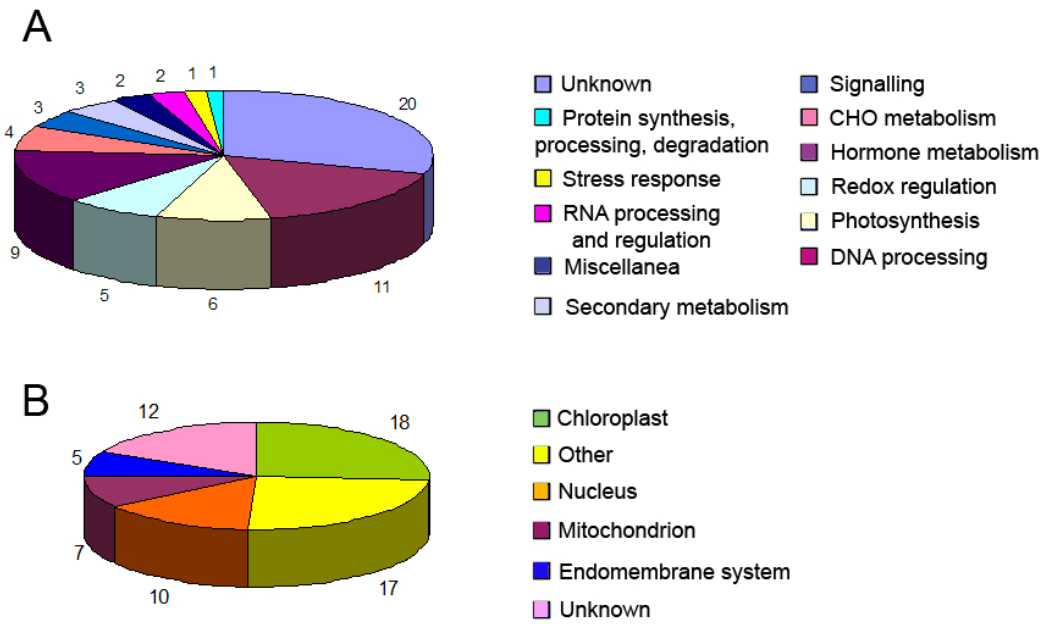


Figure 3.16. Differentially expressed genes of *prpl11* mutant. Leaf tissue of 3-week old incubator-grown plants was harvested and microarrays were performed. **A.** Functional categories, based on MapMan software. **B.** Gene localisation.

Interestingly, even though just one gene is directly involved in the photosynthetic process (*LHCB4.3*), several upregulated genes were identified whose products are either targeted to the chloroplast or involved in some chloroplast regulatory processes (Fig.3.16.B).

Gene ID	Product	Fold change	Gene ID	Product	Fold change
AT2G40100	LHCB4.3	16.33	AT3G18600	DEAD/DEAH box helicase, putative	2.93
AT5G58310	hydrolase, alpha/beta fold family protein	11.75	AT2G40750	WRKY54 | WRKY family transcription factor	2.91
AT1G23205	invertase/pectin methylesterase inhibitor family protein	6.91	AT4G08390	sAPX | L-ascorbate peroxidase, stromal	2.83
AT5G08640	FLS | flavonol synthase 1	6,86	AT5G51820	PGM | phosphoglucomutase, chloroplast	2.82
AT1G75040	PR5 | pathogenesis-related protein 5	6.24	AT4G26780	AR192 | co-chaperone grpE family protein	2.80
AT5G13930	CHS | chalcone synthase	6.00	AT1G65060	4CL3 | 4-coumarate--CoA ligase 3 / 4-coumaroyl-CoA synthase 3	2.77
AT4G01080	expressed protein	5.47	AT3G17170	RFC3 | ribosomal protein S6 family protein	2.66
AT3G44750	HD2A | histone deacetylase	5.06	AT2G33210	chaperonin, putative	2.65
AT3G16670	expressed protein	4.35	AT3G06530	BAP28-related	2.62
AT1G06000	UDP-glucoronosyl/UDP-glucosyl transferase family protein	4.29	AT3G48500	PDE312 | expressed protein	2.62
AT4G31870	ATGPX7 | glutathione peroxidase	4.09	AT2G32290	beta-amylase, putative	2.59
AT5G05270	chalcone-flavanone isomerase family protein	3.87	AT4G02930	elongation factor Tu, putative	2.55
AT4G22753	SMO1 3 | sterol desaturase family protein	3.49	AT3G56090	ATFER3 | ferritin, putative	2.51
AT5G19750	peroxisomal membrane 22 kDa family protein	3.49	AT1G56050	GTP-binding protein-related	2.48
AT1G48570	zinc finger (Ran-binding) family protein	3.39	AT1G56110	NOP56 | nucleolar protein Nop56, putative	2.41
AT3G13470	chaperonin, putative	3.21	AT3G23940	dehydratase family	2.41
AT1G09500	cinnamyl-alcohol dehydrogenase family	3.03	AT1G24020	Bet v I allergen family protein	2.35
AT1G69200	pfkB-type carbohydrate kinase family protein	3.01	AT4G12830	hydrolase, alpha/beta fold family protein	2.33
AT4G30610	BRS1 | serine carboxypeptidase S10 family protein	2.96	AT1G13270	MAP1C | metallopeptidase M24 family protein	2.29
AT4G25630	FIB2 | fibrillarin 2	2.95	AT3G53460	CP29 | 29 kDa ribonucleoprotein, chloroplast/RNA-binding protein cp 29	2.27
AT5G49910	cpHSC70 2 | heat shock protein 70	2.94	AT3G23990	HSP60 | chaperonin (CPN60)	2.26

Table 3.5. Upregulated genes in the *prpl11* mutant. The gene ID, a brief molecular function description and the fold change are reported.

These include genes involved in redox regulation, like the glutathione peroxidase (ATGPX7) and the L-ascorbate peroxidase (sAPX) and genes encoding for nucleoid targeted proteins, a kinase and a RNA binding protein (PDE312). Furthermore, genes encoding for transcriptional factors (e.g. WRKY54) and protein related to chromatin arrangement and acetylation are also represented. The complete list of the upregulated genes is shown in Table 3.5.

Among the upregulated genes, five were selected to be checked in the other genotypes, in order to analyse their expression when the translational machinery is impaired just in the mitochondrion, as in the case of *mrpl11-1* background, or in both organelles, mitochondrion and chloroplast, as in *prors1-2* and *mrpl11prpl11* double mutant. The transcript level of *At1g69200*, *At2g40100*, *At2g40750*, *At4g25630*, *At4g44750* genes was analysed by Real time PCR (Fig.3.17).

While *At2g40100* and *At2g40750*, encoding respectively for the protein LHCB4.3 and the WRKY54 transcription factor, showed an upregulation just in *mrpl11* and *prpl11*, but not in the genotypes impaired in both organelles, *At1g69200, At4g2563*0 and *At3g44750*, displayed an overall upregulation (Fig.3.17). *At1g69200* encodes a pfkB-type carbohydrate kinase, located in the nucleoid, the region of the chloroplast in which the DNA is confined. *At4g2563*0 and *At3g44750* encode respectively for the fibrillarin2 protein and the histone deacetylase2, both located in the nucleolus.

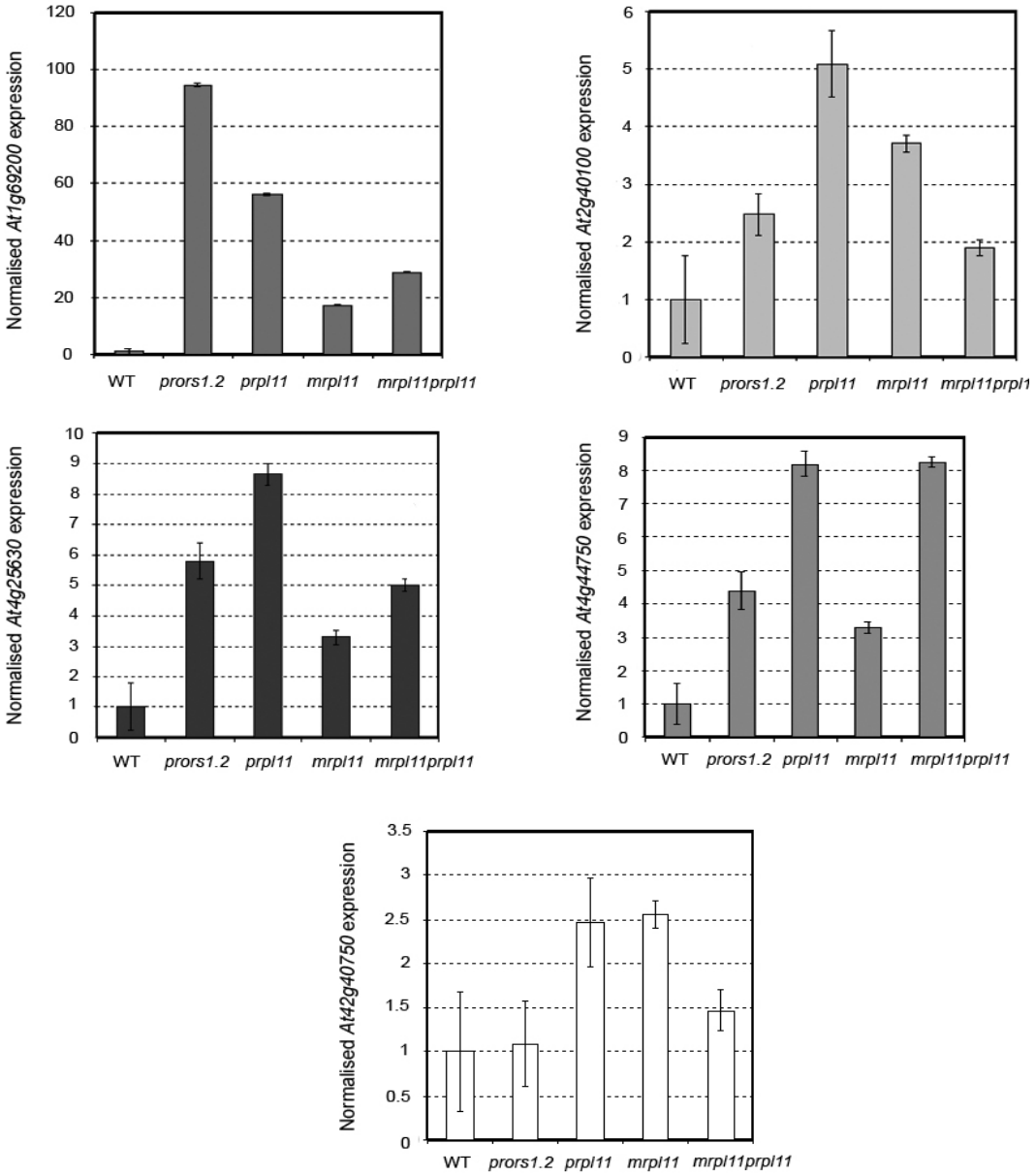


Figure 3.17. Transcription level of five genes selected from the upregulated ones shown in Table 3.4. Real time PCR was performed in *mrpl11*, *prpl11*, *mrpl11prpl11* double mutant and *prors1.2*. Left panels: *At1g69200*; *At4g25630*. Right panels: *At2g40100*; *At3g44750*. Central panel: *At2g40750*.

Due to their overexpression in all genotypes *At1g69200* and *At3g44750* were chosen to be tested in the *Alc* line expressing *MRPL11*, and a time course of these genes was performed. Given its function as transcription factor, the expression of *At2g40750* was also checked in the line (Fig.3.18).

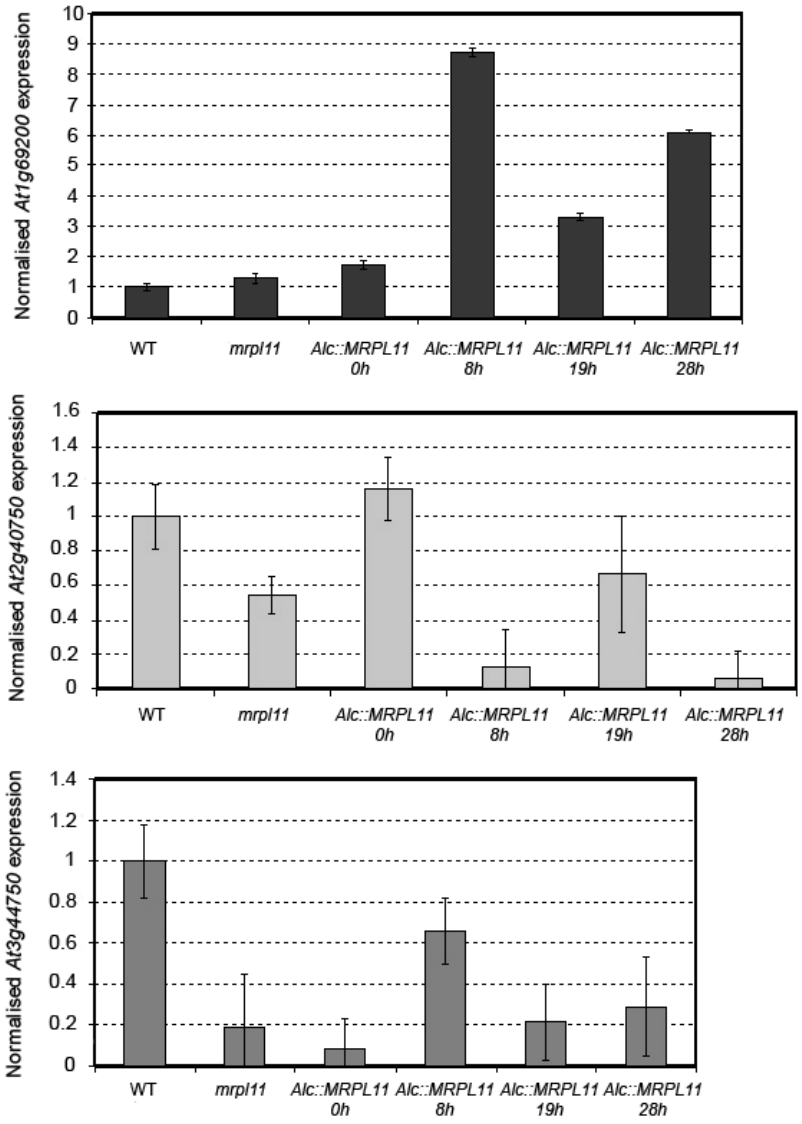


Figure 3.18. Kinetic of *At1g69200, At2g40750* and *At3g44750* transcription level of in *Alc::MRPL11* line.

The kinetic of the three tested genes did not show the expected trend: following the data collected for the knock out mutants, a higher level of transcript would have been supposed in *mrpl11* mutant at the "time 0", while WT and the *Alc* line should have shown a lower transcript level. By contrast, in mutant and in the *Alc* line, the gene expression was comparable to the WT or even less at the beginning of the experiment (Fig.3.18). Moreover, *At1g69200, At2g40750* and *At3g44750* transcript level fluctuated during the experiment, following a circadian rhythm. These observations were also confirmed by following experiments on a second

batch of plants and by screening the BAR database (http://bar.utoronto.ca/), which reported a similar expression pattern during the day (data not shown).

3.5. Generation of XVE transgenic lines

Since the ethanol induced overexpression leads to difficulties related to the phenotype rescue, a second system based on the RNA-interference (RNAi) strategy was performed, using a receptor-based chemical-inducible system. The peculiarity of the system is represented by the chimeric transcription activator, XVE, containing the DNA-binding domain of the bacterial repressor LexA (X), the acidic transactivating domain of VP16 (V) and the regulatory region of the human estrogen receptor (E) (Zuo et al, 2000 and Fig.3.19). The expression of the chimeric XVE is controlled by the strong constitutive promoter G10-90 and its transactivating activity is regulated by estrogens (e.g. 17-β-estradiol). To obtain an inducible RNAi system, a cassette consisting of two inverted copies of the genome sequence tags (GST) of *PRPL11* and *PRORS1* separated by an intron was cloned in a vector containing the XVE factor (Fig.3.19).

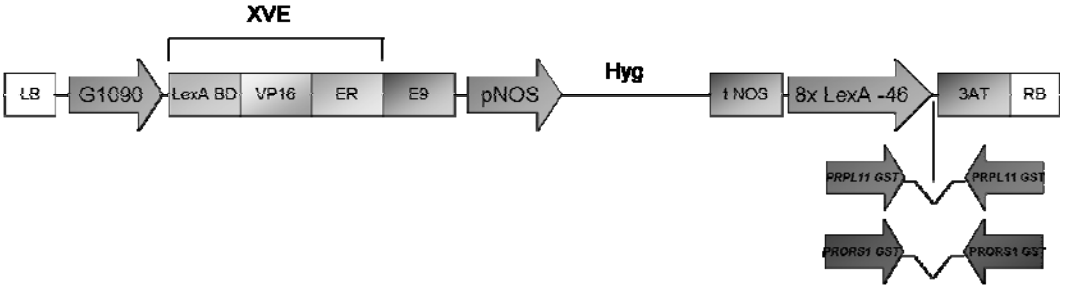


Figure 3.19. Schematic representation of the constructs obtained to generate XVE transgenic lines. In the left side *PRPL11* and *PRORS1* RNAi constructs are shown.

The cloning of *MRPL11* was unsuccessful, since the construct seemed to be toxic to *E.coli* and therefore it was not possible to generate *XVE* transgenic lines.
On the other hand, for *PRORS1* and *PRPL11* the constructs could be prepared and WT plants were transformed obtaining three and five F2 transgenic lines respectively. Every line was tested by Real time PCR, to verify the decreased gene expression: in lines RNAi-PRORS1, the transcript level of *PRORS1* showed

a 50% decrease already after 1.5 hours of induction, followed by a constant reduction after 3 hours. The expression increased after 6 hours and reached an expression level similar to WT after 24 hours. Probably this reflects the lack of inducer activity, due to its light-dependent degradation (Fig.3.20.A).

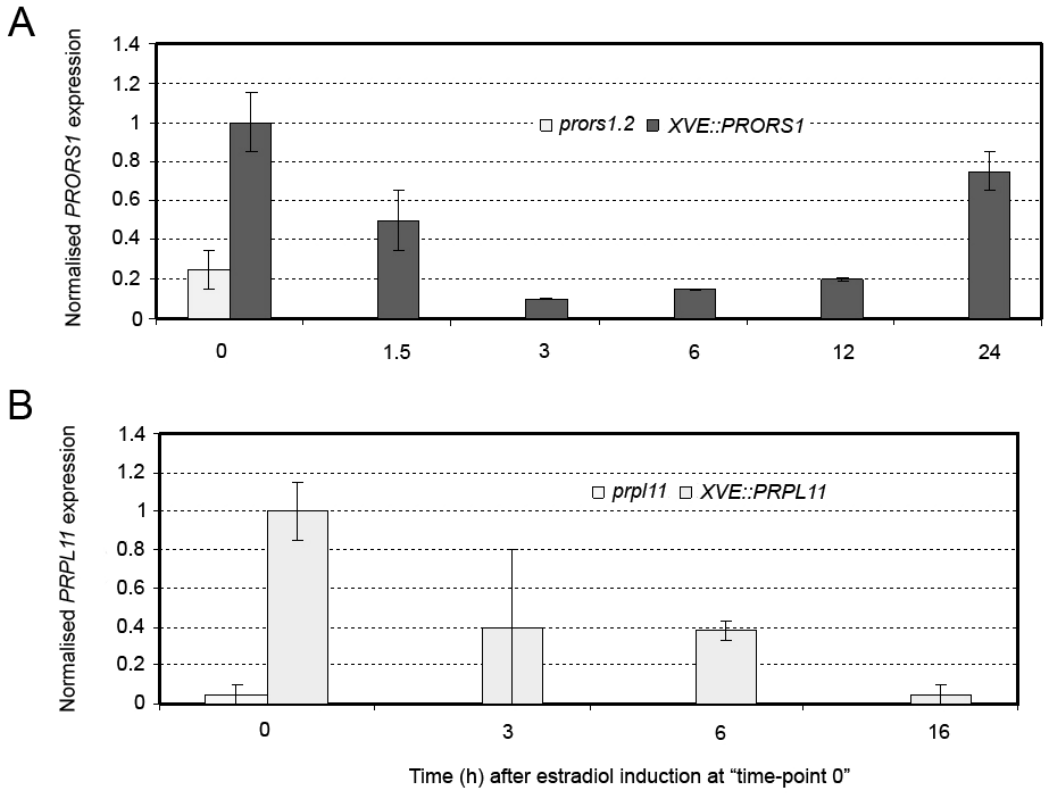


Figure 3.20. Transcript level of **A.** *PRORS1* **B.** *PRPL11* in mutant and *XVE* lines. The expression levels are shown as arbitrary units, normalized on the expression levels of *UBIQUITIN* which was used as a reference. A solution containing 1 μM 17-β-estradiol and 0.1% Triton-X was diluted in water and directly sprayed on leaves. The experiments were carried out in phytothrone and samples for qPCR were collected at the indicated time points. Errors bars are shown.

Similar expression pattern was observed for *PRPL11*: in RNAi-PRPL11 plants, its transcript showed a constant decrease during all the experiment, until reaching the *prpl11* mutant level (Fig.3.20.B). In spite of the decrease observed in the gene expression, *XVE* treated plants did not show any phenotype after 16 or 24 hours of induction and photosynthetic performances were unaltered, except for the NPQ parameter, which was higher in the transgenic plants compared to controls (Table 3.6), indicating a possible stress condition.

A

	Wt + 17-β-estradiol				XVE::PRORS1 + 17-β-estradiol			
	0h	3h	6h	24h	0h	3h	6h	24h
Fv/Fm	0.800 ± 0.003	0.797 ± 0.002	0.790 ± 0.006	0.803 ± 0.009	0.800 ± 0.010	0.780 ± 0.010	0.800 ± 0.004	0.810 ± 0.009
NPQ	0.072 ± 0.005	0.120 ± 0.030	0.070± 0.060	0.05 ± 0.020	0.117 ± 0.020	0.115 ± 0.070	0.134 ± 0.020	0.075 ± 0.030
Yeld	0.729 ± 0.009	0.729 ± 0.005	0.730 ± 0.010	0.743 ± 0.010	0.719 ± 0.008	0.704 ± 0.010	0.730 ± 0.008	0.754 ± 0.009

B

	Wt + 17-β-estradiol				XVE::PRPL11 + 17-β-estradiol			
	0h	3h	6h	24h	0h	3h	6h	24h
Fv/Fm	0.800 ± 0.003	0.797 ± 0.002	0.790 ± 0.006	0.803 ± 0.009	0.770 ± 0.020	0.766 ± 0.020	0.773 ± 0.010	0.806 ± 0.010
NPQ	0.072 ± 0.005	0.120 ± 0.030	0.070± 0.060	0.050 ± 0.020	0.199 ± 0.030	0.115 ± 0.060	0.135 ± 0.060	0.077 ± 0.010
Yeld	0.729 ± 0.009	0.729 ± 0.005	0.730 ± 0.010	0.743 ± 0.010	0.670 ± 0.020	0.688 ± 0.020	0.704 ± 0.010	0.750 ± 0.010

Table 3.6. Spectroscopic data for WT and *XVE* leaves during estradiol treatment. **A.** *XVE::PRORS1*. **B.** *XVE::PRPL11*. Photosynthetic parameters were measured on plants exposed to 1 μM 17-β-estradiol as described in Fig.3.20. Mean values ± SD (5 plants each genotype) are shown.

To verify possible secondary effects given by the inducer, photosynthetic parameters on WT plants treated and not treated with estradiol were also tested (Table 3.7): measurements of the F_V/F_M and Φ_{II} did not show any alteration in treated plants, indicating that the inducer did not affect the efficiency of PSII complexes. Also NPQ values did not displays significant changes in treated plants.

	WT not treated				WT + 17-b-estradiol			
	0 h	3 h	6 h	24 h	0 h	3 h	6 h	24 h
Fv/Fm	0.830 ±0.007	0.830 ± 0.009	0.830± 0.007	0.840 ± 0.005	0.820 ± 0.012	0.830 ± 0.007	0.840 ± 0.003	0.830 ± 0.007
NPQ	0.140 ±0.008	0.155 ± 0.010	0.140 ± 0.008	0.150 ± 0.010	0.227 ± 0.015	0.180 ± 0.014	0.177 ± 0.015	0.140 ± 0.030
Yield	0.780 ±0.008	0.780± 0.010	0.780 ± 0.010	0.780 ± 0.006	0.714 ± 0.040	0.755 ± 0.007	0.785 ± 0.003	0.780 ± 0.006

Table 3.7. Spectroscopic data for WT leaves during estradiol treatment. Photosynthetic parameters were measured on WT plants exposed and not exposed to 1 μM 17-β-estradiol, in order to evaluate possible secondary effects of the inducer. Experimental conditions are described in Fig.3.20. Mean values ± SD (5 plants each genotype) are shown.

Furthermore, *LHCB1.2* expression in treated WT did not show differences compared with the control, except a more linear decrease during the experiment (Fig. 3.21).

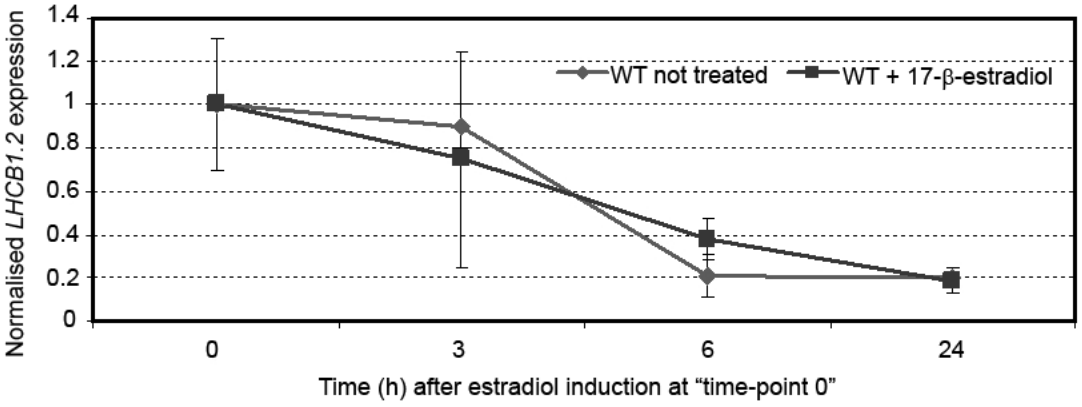


Figure 3.21. *LHCB1.2* expression in WT, treated for 24 hours with 17-β-estradiol or in control conditions. Plants were handled as described in Fig.3.20. The control plants were treated in the same conditions, but in absence of inducer. Error bars are shown.

Further analyses showed a certain level of instability in the gene expression: in fact, the gene expression did not show reproducible underexpression patterns, during the time-course, leading to the conclusion that the system was not ideal for the purpose (Fig 3.20 and Fig.3.22).

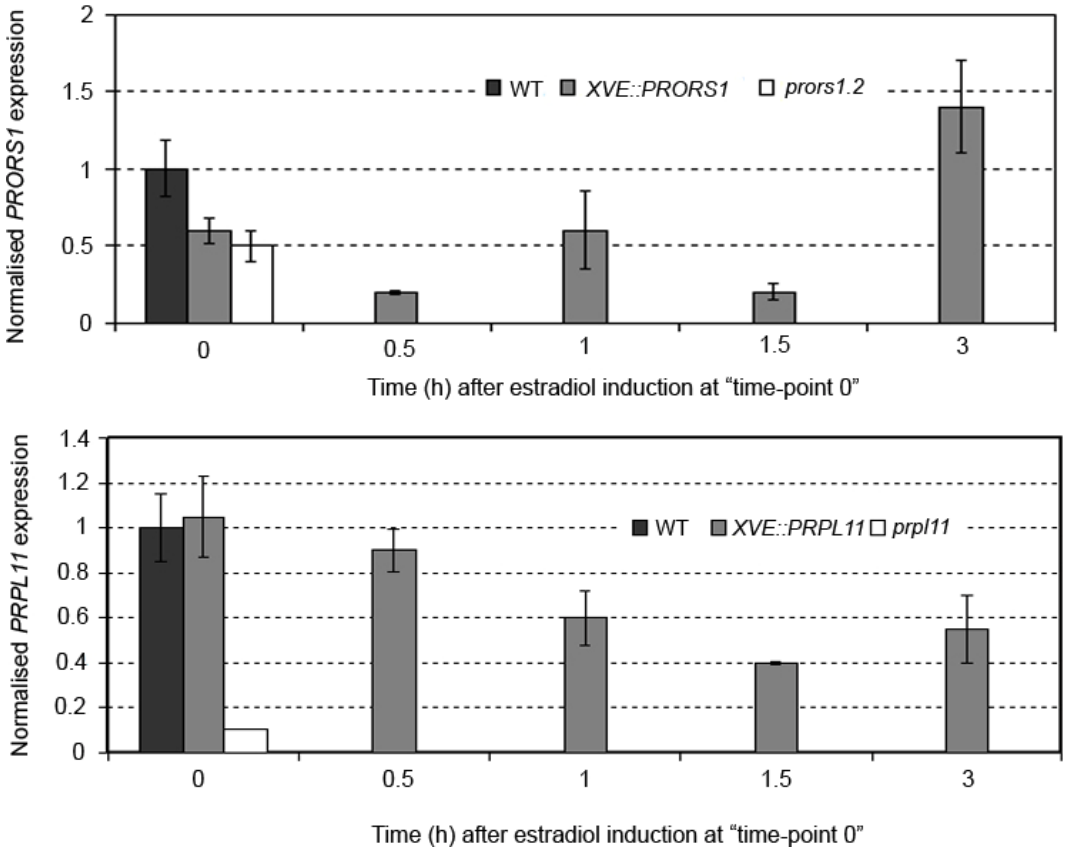


Figure 3.22. *PRPL11* expression kinetic in WT, *prpl11* and *XVE* line treated for 3 hours with 17-β-estradiol. **A.** *XVE::PRORS1*. **B.** *XVE::PRPL11*. Plants were handled as described in Fig.3.20. Error bars are shown.

To establish whether the GSTs design could have been the cause of these expression anomalies, constructs were cloned in a vector containing the cassette for a constitutive RNAi system and transgenic lines were generated. 12 and 16 lines were generated for *PRORS1* and *PRPL11*, respectively: when grown in soil, RNAi plants appeared smaller than WT and pale green, the characteristic phenotype of *prors1-2* and *prpl11-1* mutants (Fig.3.23. A and B). qPCR confirmed the downregulation of the genes, showing different transcript level in the selected lines (Fig.3.23.C and D), proving that the GSTs construct was functional.

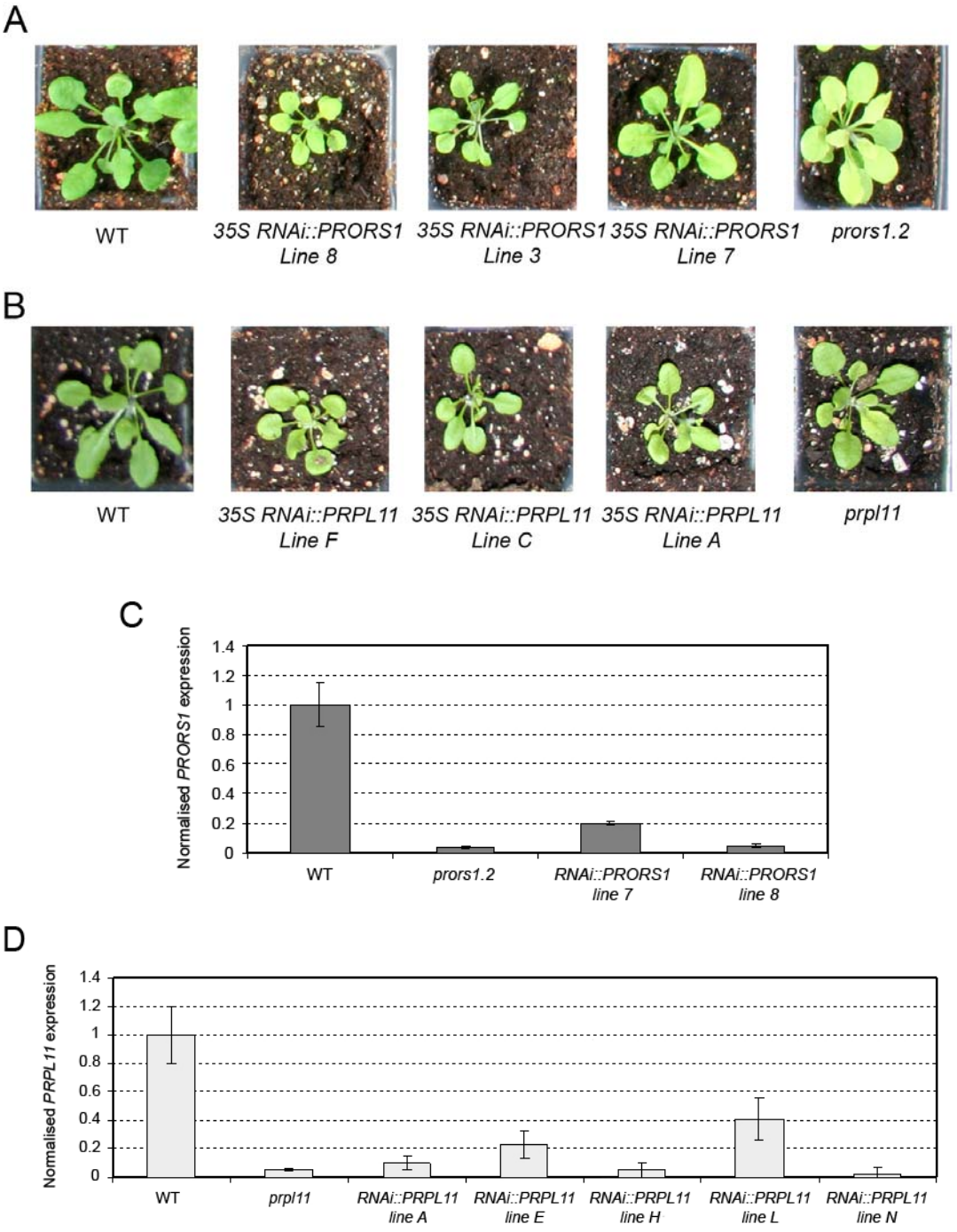


Figure 3.23. Phenotype of greenhouse-grown 3-week old *RNAi* plants. WT and the respective mutant are shown as comparison. **A.** *RNAi::PRORS1* lines. **B.** *RNAi::PRPL11* lines. **C.** *PRORS1* expression in *RNAi::PRORS1* lines. **D.** *PRPL11* expression in *RNAi::PRPL11*.

4. DISCUSSION

During evolution, a part of the organellar genetic material was gradually transferred to the cell nucleus, which nowadays encodes for the majority of organelle proteomes (Bock et al, 2008). Nevertheless, mitochondrion and chloroplast genomes encode for components of the transcription and translation machinery, such as RNA polymerase, ribosomal proteins and initiation factors, and part of the respiratory and photosynthetic complexes. The distribution of the genetic information among the three different compartments requires mechanisms that serve to integrate nuclear and organelle gene expression, including interorganellar signalling and the coordinated expression of sets of nuclear genes coding for organelle proteins (Leister, 2005). These mechanisms comprise both anterograde (nucleus to organelle) and retrograde (organelle to nucleus) signals. Investigations on mitochondrial mutants, such as *cms* (Noctor et al, 2004) or *ncs6* (Jiao et al, 2005) showed that impairments in mitochondrial metabolic activity influence the activities of nucleus and chloroplast. Additionally, the employment of mutants or inhibitors that perturbed chloroplast processes, such as tetrapyrrole biosynthesis, protein synthesis and photosynthesis, showed an influence on the expression of nuclear genes encoding photosynthetic proteins (Kleine et al, 2009; Leister, 2005).

Recently, it was shown that a simultaneous impairment in the organelles translational machinery, due to a mutation in the prolyl-tRNA synthetase PRORS1, targeted to both chloroplast and mitochondrion, induces a specific downregulation of nuclear photosynthetic genes (Pesaresi et al, 2006). However, this marked and general downregulation of nuclear photosynthetic genes was not observed in *mrpl11-1* and *prpl11-1*, impaired in the mitochondrial and plastid ribosome function, respectively, but only in the *mrpl11prpl11* double mutant, indicating that the translation rates in both chloroplast and mitochondria contribute synergistically to the regulation of nuclear gene expression (Kleine et al, 2009; Pesaresi et al, 2006). Despite these findings, primary nuclear target

genes of retrograde signalling (in particular, the pathway that originates with the expression of organelles genes) are still not characterised.
This thesis focuses on the identification of nuclear target genes, unravelling the basics of the translation-dependent-retrograde signalling in *Arabidopsis*.

4.1. The "*Alc* switch" as strategy to investigate retrograde signalling

In this thesis a strategy based on chemically regulated gene expression systems was employed to identify possible candidate regulatory proteins, involved in translation-dependent-retrograde signalling. It is known that loss-of-function mutants are powerful tools to study and define gene function, but they can present severe limitations, such as lethality, sterility or pleiotropic effects. The inducible system approach enables to avoid the deleterious effects of constitutive gene misregulation and allows to distinguish between primary effects of gene expression and long-term secondary effects.
Several chemically inducible systems, suitable for plants, have been described (Moore et al, 2006). For this study it was decided to clone *MRPL11*, *PRPL11* and *PRORS1* under the control of an ethanol inducible element, the pAlcA promoter (Caddick et al 1998, Salter et al 1998, Roslan et al 2001) **(Fig.3.1)**. This system presents a large number of advantages, including inducer low cost and low phytotoxicity (Salter et al, 1998), promoter high sensitivity to the presence of ethanol with negligible levels of back-ground expression in the absence of the inducer (Battaglia et al, 2006).
Another advantage of the *Alc* system is the wide range of inducer application methods, such as foliar sprays, root drenches, liquid growth media and ethanol vapour, which makes the system quite versatile (Li et al, 2005). The induction via ethanol vapour was chosen for the experiments because of its high efficiency in potato and *Arabidopsis*, in both soil and *in vitro* treatments (Knowles et al, 2009; Sweetman et al, 2002; Roslan et al, 2001). Ethanol vapour was also reported as suitable/ideal technique/approach for long induction periods due to the reduced fungal growth on soil (Battaglia et al, 2006). Besides, ethanol vapour treatment seems not to influence photosynthetic performances (**Table 3.1**) or photosynthetic

marker genes expression, like *LHCB1.2* (**Fig.3.3**), even when plants are continuously exposed for 24 hours, confirming that this system could be suitable for the study here described.

Although preliminary tests on *Arabidopsis thaliana* Col-0 plants, carrying the *Alc* cassette, showed a strong increase in gene expression already after 16 hours of treatment with 100% of ethanol vapour, significant differences in overexpression level were noticeable between different lines of the same gene construct (**Fig.3.2**), denoting a possible gene silencing effect. Roslan et al (2001) suggested that the observed gene silencing could be explained with the duplication of the CaMV35S minimal promoter, which is present in the AlcA-derived promoter as well as the full promoter driving AlcR. Anyway, our observations conflicted with this hypothesis, because the frequency and the extent of this effect varied both between and within lines, independently on the cassette orientation, as shown by the time course of p*Alc::MRPL11 mrpl11* in Col-0 background (**Fig.3.2.A**).

4.2. Ethanol induction *in vitro* enables complete or partial *mrpl11-1* and *prpl11-1* phenotype rescue

The crucial point in dissecting a signalling pathway is to distinguish between primary target genes, which are directly regulated, and secondary target genes, which are modulated by the product of the primary target genes. The employment of a short-term time course (i.e. measuring differential gene expression during a time-frame) could improve the possibility of identifying primary target genes. Deveaux et al (2003) and Knowles et al (2009) showed the ability of the *Alc* system in temporally regulating gene expression, using short pulses of ethanol (<24 hours). We chose to test gene expression kinetic of the *Alc* lines *in vitro*, to minimize the inducer evaporation and to have a better control on the system. Similar to what was previously observed in soil experiments with 100% ethanol vapour (Roslan et al 2001), our *in vitro* tests on p*Alc::MRPL11 mrpl11* showed that the system was not functional after 2 hours of exposure to 0.1% inducer, but after 6 hours the expression showed a four-fold increment (**Fig.3.4**). This behaviour could be due to a difficult *cuticle* permeability or to a too low inducer

concentration, which does not allow the ethanol to reach the target site in a short time, confirming the system peculiarity to respond in a dose-dependent manner. This option was tested using higher ethanol concentrations. Unluckily, the increased ethanol concentration was deleterious for the plants, that displayed a chlorosis phenotype (data not shown). Differently from previous findings (Deveaux et al, 2001 and Roslan et al, 2001), our inducible lines did not show any basal expression level when grown *in vitro*: if not exposed to ethanol vapour, p*Alc::MRPL11 mrpl11* line, as well as p*Alc::PRPL11 prpl11* line, displayed an expression level comparable to the knock out mutants (**Fig.3.4** and **Fig.3.7**).
Similar to what was reported by Battaglia et al (2006), under ethanol induction a complete or partial mutant rescue phenotype was observed (**Fig.3.5 and 3.8**) and in our p*Alc::MRPL11 mrpl11* and p*Alc::PRPL11 prpl11* lines this seems to be directly correlated to the amount of detected transcript level (**Fig.3.4 and 3.7**). Rather than a partial gene silencing effect, the incomplete phenotype rescue and the failed gene overexpression in the p*Alc::PRPL11 prpl11* lines could be due to a position effect of the transgene (**Fig.3.7**). The perfect functionality of the lines in Col-0 background chosen to obtained the *Alc* lines seems to endorse this possibility. However, the impossibility to completely rescue a mutant phenotype by the induction was already reported by Waters et al (2009), which showed how the rescued gene expression in *glk* mutants was not accompanied by WT chlorophyll content levels.

4.3. *In vitro Alc::PRORS1* shows phenotype complementation

The high level of transcript in not induced p*Alc::PRORS1 prors1.2* line could not be explained as an auto-induction, due to a possible anoxia phenomenon: in control conditions, knock down *prors1.2* mutant showed a *PRORS1* expression level comparable to the WT, not indicating a direct correlation between the growth condition and gene expression (**Fig.3.9.A**). It is known that even low concentrations of sucrose in agar can influence gene expression (Gibson, 2005) and it can be hypothesised that *PRORS1* is sensible to this influence, but further investigations are needed. Growth of *prors1.2* in culture media without sucrose

and in presence of ethanol vapour exacerbates the already severe mutant phenotype, with a strong delay in seed germination and development (data not shown).

Although phenotype complementation occurs to p*Alc::PRORS1 prors1.2* plants, after induction *in vitro* (**Fig.3.9.B, C and D**), the employment of the *Alc* switch seems to be critical for this genotype: the sterility of *Alc* plants and their severe phenotype, disclosed as growth delay and strong impairment in development, widely limit the possibility to work with this system in soil conditions.

4.4. Temporal analysis of gene expression on soil

Studies carried out on 16-day-old seedlings grown on 0% or 1% sucrose under dark or white-light conditions reported that many genes are regulated by interactions between light and sugar signalling, and that genes involved in metabolism are over-represented among sugar- and light regulated genes (Gibson, 2005). To avoid possible misleading results during gene expression analyses, the induction system was transferred to soil, whose employ is largely documented (Roslan et al, 2001; Laufs et al, 2003; Maizel and Weigel, 2004; Battaglia et al, 2006; Sakvarelidze et al, 2007). Although our work confirmed the applicability of the system in soil, several technical problems were encountered. First of all the need to work in control conditions: since ethanol is a volatile inducer, greenhouse conditions were not optimal for long-term experiments, where plants should be continuously exposed to the inducer for weeks. Also growth chambers did not seem to be the optimal solution for our experiments, because their technical characteristics do not allow the permanent induction for several days. Furthermore, continuous plants induction presents the drawback of fungal growth, which strongly affects plants development and, consequently, gene expression. The employment of *Arabidopsis* biological incubators (see Materials and Methods) overcomes these disadvantages, because temperature, humidity and light are controlled in a confined environment. Furthermore, the air flow permits to avoid possible phytotoxic ethanol effects and limits fungal growth.

As described above, the *Alc* switch strategy seems not to be suitable for p*Alc::PRORS1 prors1.2* lines: their sterility forces to employ heterozygous plants and to select homozygous, but the latter severe phenotype leads to a delay in seed germination of the already few individuals. Furthermore, ethanol application does not lead to *prors1.2* phenotype complementation in the p*Alc::PRORS1* lines which are more strongly affected in growth than *prors1.2* mutant plants. This is due probably to the pleiotropic effects of the mutation or to a too low gene overexpression in the p*Alc::PRORS1* line.

Our in soil experiments confirm *in vitro* findings, showing the complete or partial phenotype complementation (**Fig.3.10**). Furthermore, to quantitate phenotype changes, photosynthetic performance was measured after induction: parameters F_v/F_m, NPQ and ΦII in *Alc* lines show essentially mutant performance (**Fig.3.10**). As reported by Pesaresi et al (2006), *mrpl11* plants show a reduction in the abundance of all mitochondrial electron transport complexes that limits but does not arrest the electron flow. Thus, *mrpl11-1* is still able to support chloroplast activities and displays WT-like photosynthetic performance. This peculiarity is reflected by p*Alc::MRPL11 mrpl11* plants, which basically show WT efficiency. Unlike p*Alc::MRPL11 mrpl11* line, *prpl11*-like photosynthetic performances of the *Alc::PRPL11* plants are the result of a *PRPL11* transcript level substantially lower than the wild type (**Fig.3.12**).

Differential gene expression can be studied from a static or temporal viewpoint. In a static experiment, it is captured only a single moment of gene expression, irrespective of time. In a temporal experiment gene expression is monitored over a time-course, allowing one to study its dynamic behaviour. Because the regulation of gene expression is a dynamic process, it is also important to identify and characterize changes in gene expression over time (Storey et al, 2005). A crucial point in performing a time-course is the choice of the optimal time-points, which has to consider the cellular response speed to a given stimulus, the influence of the circadian rhythm and the specific characteristics of the considered system.

We observe that 8 hours after ethanol removal are still not sufficient to decrease *MRPL11* overexpression in the p*Alc::MRPL11 mrpl11* line (**Fig.3.11**), indicating

that leaves are probably still drenched with inducer, whose release is then slowed down.

Unlike *MRPL11*, the time-course of *PRPL11* in soil shows a rapid decrease in gene expression 3 hours after ethanol removal, probably because the thin leaf structure of p*Alc::PRPL11 prpl11* plants allows a faster release of the inducer (**Fig.3.12**).

4.5. Plastome analysis reveals a compensation attempt of the mutation in loss-of-function mutants

The plastid genome (plastome) is identical in all plastid types and occurs in high copy numbers with up to thousands of genome copies being present in a single cell (Rogalski et al, 2008). Gene expression in chloroplasts is controlled primarily through the regulation of translation. This regulation allows to coordinate expression between the plastid and nuclear genomes, and is responsive to environmental conditions (Manuell et al, 2007). Plastid protein biosynthesis takes place in the stroma on prokaryotic-type 70S ribosomes (Peled-Zehavi and Danon, 2007), whose just few components are encoded by the plastid genome.

We observed that in our loss-of-function and knock down mutants, a considerable part of differentially expressed genes encoded by the plastome was represented by ribosomal proteins (**Fig.3.14**). In particular, the transcript level of the small subunit proteins 14, 15, 16 and 18 of the plastid ribosomes was considerably increased in all genotypes, as an attempt to compensate the organelle translational impairment. This transcriptional change represents then an organelle adaptive response to the nuclear gene mutations caused by the T-DNA insertion and it also confirms the important role of plastid translation for the cellular viability.

Interestingly, *mrpl11-1* showed a similar plastome transcriptional profile, indicating that the impairment in the mitochondrial translational machinery influences chloroplast pathways and activities, results in an increased plastid transcription level. This influence can be attributed to the tight interdependency between plastid and mitochondrion, whose cross-talk signalling mechanism was already hypothesised. In particular, Sabar et al (2000) showed, that if the Complex

I *nad4* gene is affected, like in the *Nicotiana sylvestris nms1* mutant, photosynthesis was 50% reduced, compared to WT, emphasizing mitochondrial influence on photosynthesis.

In general, little variations were noticeable in the transcript level of genes encoding the photosystem II subunits in our loss-of-function and knock down mutants (**Fig.3.14**). In particular, the level of *psbE* transcript, encoding the α-subunit of cytochrome b559 (cyt b559), was highly increased in all genotypes. The incremented transcript level in *prpl11-1* is in contrast with results reported by Pesaresi et al (2001), whose Northern analyses showed a decreased α-cyt b559 expression in this mutant.

Unlike *psbE*, the expression of *psbF*, encoding the β-subunit of cyt b559, showed different expression pattern between the genotypes: while its transcript level remained unchanged in *mrpl11prpl11* double mutant and *mrpl11-1*, it showed a slight increase in *prors1.2* and a significant increment in *prpl11-1* mutant. The expression discrepancy between α and β subunits of cyt b559 and the unchanged transcript level of *psbL* and *psbJ*, contained in the same gene cluster, appears unclear. This might be due to technical problems occurred in performing macroarrays, such as inconsistent sequence fidelity of the spotted macroarrays, variability of differential expression, low specificity of cDNA macroarray probes, discrepancy in fold-change calculation and lack of probe specificity for different isoforms of a gene (Kothapalli et al, 2002). Further experiments have to be performed to clarify the mutants expression pattern and elucidate possible implications in retrograde signalling.

4.6. Chondriome transcriptional profiling and analysis of nuclear-encoded genes involved in mitochondrial activities.

In illuminated leaves, intracellular metabolism is dynamically modulated depending on environmental changes. Under such conditions, the function of chloroplasts and mitochondria is closely coordinated. Photosynthesis fixes atmospheric carbon dioxide and produces carbohydrates, a part of which are

partially catabolized into ATP and reductants by respiration in response to cellular energy demand (Noguchi and Yoshida, 2008).

Mitochondria have their own gene expression system that provides for the transcription and translation of the genes encoded in the mitochondrial genome (Rasmusson and Handa, 2008). Similar to what observed for plastid encoded genes, our chondriome transcriptional profiling in knock out and knock down mutants did not show a clear expression tendency (**Fig.3.15**).

As reported by Pesaresi et al (2001), in *prpl11-1* mutant the decrease in the levels of PSI, PSII core proteins, LHCII, Cyt f, the α- and β-subunits of the ATPase complex, and both subunits of Rubisco, resulted in a reduced capacity for light utilisation and an increase in photosensitivity. The increased transcript level of alternative oxidase (AOX) in this mutant could be an attempt to dissipate reductants accumulated by the chloroplast through the non-phosphorylating pathway. In contrast with what described by Pesaresi et al (2006), the transcript level of AOX did not show any significant change in *mrpl11-1* and *prors1.2* mutants. This result discrepancy could be due to the different method of analysis employed to monitor gene expression.

4.7. Transcriptional profiling of *prpl11-1* mutant as starting point to identify candidate genes in ethanol inducible lines.

The *prpl11-1* mutant shows pale green pigmentation of the leaves and a drastic reduction in growth rate under greenhouse conditions (Pesaresi et al, 2001), which makes the plant more affected than the mitochondrial counterpart *mrpl11-1*, but its phenotype is less severe than that of the *prors1.2* mutant. For this "intermediate phenotype", *prpl11-1* was chosen to perform a full expression profiling.

The transcriptional profiling showed that even if 30% of the products of the significantly differentially expressed genes were located to the chloroplast, just 2 of this 18 genes were directly involved in photosynthesis (**Fig.3.16.A and B**). The other genes belonging to this group were involved in different organelle processes, like stress response, various biosynthetic pathways and protein

synthesis and processing. Interestingly, the expression of the 18 genes whose product was related to photosynthesis or chloroplast activities was upregulated.
In particular, the most upregulated gene was *At2g40100*, encoding a protein quite similar to, but shorter than, Lhcb4, identified by Jansson (1999) and denoted Lhcb4.3. Its expression pattern varies significantly from *Lhcb4.1* and *Lhcb4.2* and usually it is not abundantly expressed in experimental conditions (Klimmek et al, 2006). The function of this protein is not clarified yet, but it was suggested to influence antenna characteristic in stress conditions (Klimmek et al, 2006).
Among the 42 upregulated genes (**Table.3.5**), several were known to be part of metabolic pathways, like *At5g08640*, *At5g13930* and *At5g05270*, encoding flavonol synthase I, chalcone synthase and chalcone-flavanone isomerase family protein, respectively: these genes are part of the flavonoid biosynthesis pathway.
The redox state of the photosynthetic electron transport chain and levels of ROS, which are continuously produced as by-products of photosynthesis when light intensities exceed photosynthetic capacity, have also been proposed as factors that activate plastid signalling (Kleine et al, 2009). In *prpl11* transcriptional profiling we observed an increase in the transcript level of three genes involved in response to reactive oxygen species (ROS), caused by oxidative stress: *At4g31870*, *At4g08390* and *At3g56090*, encoding glutathione peroxidase (AtGPX7), stromal L-ascorbate peroxidase (sAPX) and a putative ferritin (FER3). It was shown that in *Arabidopsis fluorescent* (*flu*) mutant, O_2^- and H_2O_2 induce specifically nine genes, including ascorbate peroxidase I (APXI) and FERRITIN1 genes (op den Camp et al, 2003), indicating that ROS can modulate nuclear gene expression.
Other four genes upregulated in *prpl11* are involved in the epigenetic control of the expression of other genes: fibrillarin2 (FIB2), histone deacetylase 2 (HD2A), nucleolar protein 56 (NOP56) and a putative DEAD/H box helicase. FIB2 is a key nucleolar protein for pre-rRNA processing conserved from vertebrates to archaebacteria (Barneche et al, 2000), whose activity of rRNA genes methylation, together with NOP56, is an important component of the regulatory network that controls the effective dosage of active rRNA genes (Lawrence and Pikaard, 2004). Together with fibrillarin, another protein involved in RNA processing mechanism, DEAD/H box helicase, which was reported to be essential in all aspects of RNA

metabolism at the level of expression and at the post-transcriptional level for pre-messenger RNA splicing and translation (Pause and Sonenberg, 1993; Aubourg et al., 1999). HD2A is shown to play an essential role in epigenetic control of gene expression: in particular it was reported that in plants this protein can mediate gene repression through interaction with transcription factors, such as Pti4 (Wu et al, 2003).

Furthermore, we noticed also among our genes the presence of a gene encoding a protein belonging to the family of pfkB-type carbohydrate kinases (PFKB1): Oswald et al (2001) reported that the PFKB1 protein might couple the expression of photosynthesis genes to sucrose signals in plant cells, confermed also by Pfalz et al (2006), who suggested that this protein, in association with Fe-SOD and thioredoxin, might ensure the regulation of the transcriptionally active plastid chromosome (pTAC) in response to various signals.

The expression of five upregulated genes was checked in the other translation impaired mutants: in particular, the transcript level of *At3g44750*, *At1g69200*, *At4g25630* and *At2g40750* was monitored (**Fig.3.17**). These genes were chosen on the basis of their localisation, in the nucleus in the nucleolus, and/or of their function. Besides also the gene expression of *At2g40100*, encoding Lhcb4.3, was checked. Amongst the tested genes, just *At1g69200* (PFKB1) and *At3g44750* (HD2A) displayed an increase in the transcript level in all loss-of-function or knock down mutants, indicating a possible involvement in nucleus-organelle signalling.

In the time course performed for *At1g69200* and *At3g44750* genes in *Alc::MRPL11* line, their transcript level did not show a direct relation with *MRPL11* expression (compare **Fig.3.18** to **Fig.3.11**). Furthermore, the gene expression trend seems to be influenced by the circadian rhythm, as also suggested by the data available in the BAR database.

4.8. Inducible gene silencing is a powerful tool to distinguish primary from secondary side effects.

Employment of overexpressing *Alc* lines in gene expression time courses poses the problem of phenotype rescue. In fact, to minimise the secondary side effects, caused by the T-DNA insertion in the mutant genome, it was necessary to reach the WT phenotype: at this point the inducer was removed and the gene expression decrease was monitored over the time. Inducible gene silencing is shown to be a powerful tool in dissecting signalling pathways (Waters et al, 2009), because it circumvents this problem and reduces experimental times.

The *PRPL11* and *PRORS1*expression time-courses performed on our estradiol inducible lines show that the system is suitable for short-term time courses: *PRORS1* and *PRPL11* gene expression decreases constantly for three and 16 hours after 17- β-estradiol spraying, respectively (**Fig.3.20**). Because of the short time range considered in the experiments, gene expression decrease is not followed by a decrease in photosynthetic parameters, which remain unaltered (**Table 3.6**). Furthermore, inducer application does not influence photosynthetic performances or photosynthesis gene expression (**Table 3.7 and Fig.3.21**).

However, our estradiol inducible lines show a certain grade of instability in the transcript level decrease: repeated time course experiments reveal that *PRORS1* and *PRPL11* expression decrease is very variable between biological replicates (compare **Fig.3.22 to Fig.3.20**). This behaviour is not attributable to a defect in the construct design, as shown by constitutive RNAi lines. These lines are generated cloning the same GST sequence under control of 35S promoter, which drives the constitutive silencing of *PRORS1* and *PRPL11*: with small differences due to position effects, transcript level of all lines decreases, resulting in the respective knock down and knock out mutant phenotype (**Fig.3.23**).

Taken together, these results suggest the suitability of the “Alc switch” system as a tool to investigate retrograde signalling: due to its versatility in the different experimental conditions employed, this system seems to be optimal for both short-

and long term gene expression studies. However, the use of the overexpressing construct that was used in these studies is not fully satisfying. Even the inducible overexpression of *MRPL11* and *PRPL11* in the generated transgenic lines was completely or partially achieved, encountered challenges appeared. To mimic wildtype conditions by protein overexpression requires a very accurate adjustment in the established system and to choose the time-points of sampling needs to be determined individually.

The problems described above suggest the employment of an ethanol RNAi system as a more convenient tool. In fact, regulated post transcriptional gene silencing (PTGS) could overcome the problems related to the initial phenotype recovering and the following selection of the candidate target genes.

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Ehrenwörtliche Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe. Der Autor hat zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder sich einer Doktorprüfung zu unterziehen. Die Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Ganzes vorgelegt.

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