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Mutation of the pentatricopeptide repeat-SMR protein SVR7 impairs accumulation and translation of chloroplast ATP synthase subunits in *Arabidopsis thaliana*

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Abstract RNA processing, RNA editing, RNA splicing and translational activation of RNAs are essential post-transcriptional steps in chloroplast gene expression. Typically, the factors mediating those processes are nuclear encoded and post-translationally imported into the chloroplasts. In land plants, members of the large pentatricopeptide repeat (PPR) protein family are required for individual steps in chloroplast RNA processing. Interestingly, a subgroup of PPR proteins carries a C-terminal small MutS related (SMR) domain. Here we analyzed the consequences of mutations in the *SVR7* gene, which encodes a PPR-SMR protein, in *Arabidopsis thaliana*. We demonstrate that *SVR7* mutations lead to a specific reduction in chloroplast ATP synthase levels. Furthermore, we found aberrant transcript patterns for ATP synthase coding mRNAs in *svr7* mutants. Finally, a reduced ribosome association of *atpB*/*E* and *rbcL* mRNAs in *svr7* mutants suggests the involvement of the PPR-SMR protein SVR7 in translational activation of these mRNAs. We describe that the function of SVR7 in translation has expanded relative to its maize ortholog ATP4. The results provide evidence for a relaxed functional conservation of this PPR-SMR protein in eudicotyledonous and monocotyledonous plants, thus adding to the knowledge about the function and evolution of PPR-SMR proteins.

Keywords Arabidopsis · Chloroplast · Gene expression · Pentatricopeptide repeat (PPR) protein · SMR domain · Translation

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Abbreviations

Col	Columbia (Arabidopsis accession)
Cyt b_6f	Cytochrome b_6f complex
GUN	Genomes uncoupled
Ler	Landsberg erecta (Arabidopsis accession)
LHC	Light harvesting complex
NF	Norflurazon
PPR	Pentatricopeptide repeat (protein domain)
PSI/II	Photosystem I/II
RT-PCR	Reverse transcription PCR
SMR	Small MutS related (protein domain)
WT	Wild-type

Introduction

Chloroplasts are endosymbiotic descendants of previously free living cyanobacteria (Margulis 1970). During co-evolution of host and endosymbiont, chloroplasts lost most of their genetic autonomy by gene transfer into the nuclear genome (Bock and Timmis 2008; Kleine et al. 2009; Martin 2003).The remaining chloroplast genes essentially code for parts of a bacterial type gene expression

machinery and proteins relevant for photosynthesis. However, the overwhelming majority of proteins found in chloroplasts is nuclear encoded and post-translationally imported necessitating coordinated gene expression in both the nuclear and the plastid compartments.

Compared to cyanobacteria, gene expression in chloroplasts exhibits a surprisingly complex RNA metabolism. Group I and -II intron splicing, RNA editing, 5′-, 3′-end trimming and intracistronic RNA processing are common events in chloroplast RNA maturation (Barkan 2011; Stern et al. 2010). Additionally, chloroplast gene expression is translationally regulated (Barkan 2011; Wobbe et al. 2008). Chloroplast RNA metabolism and translational regulation are realized by nuclear encoded factors (Barkan 2011). Among those factors, the pentatricopeptide repeat (PPR) protein family is outstanding in land plants because of the large number of its members (~450 in Arabidopsis) and since PPR proteins fulfill specific functions in several steps of chloroplast RNA maturation and translation processes (Schmitz-Linneweber and Small 2008). Most PPR proteins are targeted to either chloroplasts or mitochondria and their sequences are highly conserved among land plants (Lurin et al. 2004; Schmitz-Linneweber and Small 2008). PPR proteins are composed of tandem arrays of degenerated repeating units of ~35 amino acids. These tandem arrays are predicted to form helical hairpin structures that may assemble into an alpha solenoid superstructure (Delannoy et al. 2007; Small and Peeters 2000). PPR proteins bind RNA in vitro and associate with RNA in vivo (Beick et al. 2008; Gillman et al. 2007; Hammani et al. 2011; Hattori et al. 2007; Okuda et al. 2006; Pfalz et al. 2009; Prikryl et al. 2011; Schmitz-Linneweber et al. 2005; Tasaki et al. 2010; Williams-Carrier et al. 2008; Zoschke et al. 2012). PPR domains themselves are not catalytically active but guide enzymatically active domains, enzymes or ribonucleoprotein complexes to specific sequences where they perform their essential functions in organellar RNA metabolism (Delannoy et al. 2007; Schmitz-Linneweber and Small 2008).

PPR proteins can carry additional protein domains: A large subclass of PPR proteins contains a C-terminal "E" domain that is linked to organellar RNA editing (Zehrmann et al. 2011). PPR4, which contains an RNA recognition motif, and OTP51, a PPR protein with a LAGLIDAG domain typically found in homing endonucleases are involved in chloroplast splicing (de Longevialle et al. 2008; Schmitz-Linneweber et al. 2006). Furthermore several PPR proteins carry a small MutS related domain (SMR) at the C-terminus (Liu et al. 2010).

The SMR domain was originally identified as the C-terminal part of bacterial MutS2 proteins (Moreira and Philippe 1999). Bacterial SMR domains have DNA binding and endonuclease activity and act in DNA recombination and repair processes (reviewed in Fukui and Kuramitsu 2011). It was also speculated that SMR domains could function in transcription (Fukui and Kuramitsu 2011). The function of SMR domains in PPR proteins is unknown.

Out of the eight Arabidopsis PPR-SMR proteins three were analyzed experimentally so far. pTAC2 and its tobacco ortholog were found in transcriptionally active chloroplast extracts (Pfalz et al. 2006; Suzuki et al. 2004). Arabidopsis *ptac2* mutants show a strongly reduced accumulation of transcripts generated by the plastid encoded RNA polymerase (PEP) suggesting a role of pTAC2 in PEP transcription (Pfalz et al. 2006). GUN1, another PPR-SMR protein was identified in a screen for mutants with impaired retrograde chloroplast to nucleus signaling (Koussevitzky et al. 2007). Retrograde signals are elicited by changes in chloroplast physiological parameters like the photosynthetic redox status, or the accumulation of reactive oxygen species, but also by changes in chloroplast gene expression. The nucleus responds by adapting its gene expression to the requirements of the signaled plastid state. This assures a concerted gene expression in both compartments (Woodson and Chory 2008). The mutation of GUN1 disrupts several of the retrograde signaling pathways indicating a central function of this PPR-SMR protein in retrograde signaling (Koussevitzky et al. 2007). The third experimentally analyzed PPR-SMR protein is the Arabidopsis SUPPRESSOR OF VARIEGAION7 (SVR7) protein and its radish and maize orthologs P67 and ATP4, respectively (Echeverria and Lahmy 1995; Lahmy et al. 2000; Liu et al. 2010; Zoschke et al. 2012). The *SVR7* mutation suppresses the variegated phenotype of *var2* mutants by an unknown mechanism (Liu et al. 2010). The knockout of its maize ortholog ATP4 leads to a specific reduction of *atpB/E* and *atpA* translation causing the loss of the chloroplast ATP synthase (Zoschke et al. 2012). A more general reduction in chloroplast protein accumulation and a less severe effect on ATP synthase accumulation in the Arabidopsis *svr7* mutant (Liu et al. 2010) suggested that the functions of the putative orthologs in Arabidopsis and maize differ.

To get a more detailed insight in the potential functional diversification of PPR proteins during evolution, we analyzed in the current study the effects of mutations of the PPR-SMR gene *SVR7* on chloroplast gene expression in two *Arabidopsis thaliana* ecotypes. Here we show that *SVR7* mutations in Arabidopsis cause a specific, albeit less drastic reduction in chloroplast ATP synthase accumulation compared to *atp4* null mutants in maize. Additionally, a reduced ribosome association of *atpB*/*E* mRNAs was found in *svr7* mutants. Different from *atp4* mutants, *SVR7* mutations lead in addition to an impaired *rbcL* translation. Further commonalities and differences found in the accumulation of other proteins of the thylakoid membrane and

in chloroplast transcription and transcript accumulation are discussed in respect to findings in previously described mutants of the mono- and eudicotyledonous ATP4/SVR7/P67 orthologs (Lahmy et al. 2000; Liu et al. 2010; Zoschke et al. 2012).

Materials and methods

Plant material

SVR7 T-DNA insertion and transposon lines were ordered from the Nottingham Arabidopsis Stock Center and the Cold Spring Harbor Laboratory, respectively (seed stocks: CS819547 and GT20858). The locations of insertions in *SVR7* were determined by sequencing. Plants homozygous for the insertions were identified by PCR based genotyping (primers see Table 1). Wild-type tissue was obtained from the genotyped wild-type offspring of heterozygous *SVR7* mutant lines.

Plants were grown in a soil vermiculite mixture (4:1) in a growth chamber with long-day regime (16 h light [130 µmol m^{-2} s^{-1}], 23 °C/8 h dark, 23 °C). Leaf tissue was harvested 30 days after sowing.

Norflurazon treatment was essentially carried out as described by Koussevitzky et al. (2007). Plants were grown on Murashige and Skoog medium supplemented with sucrose (3 %) and with or without Norflurazon (5 µM) for 8 days with long day regime (see above) before harvesting (Murashige and Skoog 1962).

Protein analyses

Proteins were isolated as described by Barkan (1998). Protein concentrations of leaf extracts were determined by protein assay measurements following manufacturer's instructions (Bio-Rad). Proteins were size fractionated by SDS-PAGE (12 % gels), blotted onto mixed ester nitrocellulose membranes, and immuno-probed (Sambrook and Russell 2001). Antibodies used in this study were described by Roy and Barkan (1998). The quantification of immunological analyses was described previously (Zoschke et al. 2010).

Polysome analyses were carried out as described by Barkan (1993). PCR probes for polysome RNA gel blot analyses were amplified with primers listed in Table 1.

RNA analyses

RNA was extracted from leaf tissue with TRIzol reagent (Invitrogen) following manufacturer's instructions. RNA gel blot analyses were achieved as described previously (Beick et al. 2008). PCR probes were amplified with primers given in Table 1. Labeling of PCR probes was performed with [α-^{32}P]dCTP using the DecaLabel DNA labeling Kit (Fermentas) following manufacturer's instructions. Run-on analyses were carried out as described (Zoschke et al. 2007; Zubo et al. 2011). 5×10^8 chloroplasts were lysed and used for in vitro transcription reactions at 25 °C for 10 min. Primers used for macro-array probe amplification are shown in Table 1. For reverse transcription reactions, the QuantiTect Rev. Transcription Kit (Qiagen) was used following manufacturer's instructions.

Results and discussion

SVR7 mutation impairs chloroplast biogenesis and causes reduced ATP synthase levels in the *Arabidopsis thaliana* ecotypes Columbia and Landsberg erecta

Arabidopsis Ds-transposon and T-DNA lines (generated in Landsberg erecta and Columbia accessions, respectively) with insertions in the *SVR7* coding region were identified in public seed banks by database searches. The analyzed *svr7* mutant alleles carry insertions upstream of the region coding for the ten PPR domains and within the sequence of the C-terminal SMR domain and are named *svr7*-2 and *svr7*-3 (Fig. 1a), respectively. *svr7*-1 represents a previously described mutant allele of *SVR7* that leads to truncation of the reading frame within the PPR tract (Liu et al. 2010). Inbred offspring of lines carrying the mutant alleles *svr7*-2 and *svr7*-3, respectively, were PCR-genotyped and the locations of the insertions were determined by sequencing (data not shown, Fig. 1a). *svr7* mutants homozygous for the alleles *svr7*-2 and *svr7*-3 are slightly pale green and developmentally retarded (Fig. 1b). However, these *svr7* mutants are viable and fertile. Loss of *SVR7* expression was confirmed by random primed reverse transcription and subsequent PCR amplification of the *SVR7* mRNA (Fig. 2). *SVR7* cDNA amplifications with primers spanning the insertions yielded a product of the expected size in wild-type derived material. No product was detected with cDNAs derived from homozygous mutants. A control amplification with actin-mRNA specific primers demonstrated successful cDNA synthesis (Fig. 2). Contamination with genomic DNA was excluded by control reactions without reverse transcriptase (Fig. 2). Weak amplification signals were observed in the *svr7* mutants using amplification primers up- or downstream of the insertion, which could represent transcripts originating from promoters within the insertions. In any case, both insertions disrupt the *SVR7* reading frame and generate premature stop codons at protein positions 144 and 632 downstream of the start codon, respectively.

Table 1 List of primers used in this study

Oligonucleotide	Sequence (5′ → 3′)	Target	Purpose
LB1	gcctttcagaaatggataaatagccttgcttcc	T-DNA	Genotyping, sequencing
SAIL fw	gaggaaatcggtaaactcattggctgtgtcg	*SVR7*	Genotyping, sequencing, RT-PCR
SAIL rev	ccttatctggggcttcatggaaaggagc	*SVR7*	Genotyping, sequencing
CSHL fw	taattcatgaccctcttcctctctgcaatc	*SVR7*	Genotyping, sequencing
CSHL rev	ggtaacattgtagagaatcacttccctg	*SVR7*	Genotyping, sequencing
Ds5-1	gaaacggtcgggaaactagctctac	DS transposon	Genotyping, sequencing
Ds3-2	cgattaccgtatttatcccgttc	DS transposon	Genotyping, sequencing
At-atpErev	caaatcgtattgagagcctcgac	*atpE*	Northern
At-atpEfw	ctgactccgaatcgaattgtttgg	*atpE*	Northern
At-atpH fw	atgaatccactggtttctgctgc	*atpH*	Northern
At-atpH rev	cgctaatgctacaaccaggcc	*atpH*	Northern
At-atpFEx2 fw	agaagaactgcgtgaaggagc	*atpF*	Northern
At-atpFEx2 rev	ctttcatcgtaccaaacatccc	*atpF*	Northern
At-atpFIn fw	gaaagagtgcataatcccgcg	*atpF intron*	Northern
At-atpFIn rev	cccttcccgaaccaaacatg	*atpF intron*	Northern
At-psaJ-fw	ggtactaagtactctatggttcgg	*psaJ*	Northern
At-rpl33rev	cccgcggcttctttcttaatatc	*psaJ*	Northern
AtrbcL fw	caagtgttgggttcaaagctggtg	*rbcL*	Northern
AtrbcL rev	agacgtagagcagccagggc	*rbcL*	Northern
PPR52-GFP-Seqrev1	ggtaacattgtagagaatcacttccctg	*SVR7*	RT-PCR
PPR52-GFP-fwneu	taattcatgacatttcatcatctatgttcctctccc	*SVR7*	RT-PCR
GABI_779E07_rev1	caaacatcagcttcgtttggcttac	*SVR7*	RT-PCR
PPR52-GFP-Seqfor3	cggacgacagattttgtggg	*SVR7*	RT-PCR
PPR52-GFPSeqrev3	caccggattcaagagccgc	*SVR7*	RT-PCR
ActAtcDNAfw	cttaccgaggctcctcttaacc	*Actin8*	RT-PCR
ActAtcDNArev	ctgctgttgtggtgaacatgtaac	*Actin8*	RT-PCR
5′ AtatpB	aggtcctgtcgatactcgca	*atpB*	Run-on
3′ AtatpB	atctaaaggatctaccgctggata	*atpB*	Run-on
5′ AtatpF	gattctttcgtttacttgggtcac	*atpF*	Run-on
3′ AtatpF	tttaatatcctctgctttcggttatc	*atpF*	Run-on
5′ AtatpH	tttctgctgcttcggttattg	*atpH*	Run-on
5′ AtatpH	gctaatgctacaaccaggccata	*atpH*	Run-on
5′ AtpetB	tagtaaatatgttcctccgcatgtc	*petB*	Run-on
3′ AtpetB	gacggccgtaagaagaggtaat	*petB*	Run-on
5′ AtpsbA	acttctgtttttattatcgcattcattg	*psbA*	Run-on
3′ AtpsbA	tccataccaaggttagcacgg	*psbA*	Run-on
At psbD fw	gtagcggctatatttcgattcatcc	*psbD*	Run-on
At psbD rev	gccatccaagcacgaatacct	*psbD*	Run-on
At_rbcL 2 for	atatcttggcagcattccgagtaact	*rbcL*	Run-on
At_rbcL 2 rev	agtatttgcggtgaatccccc	*rbcL*	Run-on
5′ Atrrn16	attgggcgtaaagcgtctgta	*rrn16*	Run-on
3′ Atrrn16	gtaacgacttcgggcatgg	*rrn16*	Run-on
At_trnK-ex1 for	acatcaaaataagattgtaccgatcag	*trnK*	Run-on
At_trnK-ex1 rev	tgacaacagtgtatggaccaaatataa	*trnK*	Run-on
for-cbb203	cgccacaaccatctcttttt	*cbb203*	Run-on
rev-ccb203	gcgaacacagtggtctctga	*cbb203*	Run-on
Atrrn23fw	cccagagacgaggaagggc	*rrn23*	Run-on
Atrrn23rev	gccccatgctactcgggtc	*rrn23*	Run-on
25 5′At18S	aaacggctaccacatccaag	*rrn18*	Run-on
26 3′At18S	actcgaaagagcccggtatt	*rrn18*	Run-on
At-atpErev	caaatcgtattgagagcctcgac	*atpE*	Run-on
At-atpEfw	ctgactccgaatcgaattgtttgg	*atpE*	Run-on
AtpsaJ-fw	ggtactaagtactctatggttcgg	*psaJ*	Run-on
rpl33rev	cccgcggcttctttcttaatatc	*psaJ*	Run-on
CAB3_fw	cctccacaatggctctctcctcc	*LhcB1.2*	Northern
CAB3_rev	cccatgcgttgttg	*LhcB1.2*	Northern

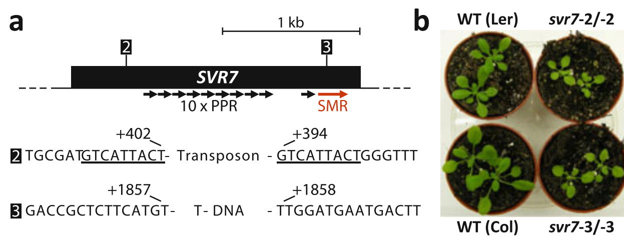


Fig. 1 Overview of *svr7* mutant alleles. **a** Map of the *SVR7* gene. The positions of the Ds transposon and T-DNA insertion in each of the two analyzed mutant alleles are indicated above the map (2 and 3, respectively). The DNA sequences flanking the insertions are given below. The 9-bp target site duplication generated by the Ds-insertion is underlined. Residue numbers indicate the positions relative to the start codon of *SVR7*. *svr7*-3 contains two inversely oriented T-DNAs at the shown insertion site. **b** Phenotype of plants of the indicated genotypes that were grown in soil in a growth chamber for 3 weeks (WT = wild-type). Note that Ds transposon lines are generated in an *Arabidopsis thaliana* Landsberg erecta background (Ler) whereas T-DNA insertion lines are generated in an *Arabidopsis thaliana* Columbia background (Col). Homozygous *svr7* mutants of both alleles show a slightly pale green and developmentally retarded phenotype in comparison to wild-type plants obtained from the offspring of heterozygous mutants

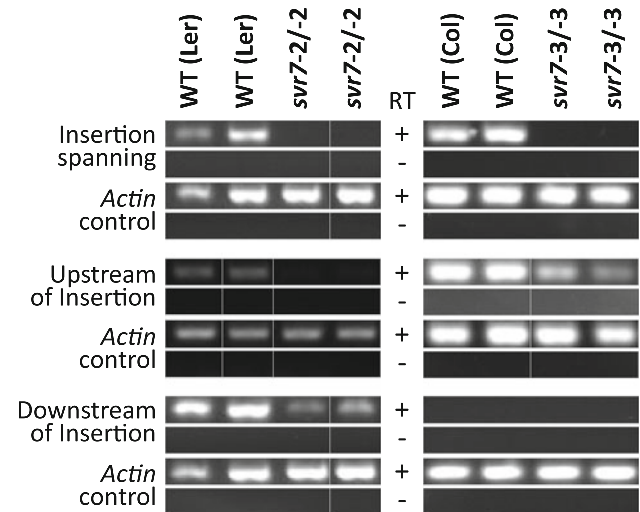


Fig. 2 Reverse-Transcription PCR analysis of *SVR7* mRNA accumulation in *svr7* mutant alleles. 2 µg total leaf RNA from 30 days old plants of the indicated genotypes were reverse transcribed with a random primed Reverse Transcriptase (RT +). The resulting cDNA was PCR amplified (30 cycles) with primers located within the *SVR7* cDNA (location as indicated, for primer sequences see Table 1) and size fractionated by agarose gel electrophoresis. The *Actin8* cDNA was amplified to control for the efficiency of the reverse transcription reaction. To check PCR reactions for genomic DNA contaminations, mock controls without Reverse Transcriptase (RT −) were carried out as well. All lanes come from the same gel; irrelevant lanes are removed from the image (vertical lines). For all analyzed regions within the *SVR7* mRNA *svr7* mutants accumulate lower amounts of the *SVR7* transcript compared to the respective wild-type plants (WT = wild type, Ler = Landsberg erecta, Col = Columbia). No amplification products could be obtained downstream of the *svr7*-3 T-DNA insertion site with cDNA derived from wild-type and mutant tissue

SVR7 and its radish and maize orthologs P67 and ATP4 were previously shown to be chloroplast localized (Lahmy et al. 2000; Liu et al. 2010; Lurin et al. 2004; Zoschke et al. 2012). ATP4 is involved in the expression of chloroplast encoded ATP synthase subunits (Zoschke et al. 2012). Thus the accumulation of the ATP synthase and the other photosystem complexes in *svr7* mutants was analyzed by quantitative immunoblot analysis of the representative chloroplast encoded subunits AtpA/-B/-E/-F (ATP synthase), PsaD (photosystem I, PSI), D1 (photosystem II, PSII), and PetD (cytochrome b_6f complex, Cyt b_6f). In *svr7* mutants a distinct and strong reduction in the accumulation of ATP synthase subunits A, B, F, and E to ~50 to ~10 % of wild-type levels was found (Fig. 3). In contrast PsaD and D1, representative subunits of PS I and II, were only slightly reduced in *svr7* mutants (to ~80–90 % of wild-type levels) and the accumulation of PetD, a component of the Cyt b_6f complex was not affected by *SVR7* mutation. A slight decrease of the large subunit of Rubisco is indicated by Ponceau-stains of Western blots (Fig. 3). The specific reduction of the ATP synthase accompanied by minor or no effects on the other photosystem complexes (PSII, PSI, Cyt b_6f) was also observed in knockouts of the SVR7 ortholog ATP4 in maize (Zoschke et al. 2012). The ATP synthase reduction accounts for the defect in chloroplast biogenesis in maize *atp4* mutants and could also cause the observed slightly impaired chloroplast biogenesis in Arabidopsis *svr7* mutants. These data are at variance with the semi-quantitative x-ray film measurements presented previously for the *svr7*-1 mutant allele, which show a reduced accumulation of AtpA, D1, PsaF, and RbcL to ~50 % of wild-type levels, without a specific effect on the ATP synthase (Liu et al. 2010). The mutant allele *svr7*-1 is chemically induced and contains amino acid exchanges and a premature stop codon within the third PPR domain (Liu et al. 2010), which should cause a knock out of SVR7 function, similar to mutations in *svr7*-2 and -3 alleles. Arabidopsis plants used for analysis of the *svr7*-1 mutant allele were grown in constant light (100 µmol m^{-2} s^{-1}) whereas plants used in this study were grown under a long day regime [16 h light (130 µmol m^{-2} s^{-1})/8 h dark]. It is known that an impaired chloroplast ATP synthase activity gives rise to acidification of the thylakoid lumen since the proton gradient generated by PSII and Cyt b_6f is not used (Maiwald et al. 2003; Rott et al. 2011). This acidification provokes the down-regulation of the expression of nuclear and plastid encoded photosystem components (Maiwald et al. 2003; Rott et al. 2011). Thus, a primary effect of *SVR7* mutation on chloroplast ATP synthase accumulation could entail secondary effects on the accumulation of other

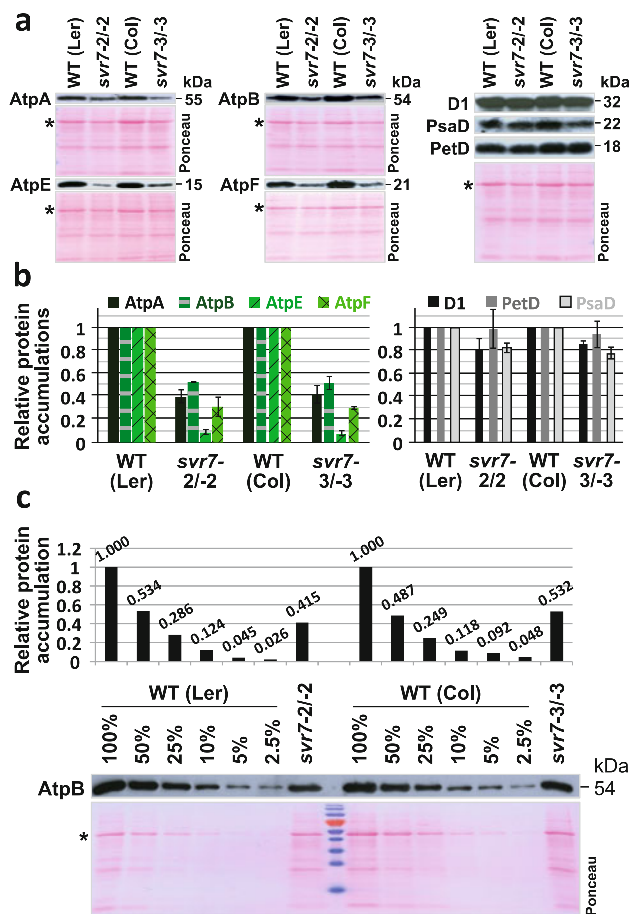


Fig. 3 Quantitative immunoblot analysis of subunits of photosynthetic enzyme complexes. **a** 10 µg of total leaf protein extracts from plants of the indicated genotypes were analyzed by immunoblot assays (WT = wild type, Ler = Landsberg erecta, Col = Columbia). The blots were probed with antisera to four subunits of the chloroplast ATP synthase (AtpA, -B, -E, -F) and also with antisera to representative subunits of photosystem I (PsaD), photosystem II (D1), and the cytochrome b_6f complex (PetD). Bottom: Ponceau S stains of the blots served as loading controls (RbcL marked by asterisks). Note that the Ponceau stained blots here and in **c** show a reduction of the large subunit of Rubisco (RbcL) in *svr7* mutants. **b** The chemiluminescence signals from the analysis shown in a and from two biological replicate experiments were recorded using a Lumi-Imager (Roche) and quantified using the Quantity One software (Bio-Rad). The signals for wild-type samples were set to one and the means of the mutant signals were calculated and plotted relative to this wild-type signal. Error bars are based on three biological replicates. **c** Dilution series of wild-type and *svr7* mutant protein extracts were analyzed side-by-side with an AtpB specific antiserum. Signals were quantified as described in **a** to verify the quantitative protein gel blot analyses shown in **a** and **b**. Upper panel Quantification of the chemiluminogram shown below. The undiluted wild-type samples were set to 1.0. Quantified signals from diluted samples correspond well with the dilution factor. Only at low sample concentrations (<10 %) is appreciable variation from the calculated values observed. Bottom: Ponceau S stain (RbcL marked by asterisks)

photosystem complexes under conditions with continuous illumination as used for growing *svr7*-1 mutants (Liu et al. 2010) and might thus explain the observed differences in the accumulation of chloroplast proteins between the previous and the current study. Future comparative studies analyzing the available *svr7* mutants under different light conditions may help to further clarify this question.

Mild effects of *SVR7* mutation on the accumulation of chloroplast *atpF*, *atpH*, and *psaJ* transcripts

The SVR7 maize ortholog ATP4 is involved in processing of *atpF* and *psaJ* transcripts (Zoschke et al. 2012). To check for a similar function of SVR7 and find the reason for the reduction of ATP synthase subunits in *svr7* mutants, the transcript accumulations of *atpB*/*E*, *atpH*, *atpF*, and *psaJ* were determined by RNA gel blot analysis (Fig. 4). In Arabidopsis the dicistronic *atpB*/*E* transcript originates from two promoters upstream of the *atpB* coding region, and additionally, a monocistronic *atpE* mRNA is transcribed from a promoter within *atpB* (Malik Ghulam et al. 2012; Schweer et al. 2006). In RNA gel blot hybridization experiments, a slight over-accumulation of the large dicistronic *atpB*/*E* transcript over other transcripts in the same sample was found in *svr7* mutants (asterisk in Fig. 4). This resembles the *atpB*/*E* over-accumulation detected in maize mutants of the SVR7 ortholog ATP4 (Zoschke et al. 2012).

In plant chloroplasts, *atpI*, -*H*, -*F*, and -*A* are encoded in one operon, which is transcribed from several promoters and the derived polycistronic transcripts undergo an extensive processing to shorter units (Malik Ghulam et al. 2012; Miyagi et al. 1998; Pfalz et al. 2009; Stahl et al. 1993; Swiatecka-Hagenbruch et al. 2007; Zhelyazkova et al. 2012). RNA gel blot analyses with *atpH*- and *atpF*-specific probes revealed changes in steady-state levels of these transcripts in *svr7* mutants (Fig. 4). A reduction of a ~1.5 kilonucleotide (knt) transcript representing the monocistronic unspliced *atpF* mRNA was detected with *atpF* exon- and intron-specific probes. The knockout of the SVR7 ortholog ATP4 in maize affects also *atpF* transcript accumulation. In *atp4* mutants all transcripts with 3′ ends in the *atpF*/-*A* intergenic region accumulate to lower levels (Zoschke et al. 2012). By contrast, *svr7* mutants display an increase in several *atpH* and *atpF* containing transcripts (Fig. 4).

PsaJ is a small non essential subunit of PSI which is encoded in an operon together with *petL*, *petG*, *rpl33*, and *rpl18* (Pfalz et al. 2009; Schöttler et al. 2007). RNA gel blot analyses showed an over-accumulation of different *psaJ* containing transcripts in *svr7* mutants (Fig. 4). Interestingly, the processed monocistronic *psaJ* mRNA, which is strongly reduced in maize *atp4* mutants (Zoschke et al. 2012) is increased in *svr7* mutants.

Altogether, the detected processing defects caused by *SVR7* mutation are mild and most likely not the only reason

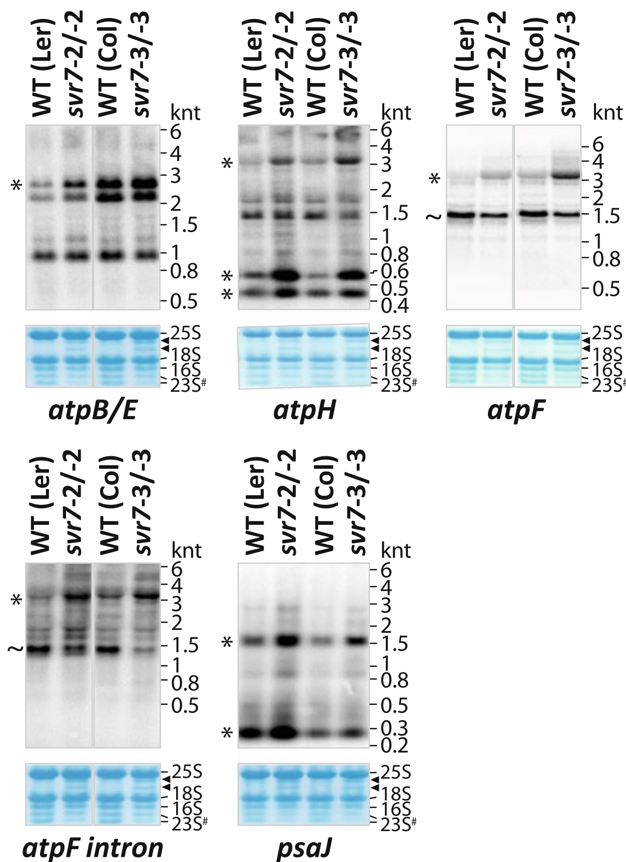


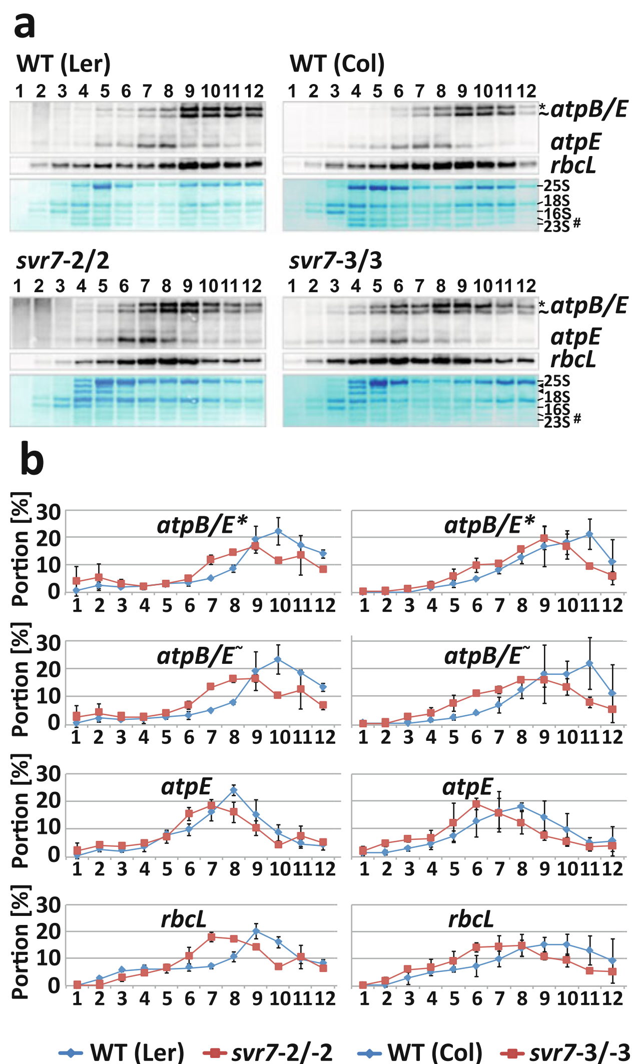
Fig. 4 RNA gel blot analysis of plastid RNAs encoding ATP synthase subunits and PsaJ. Total leaf RNA (5 μg/lane) from plants of the indicated genotypes was size fractionated on denaturing agarose gels, blotted onto Nylon membranes and analyzed by hybridization to radiolabeled DNA probes corresponding to plastid *atpB*/*E*, *atpH*, *atpF*, and *psaJ* genes (primers see Table 1). Irrelevant lanes were removed (shown by vertical lines). Transcripts over- or under-accumulating in *svr7* mutants are marked with asterisks or waves, respectively. The positions of RNA markers (knt = kilonucleotides) are shown. Methylene blue staining of rRNAs is shown below the blots and serves as loading control (bottom panels). Note the over-accumulation of chloroplast rRNA precursors (~2.9 and ~2.4 knt, marked with black triangles) and the slightly reduced accumulation of chloroplast 23S-rRNA breakdown products ($23S^{\#}$) compared to the normal accumulation of chloroplast 16S-rRNA and cytoplasmic 18S- and 25S-rRNAs in *svr7* mutants

for the determined minimum 50 % reduction of ATP synthase subunits, especially because most observed abnormalities are rather increases than reductions of transcript steady-state levels. Whether the detected dissimilarities between *svr7* and *atp4* mutants are caused by evolutionary diverged functions of the orthologous PPR-SMR proteins or by general processing differences of the analyzed operons in Arabidopsis and maize remains to be answered, e.g., by analyzing mutants of *SVR7*/*ATP4* orthologs in other mono- and eudicotyledonous model plants such as rice (*Oryza sativa*) and tobacco (*Nicotiana tabacum*).

SVR7 mutation impairs the translation of *atpB/E* and *rbcL* transcripts

Some PPR proteins are known to act as translational activators of chloroplast mRNAs (Barkan et al. 1994; Boulouis et al. 2011; Cai et al. 2011; Pfalz et al. 2009; Zoschke et al. 2012). So far, the mechanism of translational activation has been elucidated only for PPR10. PPR10 binds a region upstream of the *atpH* start codon and thereby resolves a secondary structure, which constrains ribosome entry by masking the shine dalgarno sequence (Prikryl et al. 2011). The SVR7 ortholog ATP4 activates translation of *atpB*/*E* and *atpA* mRNAs causing a drastic ATP synthase reduction in *atp4* mutants (Zoschke et al. 2012). A possible function of SVR7 in translation was examined by polysome analyses. Leaf extracts were fractionated on sucrose gradients under translational elongation inhibiting conditions (Barkan 1993). The more ribosomes are associated with an mRNA in polysomes the deeper the transcript migrates into the gradient. The distribution of *atpB*/*E* and *rbcL* mRNAs among gradient fractions was analyzed by RNA gel blot analysis (Fig. 5a). Methylene blue stained chloroplast rRNAs were found to be similarly distributed in *svr7* mutants and wild-type plants, excluding a general translation effect in *svr7* chloroplasts. By contrast, a specific shift to fractions of lower sucrose density was detected for the *atpB*/*E* and *rbcL* mRNAs in *svr7* mutants suggesting a reduced ribosome loading of these transcripts in the mutants (Fig. 5). Thus, *svr7* mutants exhibit a broader translation defect compared to *atp4* mutants, which were affected in translation of ATP synthase coding mRNAs but not in that of *rbcL* (Zoschke et al. 2012).

It was previously proposed that a general translation defect caused by the *svr7*-1 mutant allele is the reason for suppression of the variegated phenotype of *var2* mutants (Liu et al. 2010). The authors speculated that the detected chloroplast rRNA processing defects in *svr7*-1 mutants lead to impaired chloroplast translation. Indeed, also *svr7*-2 and -3 mutant alleles cause equal rRNA processing defects as seen by aberrant patterns of methylene blue stained chloroplast rRNAs in RNA gel blot analyses (Figs. 4, 5a). However, an over-accumulation of unprocessed chloroplast rRNA precursors was shown for several mutants with impaired chloroplast biogenesis (e.g. Barkan 1993; Beick et al. 2008, Schmitz-Linneweber et al. 2006, Zybailov et al. 2009). Moreover, the rRNA processing defects determined in mutants of the SVR7 ortholog ATP4 did not affect overall translation. These defects were found also in other mutants and consequently considered as unspecific effects linked to impaired chloroplast biogenesis (Zoschke et al. 2012). Similarly, compared to the reduction of ATP synthase subunits, the accumulation of D1, PsaD, and PetD proteins is almost unaffected by *svr7*-2 and -3 mutations,



◀ **Fig. 5** Polysome-analysis of ribosome association of chloroplast *atpB/E*, and *rbcL* mRNAs. **a** Total leaf extracts from plants of the indicated genotypes were fractionated in sucrose gradients under polysome maintaining conditions. The more ribosomes are associated with an mRNA, the deeper it migrates into the gradient. RNAs were isolated from 12 gradient fractions (1–12, increasing sucrose concentration). Equal proportions of the RNAs from each fraction were size fractionated on denaturing agarose gels, blotted onto Nylon membranes and analyzed by subsequent hybridization to DNA probes detecting *atpB/E*, and *rbcL* mRNAs (primers see Table 1). The two dicistronic *atpB/E* transcripts transcribed from different promoters are labeled by waves or asterisks, respectively (Malik Ghulam et al. 2012; Schweer et al. 2006). A representative blot is shown for each mRNA and genotype. The blots were stained with methylene blue to show distribution of rRNAs (bottom panels), and then probed sequentially to detect the indicated mRNAs. Note the abberant patterns of chloroplast rRNAs in *svr7* mutants as described in Fig. 4. **b** Quantification of the mRNA distribution within the sucrose fractions. The signals from the RNA gel blot analyses shown in a and from one biological replicate were recorded using **a** Phosphor-Imager (Bio-Rad) and quantified using the Quantity One software (Bio-Rad). For each transcript the percentage of the added signals in all lanes was calculated and plotted against the fractions comparing mutant and wild-type analysis (wild-type data points, blue diamonds, mutant data points, red rectangles). Error bars are based on two biological replicates. The two dicistronic *atpB/E* transcripts were analyzed separately and are shown in distinct diagrams labeled with waves or asterisks as the transcripts in **a**. The observed shifts of mRNAs to fractions of lower sucrose density in *svr7* mutants suggest a reduced ribosome association of those mRNAs

making a general defect in chloroplast translation in *svr7* mutants unlikely (Fig. 3a, b).

To precisely unravel the role of SVR7 in chloroplast translation, a detailed analysis of the events leading to translation initiation on the *atpB/E* mRNAs is needed. For this, the determination of the exact binding site of SVR7 would be helpful; however, the available ATP4 antiserum did not detect SVR7 (data not shown) and thus, identification of the RNA targets of SVR7 by RIP-Chip or related techniques is not feasible at present.

Unaltered chloroplast transcription activity in *svr7* mutants

The PPR-SMR proteins GUN1 and pTAC2 and other proteins with SMR domain were suggested to play roles in transcription (Fukui and Kuramitsu 2011; Koussevitzky et al. 2007; Pfalz et al. 2006). pTAC2 was isolated from transcriptional active chloroplast extracts and *ptac2* mutants show an impaired accumulation and transcription of PEP derived transcripts (Pfalz et al. 2006). GUN1 has an unspecific DNA binding activity and shows sub-organellar co-localization with pTAC2 (Koussevitzky et al. 2007). To examine a possible involvement of SVR7 in chloroplast transcription, run-on labeling assays were carried out with *svr7*-2 and -3 mutant and wild-type chloroplasts (Fig. 6). Equal numbers of chloroplasts were used for in vitro labeling by transcriptional incorporation of [α-^{32}P]-UTP. Labeled transcripts were hybridized to macro-arrays with probes for representative chloroplast genes encoding components of the ATP synthase, PSI, PSII, the Cyt b_6f complex, Rubisco, and the translation apparatus (Fig. 6a, b). None of the 13 analyzed chloroplast genes was found to be affected in its transcription activity in *svr7*-2 and -3 mutants (Fig. 6c). Solely the 23S rRNA gene showed a ~30 % reduction in transcription activity in one of the two analyzed *svr7* mutants, in the Landsberg background. However, the observed effects on protein accumulation, RNA processing, and translation caused by *SVR7* mutations were consistently observed in both *svr7* mutants (Figs. 3, 4, 5). Thus, it is unlikely that those defects are caused by an impaired transcription of the *rrn23* gene. Furthermore, pronounced differences in transcription activities of several genes were observed between Arabidopsis plants of Landsberg erecta and Columbia accessions (Fig. 6c). Thus it is possible that the impaired

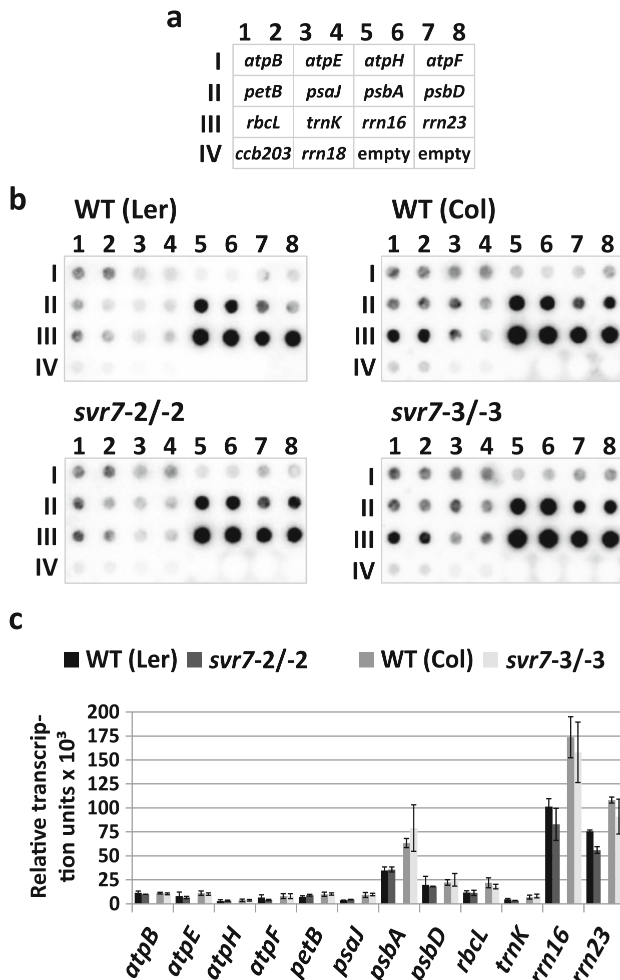


Fig. 6 Run-on analysis of chloroplast transcription activity in *svr7* mutants. **a** Overview of probe arrangement on macroarrays with duplicate dots of DNA probes for plastid genes (as indicated), and control probes for the nuclear 18S-rRNA gene (*rrn18*), and the mitochondrial cytochrome *c* biogenesis orf203 gene (*ccb203*). **b** Equal amounts of isolated chloroplasts from wild-type and *svr7* mutant plants were used for run-on transcription assays (10 min elongation). ^{32}P-labeled transcripts were isolated and hybridized to macroarrays containing plastid and control gene probes as shown in **a**. **c** Signals from macroarray analyses shown in **b** and from one biological replicate were recorded using a Phosphor-Imager (Bio-Rad) and quantified using the Quantity One software (Bio-Rad). For each probe and genotype means of 4 replicates (two biological, and two technical) were calculated and plotted against the respective gene. Data for the representative dot blots shown in **b** may be slightly different from the averages of replicate experiments shown in **c**

chloroplast gene expression caused by *SVR7* mutation leads to distinct secondary effects on transcription of the 23S rRNA gene in the analyzed Arabidopsis accessions.

SVR7 mutation does not disrupt Norflurazon induced retrograde signaling

GUN1, an SVR7 related chloroplast PPR-SMR protein, was shown to be involved in chloroplast to nucleus signaling (Koussevitzky et al. 2007). To test a similar function of SVR7 we examined the expression of a nuclear gene coding for a subunit of the chloroplast light harvesting complex (*LhcB1.2*). *LhcB* genes are under the control of retrograde signals and no longer expressed if chloroplast biogenesis is impaired (Oelmüller et al. 1986; Oelmüller and Mohr 1986). In GUN (genomes uncoupled) mutants this signaling is disrupted and *LhcB* expression still takes place despite the damage of chloroplast development (Mochizuki et al. 2001; Susek et al. 1993). We impaired chloroplast biogenesis by growing plants on Norflurazon (NF), which leads to an increased reactive oxygen stress and bleaching of plant tissue (Fig. 7). Under these conditions, expression of *LhcB* is not rescued by the *SVR7* mutation, suggesting that SVR7 is not involved in transmitting at least the NF-generated retrograde signal.

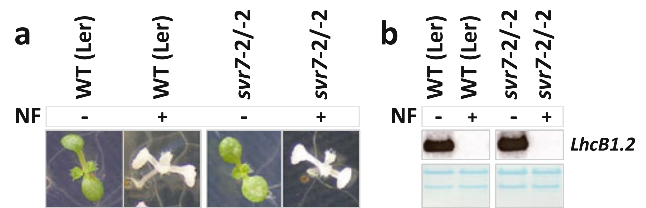


Fig. 7 Analysis of *svr7* mutants for a genomes uncoupled (GUN) phenotype after Norflurazon treatment. **a** Arabidopsis wild-type (WT; Ler = Landsberg erecta) and *svr7*-2/-2 mutant plants were grown for 8 days on Murashige and Skoog medium supplemented with Sucrose (3 %) and with (+) or without (−) Norflurazon (NF, 5 μM). Norflurazon treated plants show albinotic phenotypes caused by inhibition of carotenoid biosynthesis. **b** 5 μg total RNA isolated from Norflurazon-treated or untreated plants were size-fractionated by agarose gel electrophoresis, transferred to Nylon membrane, and probed with a DNA-probe specific to an *LhcB* mRNA (light harvesting complex B). All lanes come from the same gel, irrelevant lanes are removed from the image (vertical lines). Methylene blue staining of total RNA is shown below the blot and serves as loading control. Norflurazon treatment causes the repression of *LhcB* expression in wild-type and *svr7* mutant plants (Oelmüller et al. 1986; Oelmüller and Mohr 1986). An uncoupling of this feedback is typical for the GUN phenotype (Mochizuki et al. 2001; Susek et al. 1993), but was not observed in *svr7* mutants. The assay was repeated once with the same result

SVR7 and its maize ortholog ATP4: evolutionary implications of conserved and diverged mutant defects

In the few instances in which orthologous proteins involved in chloroplast biogenesis have been studied in Arabidopsis and maize so far, their functions are largely conserved (Asakura and Barkan 2006, 2007; Asakura et al. 2008). In case of the orthologous PPR-SMR proteins SVR7 and ATP4 this appears to be only partially true. Both maize *atp4* and Arabidopsis *svr7* mutants exhibit impaired *atpF* transcript patterns, reduced accumulation of ATP synthase subunits and diminished ribosome association with the

atpB/E mRNA. The observed common mutation effects together with the high degree in identity/similarity of the sequences strongly substantiate orthology (Zoschke et al. 2012). However, there are also remarkable differences in chloroplast gene expression between *svr7* and *atp4* mutants (Zoschke et al. 2012): Several *atpF/-H* and *psaJ* transcripts, which over-accumulate in *svr7* mutants, are in fact not altered or even reduced in *atp4* mutants. Additionally, the reduced accumulation of ATP synthase subunits is less pronounced in *svr7* mutants compared to *atp4* mutants and the translational defects caused by *SVR7* mutation include also RbcL and thus appear to be broader than that of *atp4* mutants.

Furthermore, the phenotypic consequences of *SVR7* and *ATP4* mutations are remarkably different. Whereas the knockout of ATP4 causes pale green phenotypes and seedling lethality, the mutations of *SVR7* account only for slightly pale green and developmentally retarded but viable and fertile plants (Liu et al. 2010; Zoschke et al. 2012). These phenotypic variations could be explained by differences in the accumulation of ATP synthase subunits. In *svr7* mutants, the ATP synthase is much less reduced than in *atp4* mutants and it is known that even strongly reduced ATP synthase contents are sufficient for the plant's energy supply by an upregulated activity of the remaining ATP synthase complexes (Rott et al. 2011). Consequently, reduced ATP synthase levels in *svr7* mutants could be sufficient for viability of the plants whereas the severe ATP synthase reduction in *atp4* mutants causes seedling lethality after exhaustion of the seed storage. Nevertheless, it is remarkable that the phenotypic effects of the Arabidopsis mutants are far less severe than the ones in maize, because in other cases, where knock-outs of PPR proteins have been investigated in orthologous genes between maize and Arabidopsis, the phenotypes in Arabidopsis were more drastic (e.g. Lurin et al. 2004; Schmitz-Linneweber and Small 2008; Tzafrir et al. 2004). For example, loss of PPR4 and -5 lead to albino seedlings in maize, while in Arabidopsis, plants die as early embryos (Beick et al. 2008; Cushing et al. 2005; Schmitz-Linneweber et al. 2006). These mutants have global defects in chloroplast translation, whereas *ATP4/SVR7* mutations cause specific defects. Possibly, the ATP synthase activity is differently regulated in eudicotyledonous plants and thus less susceptible to changes in its expression levels than in monocotyledonous plants.

Alternatively, the protein functions of SVR7 and ATP4 maybe diverged during evolution. Finally, SVR7 functions in Arabidopsis might overlap with one or more other protein(s), which partially complement *SVR7* mutation thus extenuating the effects compared to *ATP4* mutations. Further studies are needed to discriminate between these alternatives.

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