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Tobacco plastid ribosomal protein S18 is essential for cell survival

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ABSTRACT

Plastid genomes contain a conserved set of genes most of which are involved in either photosynthesis or gene expression. Among the ribosomal protein genes present in higher plant plastid genomes, *rps18* is special in that it is absent from the plastid genomes of several non-green unicellular organisms, including *Euglena longa* and *Toxoplasma gondii*. Here we have tested whether the ribosomal protein S18 is required for translation by deleting the *rps18* gene from the tobacco plastid genome. We report that, while deletion of the *rps18* gene was readily obtained, no homoplasmic Δ*rps18* plants or leaf sectors could be isolated. Instead, segregation into homoplasmy led to severe defects in leaf development suggesting that the knockout of *rps18* is lethal and the S18 protein is required for cell survival. Our data demonstrate that S18 is indispensable for plastid ribosome function in tobacco and support an essential role for plastid translation in plant development. Moreover, we demonstrate the occurrence of flip-flop recombination on short inverted repeat sequences which generates different isoforms of the transformed plastid genome that differ in the orientation a 70 kb segment in the large single-copy region. However, infrequent occurrence of flip-flop recombination and random segregation of plastid genomes result in the predominant presence of only one of the isoforms in many tissue samples. Implications for the interpretation of chloroplast transformation experiments and vector design are discussed.

INTRODUCTION

Protein biosynthesis in plastids relies on prokaryotic-type 70S ribosomes. The RNA components of these ribosomes are exclusively encoded by the plastid genome (plastome) whereas only part of the ribosomal proteins is encoded in the plastome. The chloroplast genomes of higher plants harbor a conserved set of 21 ribosomal protein genes [reviewed e.g. in (1–3)] which, with only few exceptions, are also found in the plastomes of all other plastid-containing taxa. A notable exception is the gene for the ribosomal protein 18 of the 30S subunit of the plastid ribosome (S18) which was recently found to be absent from plastid genomes of several non-green unicellular organisms, including the colorless alga *Euglena longa* [= *Astasia longa*; Ref. (4); database accession no. AJ294725] and the parasitic protozoans *Eimeria tenella* (database accession no. NC_004823), *Theileria parva* (database accession no. NC_007758) and *Toxoplasma gondii* [database accession no. NC_001799; Refs (5–7)].

Absence of *rps18* from the plastid genomes of non-green organisms as diverse as *E.longa* and *Toxoplasma gondii* raises the possibility that the ribosomal protein S18 is not required for translation in non-photosynthetic plastids. This could be a reasonable assumption, because photosynthesis is the main function of plastids and most plastid genes are either directly or indirectly involved in supporting photosynthesis. Thus, the absence of photosynthesis results in a much lower demand for plastid protein biosynthesis and ribosomes without S18 could provide sufficient basal translational activity to support the very few extraphotosynthetic functions of the chloroplast [such as, fatty acid biosynthesis for which a plastid-encoded acetyl-CoA carboxylase subunit is required; Ref. (8)]. In addition, S18 does not bind directly to the RNA component of the small ribosomal subunit, the 16S rRNA, and thus assembles only relatively late into the 30S subunit (9) which would also be compatible with a non-essential role of the S18 protein.

To address the requirement for S18 in plastid translation directly, we constructed knockout alleles and introduced them into the tobacco plastid genome by genetic transformation. A second purpose of the present study was to test directly the requirement for plastid translation by generating a possibly translation-deficient *rps18* knockout mutant. Earlier studies in barley (10,11) and Brassica (12) have provided evidence for plastid translation being dispensable under heterotrophic growth conditions (i.e. in the presence of sucrose). In contrast, recent transgenic experiments have suggested that plastid translation is essential in tobacco: Recombination-induced elimination of a spectinomycin resistance gene led to arrested cell division in the presence of the antibiotic

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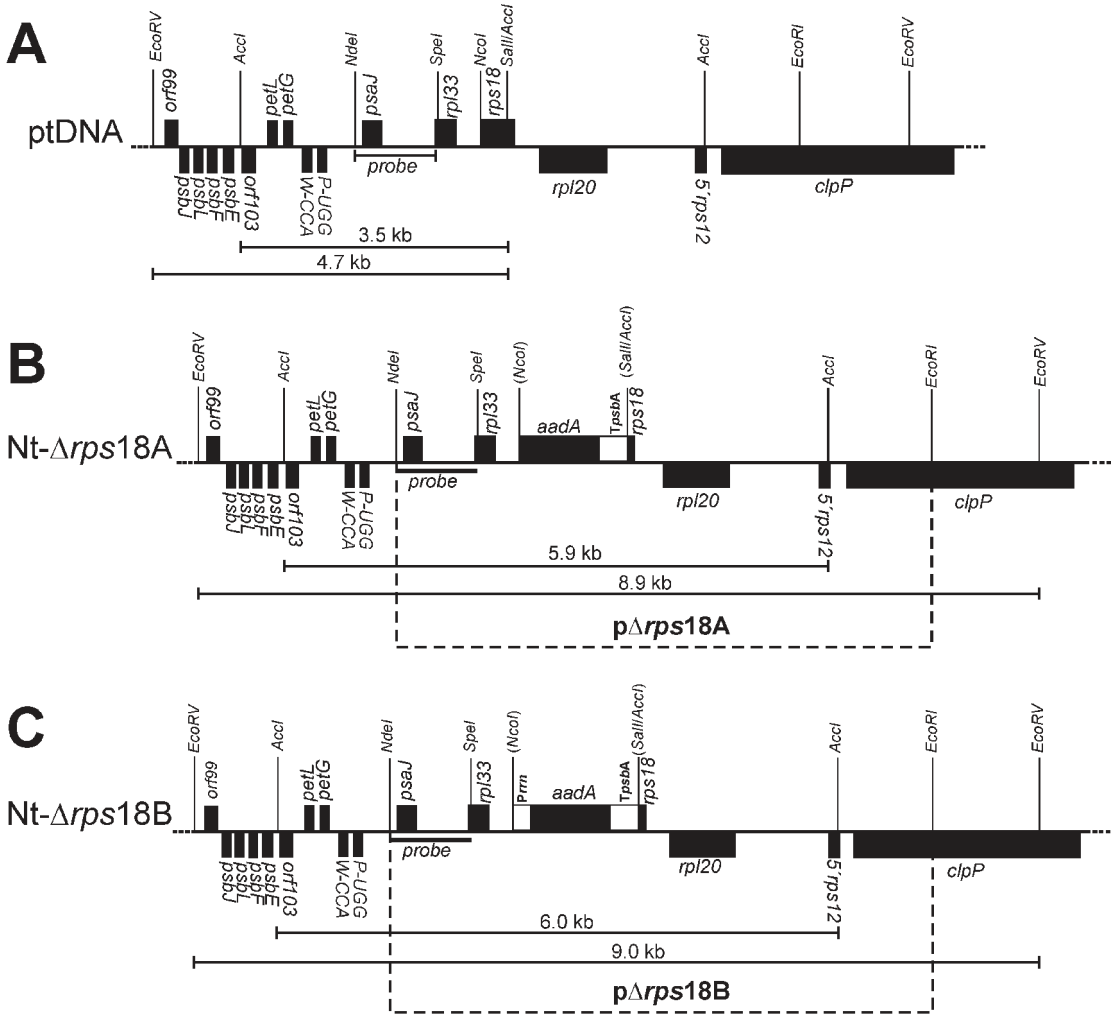

Figure 1. Construction of plastid transformation vectors for disruption of *rps18*. (**A**) Physical maps of the *rps18* region in the tobacco plastid genome [ptDNA; Ref. (15)]. Genes above the lines are transcribed from the left to the right, genes below the lines are transcribed in the opposite direction. (**B**) Map of transformation vector pΔrps18A. Note that the spectinomycin resistance gene *aadA* is driven by the endogenous *rps18* promoter. (**C**) Map of transformation vector pΔrps18B. In this vector, the rRNA operon-derived chimeric Prrn promoter (16) drives the selectable marker gene *aadA*. Restriction sites used for cloning, RFLP analysis and/or generation of hybridization probes are indicated. Sites lost owing to ligation to heterologous ends are shown in parentheses. The hybridization probe (NdeI/SpeI fragment) is indicated and the expected sizes of hybridizing bands in the two RFLP analyses (Figure 2) is shown below each map. The chloroplast targeting fragment in transformation vectors pΔrps18A and pΔrps18B is marked by dashed lines (NdeI/EcoRI fragment cloned into pUC18).

which in turn resulted in severe defects in leaf and floral development (13).

MATERIALS AND METHODS

Plant material, growth conditions and phenotypical assays

Sterile tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were grown on agar-solidified MS medium containing 30 g/l sucrose (14). Transplastomic lines were rooted and propagated on the same medium. To obtain seeds and analyze the phenotype of *rps18* knockout plants upon growth in soil, transplastomic plants were grown to maturity under standard greenhouse conditions. Seedling phenotypes were analyzed by germination of seeds in a spectinomycin-containing MS medium (500 mg/l spectinomycin). Spectinomycin re-exposure experiments were performed on the same medium interrupted by a period of growth in the absence of spectinomycin which was performed by plant transfer to a box with spectinomycin-free MS medium.

Construction of Δ*rps18* plastid transformation vectors

The region of the tobacco plastid genome containing the *rps18* gene was isolated as a 3.7 kb NdeI/EcoRI fragment [Ref. (15); Figure 1] and cloned into the similarly cut pUC18 vector producing plasmid pMRG2. To generate transformation vector pΔrps18A, pMRG2 was linearized with SalI followed by blunting of the protruding single-stranded overhangs using the Klenow fragment of DNA polymerase I. The DNA was subsequently digested with NcoI deleting most of the *rps18* reading frame. (The *rps18* start codon is part of the NcoI restriction site.) A promoterless selectable marker gene *aadA* conferring resistance to aminoglycoside antibiotics (16) was then ligated into the *rps18* deletion as

NcoI/DraI fragment with the NcoI site harboring the *aadA* start codon and DraI generating blunt ends downstream of the *psbA* 3′-untranslated region (3′-UTR) of the *aadA* cassette (Figure 1). Transformation vector pΔrps18B was produced by digestion of plasmid pMRG2 with NcoI and SalI followed by a fill-in reaction with the Klenow fragment of DNA polymerase I. A blunt-end chimeric *aadA* cassette consisting of the ribosomal RNA promoter (Prrn), the *aadA* coding region and the *psbA* 3′-UTR was then ligated into the *rps18* deletion as Ecl136II/DraI fragment. A plasmid clone carrying the *aadA* gene in the same orientation as the *rps18* gene in the wild type yielded the final transformation vector pΔrps18B (Figure 1).

Plastid transformation and selection of homoplasmic transformed tobacco lines

Young leaves from sterile tobacco plants were bombarded with plasmid-coated 0.6 μm gold particles using a biolistic gun (PDS1000He; BioRad). Primary spectinomycin-resistant lines were selected on RMOP regeneration medium containing 500 mg/l spectinomycin (16,17). Spontaneous spectinomycin-resistant plants were eliminated by double selection on medium containing spectinomycin and streptomycin [500 mg/l each; Refs. (16,18)]. Several independent transplastomic lines were subjected to four additional rounds of regeneration on RMOP/spectinomycin to enrich the transplastome and select for homoplasmic tissue.

Isolation of nucleic acids and hybridization procedures

Total plant DNA was isolated by a rapid cetyltrimethylammoniumbromide (CTAB)-based miniprep procedure (19). DNA samples were digested with restriction enzymes, separated on 0.8% agarose gels and blotted onto Hybond N nylon membranes (Amersham). For hybridization, [α-^{32}P]dATP-labeled probes were generated by random priming (Multiprime DNA labeling kit, Amersham). A restriction fragment covering part of the *psaJ*/*rpl33* region was used as probe for the restriction fragment length polymorphism (RFLP) analyses. Hybridizations were carried out at 65°C in Rapid Hybridization Buffer (Amersham) following the manufacturer's protocol.

PCR and DNA sequencing

Presence of the products of flip-flop recombination in transplastomic lines was confirmed by PCR amplification using the four combinations of primers indicated in Figures 3 and 4. Primer sequences were as follows:

PaadA25: 5′-AGA TCA CCA AGG TAG TCG GCA A -3′; Prpl20: 5′-AAC CGT AAA ATA CTT GCA CAA ATA GCT-3′; PtrnH: 5′-CTT GAT CCA CTT GGC TAC ATC C-3′; PpsbA: 5′-AAT GCT CAC AAC TTC CCT CTA G-3′.

In 50 μl reactions, 50 ng total genomic DNA was amplified in a reaction mixture containing 200 μM of each dNTP, 2.0 mM $MgCl_2$, 10 pmol of each primer and 1 U *Taq* DNA polymerase (Promega). The standard PCR program was 40 cycles of 40 s at 94°C, 90 s at 52–55°C and 90 s at 72°C with a 3 min extension of the first cycle at 94°C and a 5 min final extension at 72°C. PCR products were analyzed by electrophoretic separation in 1.5% agarose gels. For direct sequencing, amplification products were purified by electrophoresis on 1.5% agarose gels and subsequent extraction from gel slices using the GFX kit (Amersham). Sequencing of amplification products was performed by cycle sequencing according to standard protocols followed by automated analysis in a capillary sequencer.

RESULTS

Targeted disruption of the plastid *rps18* gene

To facilitate a functional analysis of the plastid *rps18* gene by reverse genetics, two knockout alleles were constructed (Figure 1). In both alleles, the selectable marker gene *aadA* conferring resistance to the aminoglycoside antibiotics spectinomycin and streptomycin was used to replace *rps18*. In plasmid pΔrps18A, the *rps18* coding region was excised and a promoterless *aadA* gene was fused to the *rps18* gene promoter. In contrast, the *aadA* gene is driven by a foreign (ribosomal RNA operon-derived) promoter in plasmid pΔrps18B (Figure 1).

With both transformation vectors, biolistic transformation of tobacco leaves and selection for chloroplast transformants resulted in numerous spectinomycin-resistant lines (26 for transformation plasmid pΔrps18A and 52 for vector pΔrps18B). Chloroplast transformation was confirmed by double resistance tests on medium containing spectinomycin and streptomycin, a test which eliminates spontaneous spectinomycin-resistant lines (16–18). Six independently generated lines from each transformation construct were selected for further analyses (designated Nt-Δrps18A-9, -13, -14, -17, -18, -19 and Nt-Δrps18B-2, -15, -18, -27, -43 and -45, respectively). The lines were subjected to several additional rounds of regeneration and selection to enrich the transgenic genome and select against residual wild-type genomes. Typically, this procedure results in homoplasmic transplastomic lines (i.e. regenerants lacking residual wild-type genome copies) after two to three rounds of selection and regeneration (16,18,20).

Transplastomic Δ*rps18* plants remain heteroplasmic

We next wanted to test whether several rounds of stringent antibiotic selection had successfully eliminated all wild-type genomes. We therefore analyzed DNA from transplastomic lines by Southern blot hybridizations (Figure 2A and B). Using different restriction enzymes, these analyses revealed novel fragments that were larger than the corresponding restriction fragments in the wild type suggesting that they carry the *aadA* gene inserted into the *rps18* locus. In addition, all transplastomic lines showed a hybridization signal for the wild-type fragment indicating that the *rps18* gene cannot be eliminated from all plastid genomes in the cell. The ratio between wild-type signal and signal for the transplastome varied only little between the different lines and in different regeneration rounds (Figure 2A and B and data not shown) suggesting a balancing selection in which the cell must maintain both genome types: the transplastomic genome to provide the antibiotic resistance and the wild-type genome to provide the S18 protein. A similar situation has been observed before in knockout experiments for two essential open reading frames encoded in the tobacco plastid genome, *ycf1* and *ycf2* (21), as well as for the *clpP* gene encoding

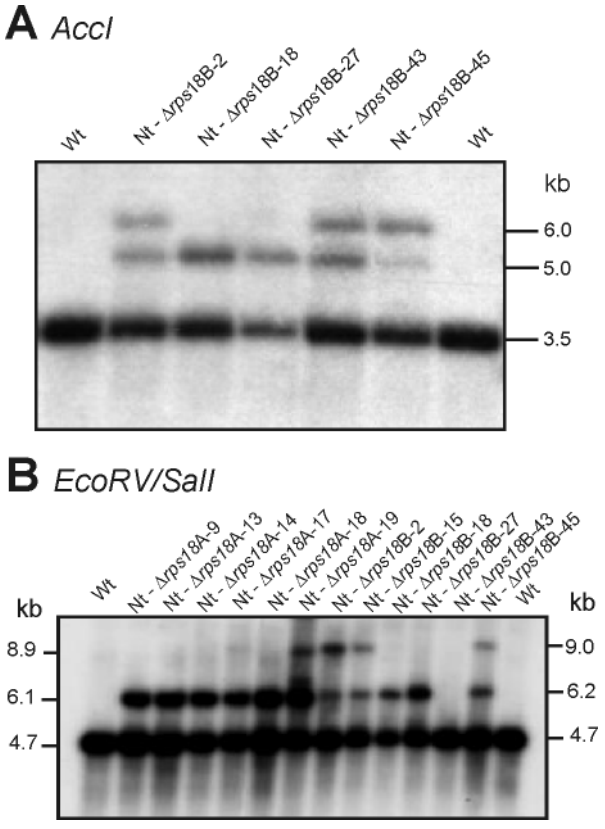

Figure 2. RFLP analysis of chloroplast transformants obtained with *rps18* knockout constructs. Equal amounts of extracted total cellular DNA (5 µg per sample) were digested with the restriction enzymes indicated, separated in 0.8% agarose gels, blotted and hybridized to a radiolabeled restriction fragment derived from cloned tobacco plastid DNA (NdeI/SpeI fragment; Figure 1). (**A**) RFLP with AccI. (**B**) RFLP with EcoRV and SalI. The probe detects, in addition to the expected fragments (Figure 1), an unexpected band in most samples which is the product of flip-flop recombination (see text for details and Figures 3 and 4). Fragment sizes of the molecular weight marker are given in kb. Lane Nt-Δrps18B-43 is positive in the AccI RFLP and negative in the EcoRV/SalI RFLP most probably because the leaf sample harvested for DNA extraction for the AccI blot was heteroplasmic, whereas the sample harvested for isolating the DNA for the EcoRV/SalI blot had segregated into virtual homoplasmy for the wild-type genome. Wt: wild type.

an essential subunit of a chloroplast ATP-dependent protease (22,23). This suggests that *rps18* is an essential gene in tobacco and that plastid translation may strictly depend on ribosomal protein S18.

Flip-flop recombination in transplastomic lines

The hybridization pattern for several transplastomic lines was unexpected. Digestion with the restriction enzyme AccI was expected to produce a fragment of 6 kb in *rps18* knockout plants (Figures 1 and 2A). While several lines indeed showed this fragment, most lines showed an additional hybridizing restriction fragment that was substantially smaller (~5 kb; Figure 2A). The ratio between the expected 6 kb and the unexpected 5 kb fragments was variable with some lines showing barely detectable amounts of either the one or the other. This finding was confirmed for a second RFLP using the restriction enzyme combination SalI and EcoRV (Figure 2B). This digest produced besides the expected 9 kb fragment a significantly smaller fragment of 6.2 kb which was present in nearly all lines.

We suspected the additional bands to have arisen from a recombination event. As recombination in chloroplasts is restricted to homologous recombination, we reasoned that the recombination event must involve a sequence that is duplicated in the transplastome. Two such sequences are present: The rRNA operon-derived Prrn promoter driving the *aadA* selectable marker gene (148 bp) and the 3′-UTR of the *aadA* derived from the plastid *psbA* gene (232 bp). We assembled all possible recombination products between the two Prrn promoters and the two *psbA* 3′-UTRs and digested them *in silico* with the enzymes used in our RFLP assays. This analysis revealed that flip-flop recombination between the two copies of the *psbA* 3′-UTR can explain all bands seen in our Southern blot experiments (Figure 3). It is important to note that the two copies of the *psbA* 3′-UTR are present as inverted repeats (Figure 3A) and therefore, recombination between them is expected to result in an inversion, whereas a partial genome deletion would require their presence as direct repeats.

To provide direct proof for flip-flop recombination occurring in our transplastomic lines, we designed PCR primers suitable to amplify all recombination products (Figure 3B and C). PCR analyses with these four different primer combinations and sequencing of selected amplification products confirmed that indeed, flip-flop recombination produces two different isoforms of the transgenic chloroplast genome (Figure 4). Unambiguous identification of all recombination sites and products ultimately confirms the recombination via the two *psbA* 3′-UTRs in all lines analyzed. The two genome isoforms differ in the orientation of a 70 kb segment in the large single copy region of the plastid genome (Figure 3A). However, the two isoforms are functionally equivalent in that they both carry the knockout allele for *rps18* and do not affect the integrity of other chloroplast genes. It is noteworthy that the two isoforms are not always present in similar amounts (Figure 2A) and in some samples, one of the isoforms is much less abundant than the other or even undetectable with the sensitivity of DNA gel blot hybridization. However, PCR assays, which are much more sensitive, always detected both isoforms (Figure 4) suggesting that their varying abundance in different samples is due to random segregation of the isoforms during cell and organelle division. As always both isoforms were readily detectable by PCR and only PCR products of sizes that exactly corresponded to the predicted sizes of the recombination products were obtained, it is highly unlikely that template switching or similar PCR artifacts are involved here.

Rps18 knockout plants exhibit defects in leaf development

Having obtained circumstantial evidence for *rps18* being an essential gene, we were interested in providing direct proof for the essentiality of *rps18* and the requirement for plastid protein biosynthesis in tobacco plants. We therefore germinated large numbers of seeds from several transplastomic lines on antibiotic-containing medium. Transmission of the *rps18* knockout allele into the next generation turned out to

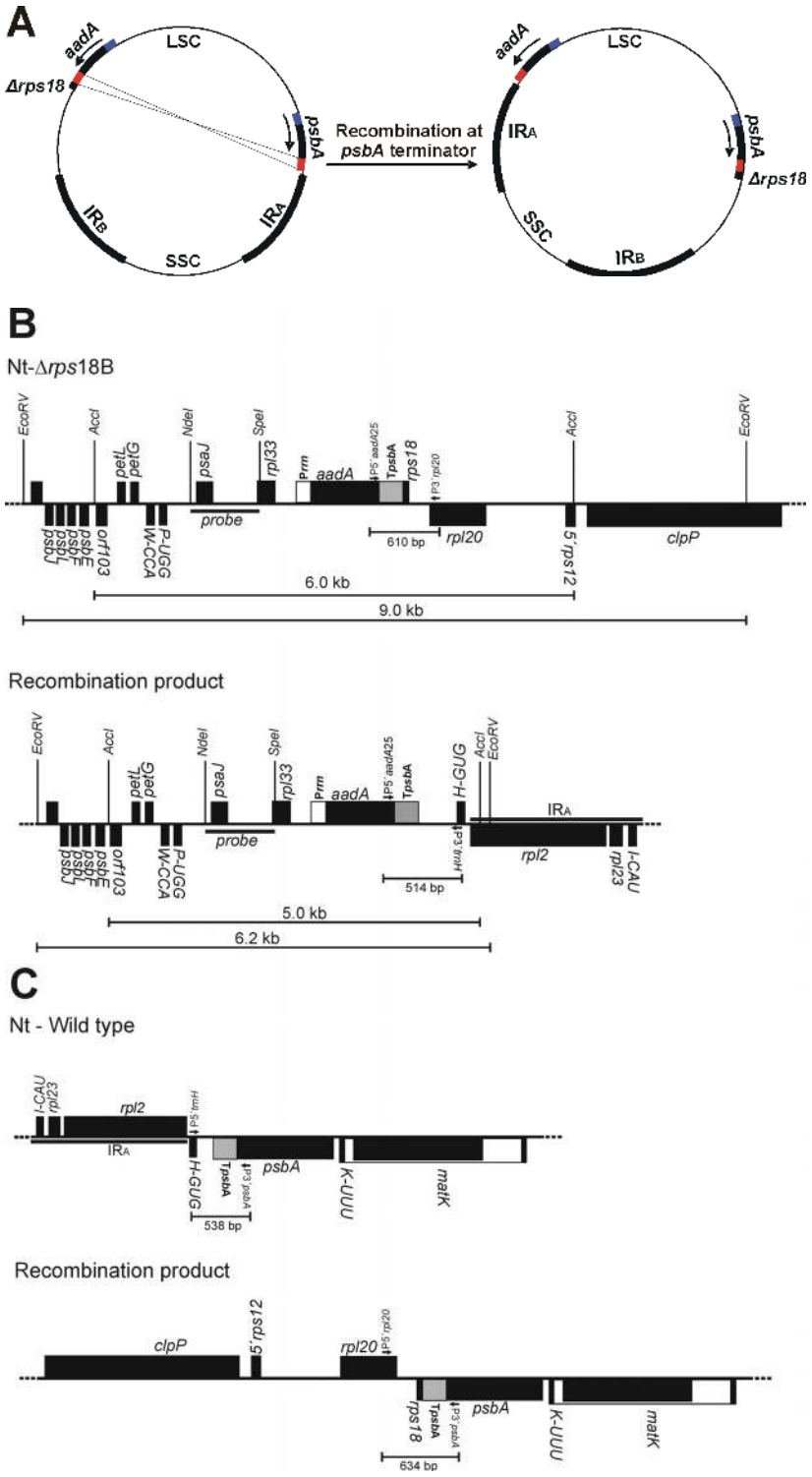

Figure 3. Model for flip-flop recombination on the two copies of the *psbA* 3′-UTR present in the transplastomes. (**A**) Inversion of a large, 70 kb segment of the large single copy region (LSC) by recombination between the two homologous sequences. IR_A and IR_B, inverted repeat regions; SSC, small single copy region. (**B**) Maps of the two different genome conformations in the *psaJ*/*rpl33* region of the plastid genome. Restriction fragments hybridizing to the probe are indicated below each map (cp. Figure 2). (**C**) Maps of the two different genome conformations in the *psbA*/*trnK*/*matK* region of the plastid genome. PCR primers and expected sizes of amplification products in PCR assays diagnostic for the different genome conformations are also indicated.

be low, varying between 0.3 and 2.3% of the seedlings in the six transplastomic plants analyzed (Figure 5; Table 1). This finding indicates that the knockout allele is strongly selected against. In the absence of selective pressure exerted by spectinomycin, the transplastome is quickly lost and displaced by wild-type genome copies.

Interestingly, the cotyledons of the surviving seedlings were variegated suggesting that the seedlings were

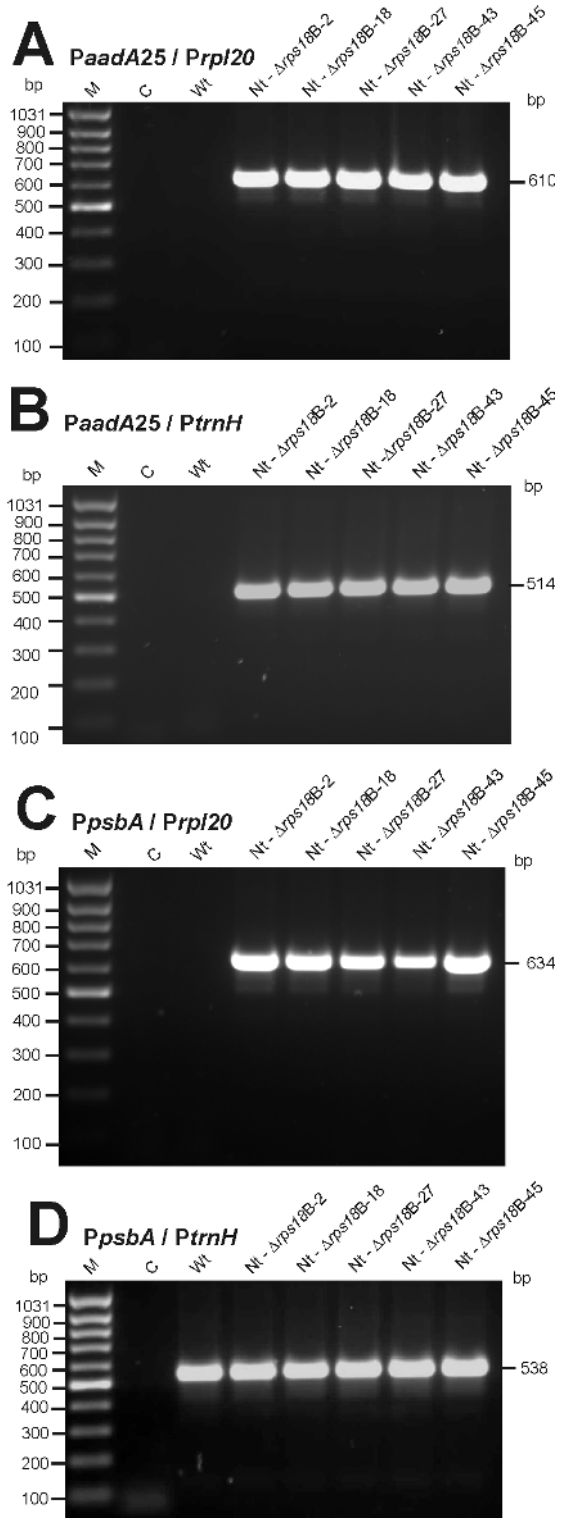

Figure 4. Confirmation of flip-flop recombination by PCR. For location and orientation of PCR primers, see Figure 3. M, molecular weight marker; C, buffer control; Wt, wild type control. Sizes of obtained amplification products are indicated at the right in base pair. (**A**) Test for the expected genome conformation in transplastomic lines (see Figure 3B upper panel). (**B**) Test for the product of flip-flop recombination in the *psaJ*/*rpl33* region of transplastome (see Figure 3B lower panel). (**C**) Test for the product of flip-flop recombination in the *psbA*/*trnK*/*matK* region of transplastome (see Figure 3C lower panel). (**D**) Control amplification of the *psbA* 3′-UTR from the wild-type genome (see Figure 3C upper panel).

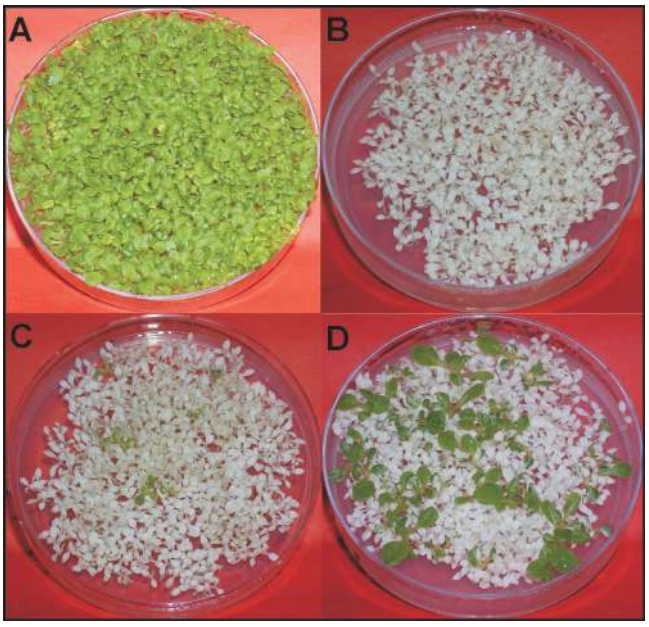

Figure 5. Seed assays confirming heteroplasmy and random genome segregation in *rps18* knockout lines. (**A**) Seed germination in the absence of spectinomycin. (**B**) Wild-type control on medium with spectinomycin. (**C** and **D**) Seeds from two independently generated transplastomic lines germinated in the presence of spectinomycin (C: Nt-Δrps18B-27; D: Nt-Δrps18B-15). In white seedlings, the transplastome has been lost owing to random sorting out of plastid genomes. In contrast, green and variegated seedlings contain the transplastome and hence, express spectinomycin resistance. Approximately 1500 seeds were sown per plate.

Table 1. Segregation of the *aadA* antibiotic resistance gene disrupting *rps18* in the T1 generation

Plant line	Resistant seedlings		
	Test 1	Test 2	Total
Wild type	0 (0.0%)	0 (0.0%)	0 (0.0%)
Nt-Δ*rps18*A-13	6 (0.4%)	13 (0.9%)	19 (0.6%)
Nt-Δ*rps18*A-14	22 (1.5%)	29 (1.9%)	51 (1.7%)
Nt-Δ*rps18*A-18	20 (1.3%)	12 (0.8%)	32 (1.1%)
Nt-Δ*rps18*B-2	17 (1.1%)	16 (1.1%)	33 (1.1%)
Nt-Δ*rps18*B-15	30 (2%)	39 (2.6%)	69 (2.3%)
Nt-Δ*rps18*B-27	7 (0.5%)	2 (0.1%)	9 (0.3%)

Approximately 3000 seeds per line (obtained from self-fertilized plants) were sown on spectinomycin-containing medium and assayed for spectinomycin resistance in two independent experiments for each line.

heteroplasmic and contained sectors with wild-type chloroplasts only, which bleach out in the presence of the antibiotic (Figure 5C and D). When plants were grown in soil, most of them displayed phenotypic abnormalities. Misshapen leaves were seen frequently and many leaves lacked parts of the leaf blade (Figure 6). In the most extreme cases, almost the entire leaf blade was missing giving the leaves a needle-like appearance (Figure 6C). In addition, irregular leaf branching occurred quite frequently (Figure 6A and C). In general, we observed a positive correlation between the severity of the phenotype in the parent plant and the transmission frequency of the knockout allele into the next generation (Figures 5 and 6, Table 1 and data not shown).

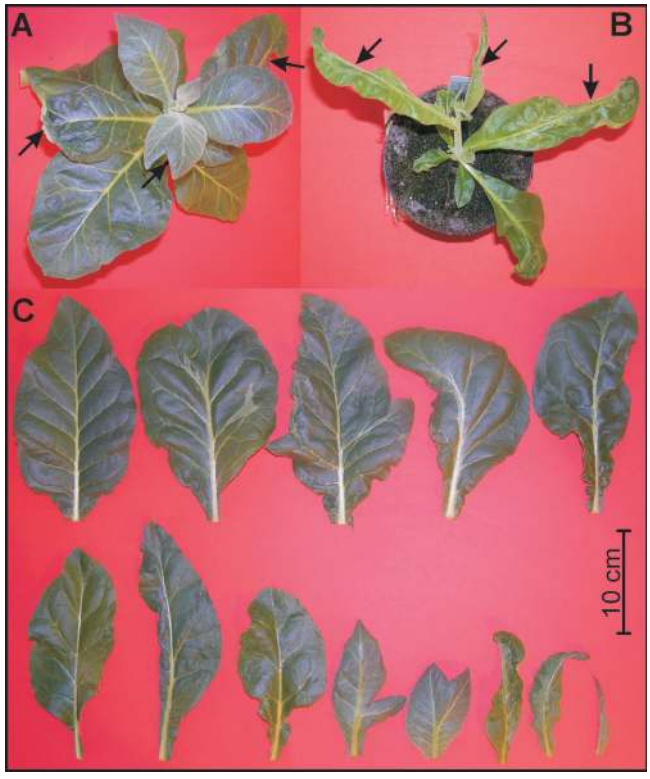

Figure 6. Mutant phenotypes of Nt-Δrps18 tobacco plants. (**A** and **B**) Leaf abnormalities in greenhouse-grown plants (T_0 generation). Arrows point to misshapen leaves that lack parts of the leaf blade. The phenotype is variable and depends on the frequency of somatic segregation towards homoplasmy for the *rps18* knockout. (**A**) Example of a plant with a relatively mild phenotype. (**B**) Example of a plant with a severe phenotype. (**C**) Spectrum of leaf phenotypes observed in Nt-Δrps18 plants. Phenotypes range from nearly normal leaves (top row left) to needle-like mutant leaves lacking the entire leaf blade (bottom row right).

Developmental defects result from somatic segregation into homoplasmy

Similar leaf phenotypes have been described recently in plants that have lost the spectinomycin resistance gene by loop-out recombination and are continuously grown in the presence of the antibiotic (13). This has been attributed to a loss of cell division upon inhibition of plastid translation by spectinomycin. Similarly, loss of plastid translation upon knockout of *rps18* could explain the phenotypic abnormalities in the transplastomic plants: If random segregation of chloroplast genomes produces homoplasmic *rps18* knockout cells and these cells do not survive or cannot divide, the lack of cell lines during leaf development can be expected to produce the observed phenotypic aberrations.

To visualize plastid genome segregation in *rps18* knockout plants, we performed media-shift experiments in which plants were grown for some time in the absence of spectinomycin and then transferred back to medium with antibiotic. Growth in the absence of the antibiotic should allow random sorting out of genomes and thus result in segregation into wild-type sectors and heteroplasmic transplastomic sectors. The wild-type sectors can then be visualized by back-transfer to spectinomycin-containing medium on which they should bleach out. When *rps18* knockout plants were grown under

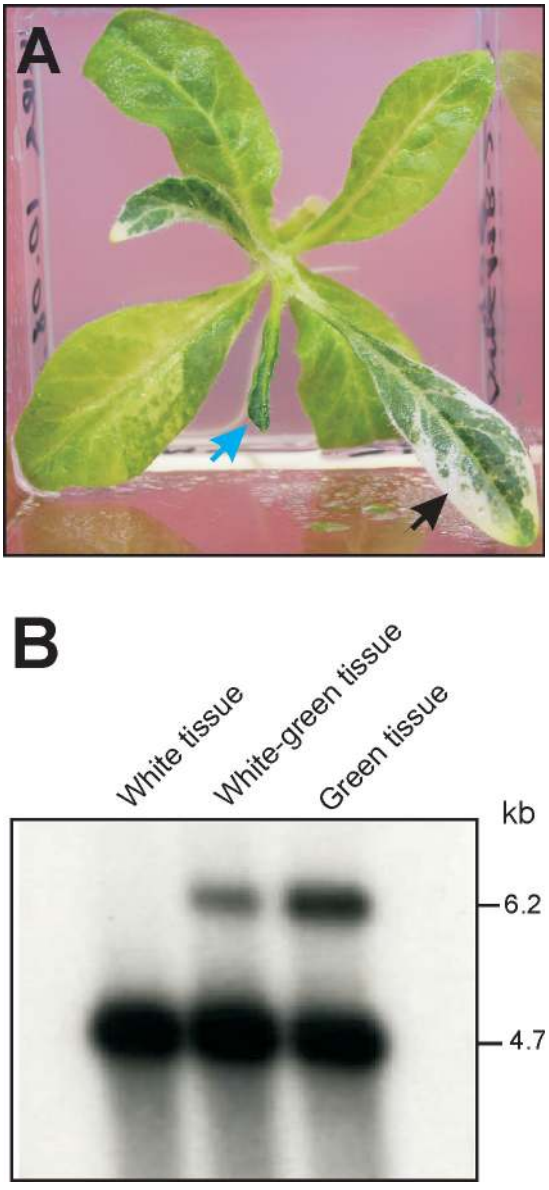

Figure 7. Genome segregation in the absence of selection for the transplastome (**A**) Leaf phenotype in the spectinomycin re-exposure experiments. Plants were initially raised on spectinomycin-containing medium (note blue arrow pointing to a misshapen leaf), then transferred to spectinomycin-free medium for 3 weeks followed by re-exposure to spectinomycin-containing medium. Spectinomycin re-exposure visualizes sectors that have lost the transplastome and, therefore. lack the *aadA* spectinomycin resistance gene (bleaching sectors indicated by the black arrow). (**B**) RFLP analysis to confirm the absence of transformed Δ*rps18* genomes in white sectors of Nt-Δrps18 leaves grown in the absence of spectinomycin and then re-exposed to the drug. The restriction enzyme combination used for the RFLP was EcoRV/SalI (cp. Figure 2B). Of total cellular DNA 1 μg was digested and separated in a 0.8% agarose gel. Whereas green sectors show the 6.2 kb transplastome-specific band, white sectors show only a signal for the 4.7 kb wild-type band.

this regime, sectorial bleaching was indeed seen, confirming that release of selection pressure allows for rapid sorting out of genome types (Figure 7A). To confirm that the white sectors are homoplasmic for the wild-type genome, we analyzed green, white and mixed sectors for their genome composition by Southern blot analysis (Figure 7B). Whereas the green

and mixed sectors contained both genome types, only the wild-type genome was detectable in the white sectors (Figure 7B), demonstrating that their bleaching is due to the loss of the *aadA* spectinomycin resistance gene by random genome segregation.

DISCUSSION

In this work, we have shown that the plastid genome-encoded gene for the plastid ribosomal protein S18 is essential. Knockout of the corresponding chloroplast gene results in stable heteroplasmy under antibiotic selection (balancing selection). In contrast, release of the selective pressure for the transplastome by growth in the absence of the antibiotic results in rapid loss of the *rps18* knockout allele by random plastome segregation.

Genome segregation into homoplasmy produces two distinct phenotypes: White sectors in otherwise normally shaped leaves are seen in cotyledons and in spectinomycin re-exposure experiments (Figures 5C, D and 7A). In contrast, the true leaves are green but lack parts of their leaf blades (Figures 6 and 7A). The appearance of misshapen true leaves is independent of treatment with spectinomycin in that it occurs upon continuous growth in either the presence or the absence of the antibiotic (Figures 6 and 7A and data not shown). The white sectors seen in the cotyledons of variegated *rps18* knockout seedlings (Figure 5C and D) are not seen in true leaves that develop continuously either in the presence or absence of spectinomycin. This is explained with the development of the cotyledons taking place already during embryogenesis and seed development (i.e. in the absence of antibiotic exposure) and suggests that white sectors in the cotyledons are not equivalent to missing sectors in true leaves: While the white sectors in the cotyledons (and in our spectinomycin re-exposure experiments) are homoplasmic for the wild-type genome, the missing sectors in the misshapen true leaves result from segregation into homoplasmy for the transplastome.

Our data suggest that S18 is an essential component of the plastid ribosome and translation in the absence of S18 is unlikely to be possible. As sucrose was present in all our selection media and, therefore, photosynthesis is dispensable under *in vitro* culture conditions (24,25), it seems also unlikely that S18 could be dispensable in non-photosynthetic tissues. The strict requirement for S18 for plastid ribosome function and the absence of *rps18* genes from the plastid genomes of *E.longa* and protozoans may indicate that the gene was transferred to the nucleus in these species. Precedence for the repeated transfer of a plastid gene to the nucleus is the gene for the translation initiation factor *infA* which was transferred to the nucleus many times independently during angiosperm evolution (26). However, whether or not this is also the case for *rps18* in the protozoan lineage, remains to be investigated experimentally.

Supporting earlier results obtained from loss of plastid antibiotic resistance by loop-out recombination (13), our data on the targeted inactivation of *rps18* demonstrate directly an essential role of plastid translation for cell survival in tobacco. Interestingly, this contrasts the situation in barley (10,11) and *Brassica* (12) where plastid translation appears to be dispensable under heterotrophic growth conditions. In *Brassica*, it has been suggested that the presence of a second, nuclear-encoded acetyl-CoA carboxylase (ACCase) makes plastid translation non-essential by compensating the loss of expression of the plastid-encoded *accD* gene (8). This explanation would be compatible with the non-essentiality of plastid translation in barley, because *accD* is not encoded in the chloroplast genome of cereals which, instead, have a eukaryotic-type ACCase encoded in their nuclear genome (27). However, whether *accD* is the only truly essential gene in the tobacco chloroplast genome and thus can indeed explain the differences in the essentiality of plastid translation between different plant species, remains to be established.

Another interesting observation made in this study was the appearance of unexpected genome conformations which could be explained by flip-flop recombination on the *psbA* 3′-UTR which occurs twice in the transplastomes. It is well established that the two large inverted repeat regions of the chloroplast genome, which contain, for example, the ribosomal RNA operon, constantly undergo homologous recombination, thus producing two isoforms of the chloroplast genome (28,29). However, flip-flop recombination on inverted repeats as short as the *psbA* 3′-UTR (∼200 bp) has not been directly demonstrated before. Is this a peculiarity of the *rps18* knockout lines or a more common phenomenon in chloroplast transformation experiments that has been overlooked before? Interestingly, already the very first chloroplast transformation experiments with chimeric selectable marker genes reported in 1993 produced several lines that showed rearranged plastid genomes (16). Although involvement of the 3′-UTR of the *aadA* marker in the rearrangements was demonstrated, the mechanisms generating the rearranged genomes could not be fully explained (16). We therefore re-analyzed the data and tested whether flip-flop recombination could explain the rearrangements. This was indeed the case, although the fact that the recombined clones could not be brought to homoplasmy could indicate that, in these lines, additional rearrangements (e.g. deletions) had occurred. Whether or not such possible deletions could be explained by genome multimerization and/or template switching during chloroplast DNA replication (30), which could bring short repeats in directly repeated orientation, remains to be investigated.

Taken together, these data indicate that the occurrence of flip-flop recombination could be a common phenomenon in chloroplast transformation experiments and, as plastome-derived 3′-UTRs are required to stabilize mRNAs made from plastid transgenes, probably cannot be avoided entirely. Being aware of this type of recombination occurring in transgenic chloroplast genomes is important for the interpretation of RFLP analyses which are commonly conducted to demonstrate transgene integration and homoplasmy of transplastomic plants. It is also important to know that flip-flop recombination might result in genome instability when, for example, a selectable marker gene cassette with the *psbA* 3′-UTR is integrated into the small single copy region of the plastid genome. In this case, flip-flop recombination between the two *psbA* 3′-UTRs (one in the large single copy region and one in the small single-copy region) would invert one of the inverted repeat regions of the chloroplast genome (Figure 4). This, in turn, would result in the two

inverted repeat regions now being direct repeats which can recombine with each other producing a large deletion in the genome. Thus, the occurrence of flip-flop recombination has important implications for the design of optimum chloroplast transformation vectors that are suitable to produce genetically stable transplastomic plants.

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