Mol Gen Genet (1996) 251:373–380

ORIGINAL PAPER

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Selectable marker recycling in the chloroplast

Received: 2 January 1996 / Accepted: 1 February 1996

Abstract The bacterial gene *aadA* is an important and widely used selectable marker for manipulation of the chloroplast genome through biolistic transformation. Because no other such marker is available, two strategies for recycling of the *aadA* cassette have been developed. One utilizes homologous recombination between two direct repeats flanking the *aadA* cassette to allow its loss under non-selective growth conditions. A second strategy is to perform co-transformation with a plasmid containing a modified, non-essential chloroplast gene and another plasmid in which the *aadA* cassette disrupts a chloroplast gene known to be essential for survival. Under selective growth conditions the first mutation can be transferred to all chloroplast DNA copies whereas the *aadA* insertion remains heteroplasmic. Loss of the selectable marker can be achieved subsequently by growing the cells on non-selective media. In both cases it is possible to reuse the *aadA* cassette for the stepwise disruption or mutagenesis of any gene in the same strain.

Key words *Chlamydomonas reinhardtii* · Chloroplast transformation · *aadA* · Recombination

Introduction

Since the first report of successful particle-gun mediated transformation of the chloroplast of *Chlamydomonas reinhardtii* (Boynton et al. 1988), this technology has been steadily improved. In most cases, the transformants were selected for photo-autotrophic growth by rescue of mutants carrying a deletion in a photosynthetic gene with wild-type DNA fragments (Boynton et al. 1988, 1990). Other approaches have used mutations in the 16S and 23S rRNA genes that confer resistance to antibiotics (Newman et al. 1990) or mutations in the *psbA* gene, which confer resistance to certain herbicides (Boynton et al. 1990). The bacterial *aadA* gene encodes an aminoglycoside adenyl transferase; when fused to the appropriate promoter and 5′UTR on *C. reinhardtii* and tobacco cells resistance to spectinomycin and streptomycin *aadA* is expressed in the chloroplast and confers (Goldschmidt-Clermont 1991; Svab and Maliga 1993). This selectable marker has been shown to be very powerful for chloroplast transformation, facilitating disruption and mutagenesis of any chloroplast gene (Takahashi et al. 1991; Rodday et al. 1995). One limitation for mutagenesis studies of chloroplast genes is that no other selectable drug-resistance marker is available, precluding mutagenesis of different regions of the chloroplast genome in the same strain. It would therefore be very useful to be able to re-use the *aadA* cassette several times. Here we present two different methods for recycling of the *aadA* cassette, allowing its use for sequential transformation of the same strain.

Integration of transforming DNA into the chloroplast genome occurs via homologous recombination (Boynton et al. 1988, 1992). It has been shown that recombination between two direct repeats can occur within the chloroplast of *C. reinhardtii* (Cerutti et al. 1995; Künstner et al. 1995), leading to the loss of the DNA fragment between the two repeats. Here we have used an *aadA* cassette flanked by two direct repeats to introduce a first mutation into the chloroplast genome. Once the mutation is homoplasmic, the transformants can be transferred to growth media lacking antibiotic, allowing excision of the *aadA* cassette to occur and the deletion to become homoplasmic. The resulting strain carries the desired primary mutation but has lost the resistance gene, and one of the two repeats is left in the chloroplast genome (Fig. 1A). A similar strategy had

Communicated by R. G. Herrmann

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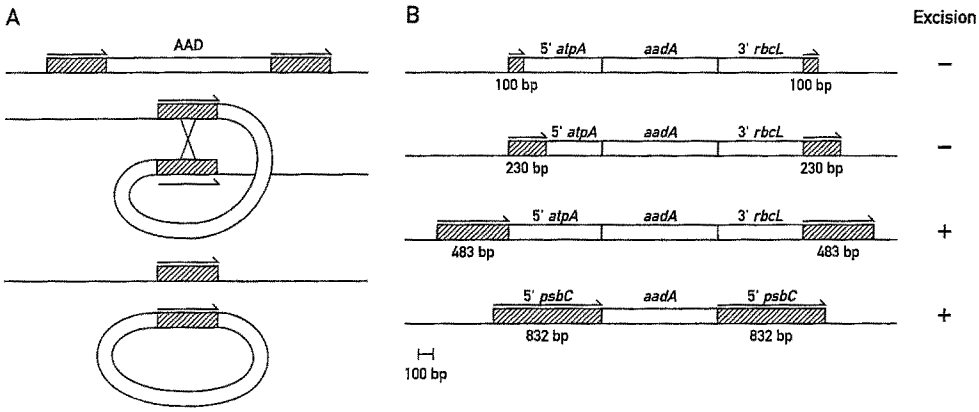

Fig. 1A Excision of *aadA* by homologous recombination. A homologous recombination event between two direct repeats (*hatched bars and arrows*) leads to the excision of the selectable marker *aadA* (*open bar*). **B** Structure of the various constructs containing the *aadA* cassette flanked by two direct repeats. The 100- and 230-bp repeats are part of the *atpA* promoter; the 483-bp repeat originates from the pACYC184 plasmid. The 832-bp repeat includes the *psbC* promoter and 5′ UTR; this promoter in the first repeat drives *aadA* expression.

been successfully used in *Saccharomyces cerevisiae* allowing the repeated use of the *URA3* marker (Alani et al. 1987).

The second strategy adopted to allow recycling of the *aadA* gene exploits the fact that the chloroplast genome of *C. reinhardtii* contains several genes that are essential for cell viability (Goldschmidt-Clermont 1991; Huang et al. 1994). One example is ORF472; previous attempts to disrupt this open reading frame (ORF) with the *aadA* cassette resulted in the production of a heteroplasmic population of chloroplast DNA molecules, containing both the wild-type and the inactivated allele (Goldschmidt-Clermont 1991). We reasoned that this property of ORF472 could be exploited to allow subsequent loss of the *aadA* cassette inserted at that locus, upon relief of the selective pressure. Previous work has revealed that chloroplast cotransformation occurs readily (Roffey and Sayre 1990; Kindle et al. 1991; Newman et al. 1991). It therefore seemed possible that a second plasmid, containing the modified gene would be integrated as well and could possibly be driven to homoplasmicity, following cotransformation with the ORF472::*aadA* plasmid.

Materials and methods

Strains and media

C. reinhardtii wild-type and mutant strains were grown as described by Harris (1989). Where necessary, the media [TRIS-acetate phosphate medium (TAP) and high salt minimal medium (HSM)] were solidified with 2% Bacto agar (Difco) and supplemented with spectinomycin (Sigma).

The recipient strain (suF15) for the essential ORF strategy is a nuclear mutant (F15) affected in the translation of the *psaB* mRNA; this same strain contains a chloroplast suppressor mutation mapped to the 5′ UTR of the *psaB* mRNA (O. Stampacchia, J.-L. Zanasco, W. Zerges, P. Bennoun, J. Girard-Bascou and J.-D. Rochaix, manuscript in preparation).

Plasmids

Five plasmids designed to disrupt the *psaA*, *psaB* and *psaC* genes were constructed with different repeats flanking *aadA*. A 5.8 kb *Eco*RI-*Pst*I fragment from the chloroplast DNA fragment R23 (Rochaix 1978), in which the coding region of the *psaC* gene had been precisely deleted and replaced by a *Bam*HI site using specific oligonucleotides and PCR amplification, was cloned into a pBluescript plasmid (Stratagene). An *aadA* cassette driven by the *atpA* promoter and containing the *rbcL* 3′ region downstream of the coding region (Goldschmidt-Clermont 1991) was cloned at a *Sal*I site 800 bp upstream of the *psaC* gene. The two first transformation vectors were obtained by inserting either a 100-bp or a 230-bp PCR-amplified fragment of the *atpA* promoter in direct orientation at the *Kpn*I site at the 3′ end of the *aadA* cassette. Another plasmid was created by inserting a 483-bp *Nru*I-*Bsp*HI fragment from the pACYC184 plasmid (New England Biolabs) at the *Not*I and *Cla*I sites located at the 3′ and 5′ ends of the *aadA* cassette, respectively (Fig. 1B).

Plasmid cg23 was a gift from W. Zerges (Univ. of Geneva); it is identical to cg20 (Zerges and Rochaix 1994), except that it contains an additional 100 bp of sequence at the 5′ end. This results in placing the *psbC* promoter and 5′ UTR upstream of the *aadA* gene, followed by the *rbcL* 3′ UTR. The *psbC* 5′ region was isolated from plasmid cg23 as an 840-bp *Cla*I-*Nco*I fragment, blunted with Klenow fragment, and cloned back into cg23 cut with *Pst*I and blunted with T4 polymerase; this results in the replacement of the *rbcL* 3′ region by the *psbC* promoter and 5′ UTR (832 bp). The *aadA* gene flanked by the *psbC* repeats (Fig. 1B) was then subcloned as an *Sph*I(blunted)-*Cla*I fragment into pBluescript cut with *Cla*I and *Eco*RV, resulting in plasmid pKR104. This plasmid was cut with *Cla*I and *Xho*I, and the *rbcL* gene was inserted into it as a 2.7-kb *Hin*PI-*Xho*I fragment from chloroplast DNA fragment R15 (Rochaix 1978); the ligation product was cut with *Eco*RI, blunted with T4 polymerase, and a 2.5 kb *Pvu*I-*Bsp*HI blunted fragment from the chloroplast genomic Ba7 fragment (Rochaix 1978) was inserted. The resulting plasmid, pKR115, contains a deletion of the *psaB* gene between the *Bsp*HI and *Hin*PI sites into which the *psbC-aadA-psbC* cassette has been inserted. The chloroplast DNA fragment Ba3 (Rochaix 1978) was subcloned into pBR322, and a 2.4-kb *Afl*II fragment containing the *psaA* exon 3 was removed and replaced by a *Hin*cII-*Eco*RI fragment from pKR104, inserted as a blunted fragment. This deletes the third exon between the *Afl*II sites and replaces it with *psbC-aadA-psbC*, resulting in plasmid pKR114. A 1.9 kb *Pvu*I(blunted)-*Eco*RI fragment from the chloroplast DNA fragment Ba7 (Rochaix 1978) was subcloned into pBluescript cut with *Hin*cII and *Eco*RI. This plasmid

was then cut with *Xba*I and *Sac*I, blunted with T4 polymerase, and ligated to the 2.7-kb *Hin*PI-*Xho*I blunted fragment containing the *rbcL* gene. The resulting plasmid, pKR132, lacks the *psaB* gene from the *Eco*RI to the *Hin*PI site. Plasmid pORF472::*aadA* has been described previously (Goldschmidt-Clermont 1991) and lacks a fragment of ORF472, which is replaced by an insertion of the *aadA* marker.

Chloroplast transformation

Chloroplast transformation in *C. reinhardtii* wild-type cells was carried out as described (Boynton et al. 1988) with a helium-driven particle gun adapted from the one designed by Finer et al. (1992). Wild-type cells were grown at 25° C in liquid TAP medium and plated on TAP plates supplemented with 100 μg/ml spectinomycin. Once the plates were dry, cells were bombarded with tungsten microprojectiles coated with the appropriate plasmid DNA. The bombarded cells were incubated for two weeks at 25° C under dim light (0.5 $\mu E/m^2/s$). Primary transformants were restreaked three times on higher spectinomycin concentrations (up to 500 μg/ml) and characterized.

Nucleic acid techniques

Procedures for the preparation of recombinant DNA plasmids and DNA amplification by polymerase chain reaction were as described (Sambrook et al. 1989). The bacterial host used was *E. coli* DH5α. *C. reinhardtii* total DNA was isolated as described previously (Rochaix et al. 1988). Southern blotting and hybridization were carried out as described (Southern 1975; Sambrook et al. 1989).

Results

Direct repeat method

Five plasmids were constructed to disrupt the *psaA*, *psaB* and *psaC* chloroplast genes. These constructs were introduced into wild-type *C. reinhardtii* chloroplasts via biolistic transformation. Analysis of dark-adapted transformant colonies showed that most of them displayed fluorescence transients characteristic of photosystem I (PSI)-deficient mutants (data not shown) (Chua et al. 1975; Bennoun et al. 1977). This was expected because the products of the targeted genes are core components of the PSI complex. PSI-deficient colonies were restreaked three times on plates containing spectinomycin and grown in dim light. Total genomic DNA was isolated and used for Southern blot hybridization and PCR amplification to examine both the disruption of the target gene and the presence of the *aadA* cassette (see Figs. 2, 3).

The three plasmids designed for disrupting *psaC* all lack its coding region, which is replaced by a *Bam*HI site. They carry an *aadA* cassette inserted 800 bp upstream of *psaC* and flanked by repeats of different lengths and origins. Two of them carried 100-bp or 230-bp repeats of the *atpA* promoter, while the third contained 483-bp repeats from the pACYC184 plasmid (Fig. 1B; see Materials and methods for details). These constructs gave different results with regard to the frequency of loss of *aadA*. The cassettes flanked by the 100-bp and 230-bp repeats of the *atpA* promoter did not allow efficient excision of *aadA* and generated no Spc^S colonies. In contrast, the cassette with the 483-bp repeats from pACYC184 led to the efficient loss of *aadA*. Colonies homoplasmic for the deletion of *psaC* were transferred to TAP plates (nonselective media) and then grown in liquid TAP media without antibiotic. Cells were plated to obtain single colonies and the sensitivity to spectinomycin was tested by replica plating the cells to TAP plates supplemented with 500 μg/ml spectinomycin. None of the 10 000 colonies screened that contained the *aadA* cassette with the 100-bp and 230-bp *atpA* repeats became sensitive to spectinomycin even after long growth periods on non-selective media. This result suggests that recombination between these repeats occurs at very low frequency, if at all. Approximatively 40% of the colonies containing the construct with the 483-bp repeats did not grow on TAP containing 500 μg/ml spectinomycin after three rounds of growth on TAP plates without antibiotic. Selected colonies were restreaked once more on TAP plates and most of them then became sensitive to 100 μg/ml spectinomycin. PCR analysis of genomic DNA, and Southern blot analysis showed that these cells had lost all the copies of the *aadA* cassette and that one pACYC184 repeat was left in the chloroplast genome (Fig. 2). The conditions used for PCR amplification allow detection of a single copy of a gene per cell (data not shown; see also Fig. 3B, lane WT 1/100).

We also constructed two plasmids to delete the *psaA* third exon (*psaA-3*) and *psaB* gene. They contain the *aadA* gene flanked by two copies of the *psbC* promoter and 5′ UTR (Fig. 1B), thus creating an 832-bp direct repeat. After biolistic transformation with these plasmids, Spc^R transformants were selected and propagated as described above. The selective pressure was released after the homoplasmic condition of the deletions of *psaA-3* and *psaB* had been verified by PCR (Fig. 3B) and Southern analysis (data not shown). After a single passage on TAP plates, greater than 90% of the transformants could no longer grow on 500 μg/ml spectinomycin. After two more passages on TAP plates, greater than 90% of the transformants could no longer form colonies when replica plated to plates containing 150 μg/ml spectinomycin. After one further round of growth on TAP, the Spc^S colonies were tested by plating approximately 10^7 cells on plates containing 100 μg/ml spectinomycin; none formed any colonies.

PCR analysis demonstrates that the starting Spc^R transformants had already lost either *psaA-3* or *psaB*, but still retained the *aadA* gene (Fig. 3B). After they had become sensitive to 100 μg/ml spectinomycin (Spc^S), no copies of the *aadA* gene could be detected (Fig. 3B). Southern analysis of the Spc^S strains indicated that the excision had occurred exactly as expected, leaving behind a single copy of the *psbC*

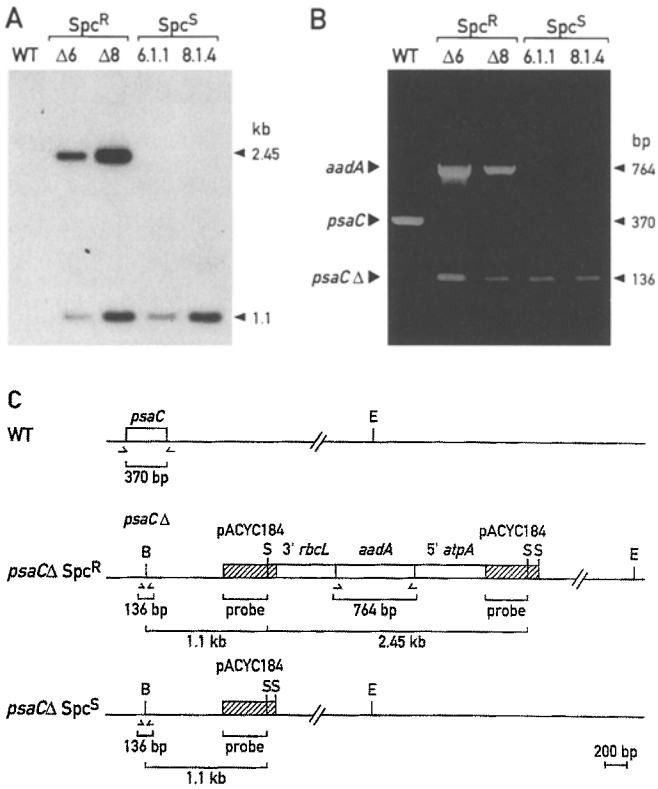

Fig. 2A–C Deletion of the *psaC* gene. **A** Southern analysis performed on total genomic DNA from wild-type cells, transformants deleted for the *psaC* gene (lanes Δ6 and Δ8), and the same transformants after four rounds of growth on non-selective medium (lanes 6.1.1 and 8.1.4). The DNA was digested with *Bam*HI and *Sal*I restriction enzymes, separated by agarose gel electrophoresis, blotted onto Hybond N+ (Amersham) membrane and hybridized with a radioactive probe containing a *Sal*I-*Sac*I fragment of the 483-bp repeat (Fig. 1). **B** Ethidium bromide-stained agarose gels of PCR amplifications performed with two pairs of primers (for *psaC* and *aadA*) on total genomic DNA of the same strains as in **A**. Lane WT, expected (370-bp) product of *psaC* amplification; lanes Δ6 and Δ8 expected 764-bp and 136-bp products of amplification of *aadA* and the deletion derivative of *psaC*; lanes 6.1.1 and 8.1.4, expected 136-bp product of amplification of the deleted *psaC*; no *aadA* copy is left in the genome. **C** Predicted structure of the chloroplast genome in the *psaC* region in the different strains analysed. Primers used for PCR (*arrows*), the probe used for Southern hybridisation and expected DNA fragment sizes after digestion or PCR amplification are indicated. Restriction enzyme site abbreviations are: E, *Eco*RI; S, *Sal*I; B, *Bam*HI

sequence (Fig. 3A). As the probe consisted of the *psbC* repeat, it was detected in both the wild-type and the mutant strains at the expected size for the *psbC* locus (36 kb, the size of the Bam1 fragment; Harris 1989). Note that in the *Eco*RI digests of the *psaB*Δ mutants, two fainter 4.6- and 3.0-kb bands are visible, one above the *psbC* band and the other below the *psaB*Δ band. We believe that these result from recombination between the *psbC* locus and the *psaB*Δ locus, mediated by the 832-bp *psbC* sequence left at *psaB*Δ (see Discussion). The fragment sizes are consistent with this hypothesis, and they are inconsistent with other explanations, such as persistence of the transformed plasmids (data not shown). We do not observe extra bands in the *psaA-3*Δ mutants. However, a similar recombination event between *psbC* and *psaA*-3Δ would have produced new *Bam*HI fragments of approximately 20–21 kb, and these would probably not have been resolved from the 36-kb band under our conditions of electrophoresis.

Essential ORF method

The strategy adopted for this method is outlined in Fig. 4. We co-transformed the strain with two plasmids. Plasmid pORF472::*aadA* contains a disruption of ORF472 due to insertion of the *aadA* gene (Goldschmidt-Clermont 1991). Plasmid pKR132 contains regions flanking the *psaB* gene on both sides, integration of which should result in deletion of the entire *psaB* ORF from the chloroplast genome. The ratio of pKR132 to pORF472::*aadA* was 2:1, to increase the possibility of cointegration of the non-selected plasmid. Transformants were selected on TAP plates containing 100 μg/ml spectinomycin. Single colonies were restreaked five times on higher concentrations of spectinomycin (up to 300 μg/ml). Analysis of the fluorescence transients of different clones was used to identify the *psaB* deletion candidates. Out of 40 initial transformants analyzed, six had a fluorescence profile typical of PSI mutants. These candidates were further restreaked, and PCR analysis was undertaken to assess the degree of homoplasmicity of the *psaB* deletion.

Another round of restreaking produced clones that had completely lost the *psaB* gene (Fig. 5A); even under conditions that allowed us to detect a single wild-type copy per cell by PCR analysis (Fig. 5A, WT lane), no *psaB* signal could be detected (compare the WT lane with the Spc^R lanes). Control primers for the *psaC* and *aadA* genes gave the expected bands (370 and 764 bp, respectively). Southern analysis (Fig. 5B) showed that ORF472 is present as two distinct alleles in the Spc^R clones: one is the wild-type allele, and the other is the allele inactivated by the *aadA* insertion. The Southern analysis also shows that the deletion of the *psaB* gene has reduced the size of the R15 chloroplast DNA fragment from 5.8 (WT lane) to 2.8 kb (Spc^R lanes). The same two clones were then inoculated in liquid TAP medium without the antibiotic, to allow loss of the *aadA* cassette to occur. After growth to saturation, single colonies were streaked from the liquid culture. They were then tested for spectinomycin sensitivity on 100 μg/ml spectinomycin. All the clones tested were Spc^S, indicating that the loss of the inactivated allele of ORF472 occurs rapidly in non-selective conditions. When 10^7 cells were plated onto medium containing 100 μg/ml spectinomycin, no resistant colonies appeared. Complete loss of the *aadA* cassette was confirmed by PCR and Southern analysis of the Spc^S

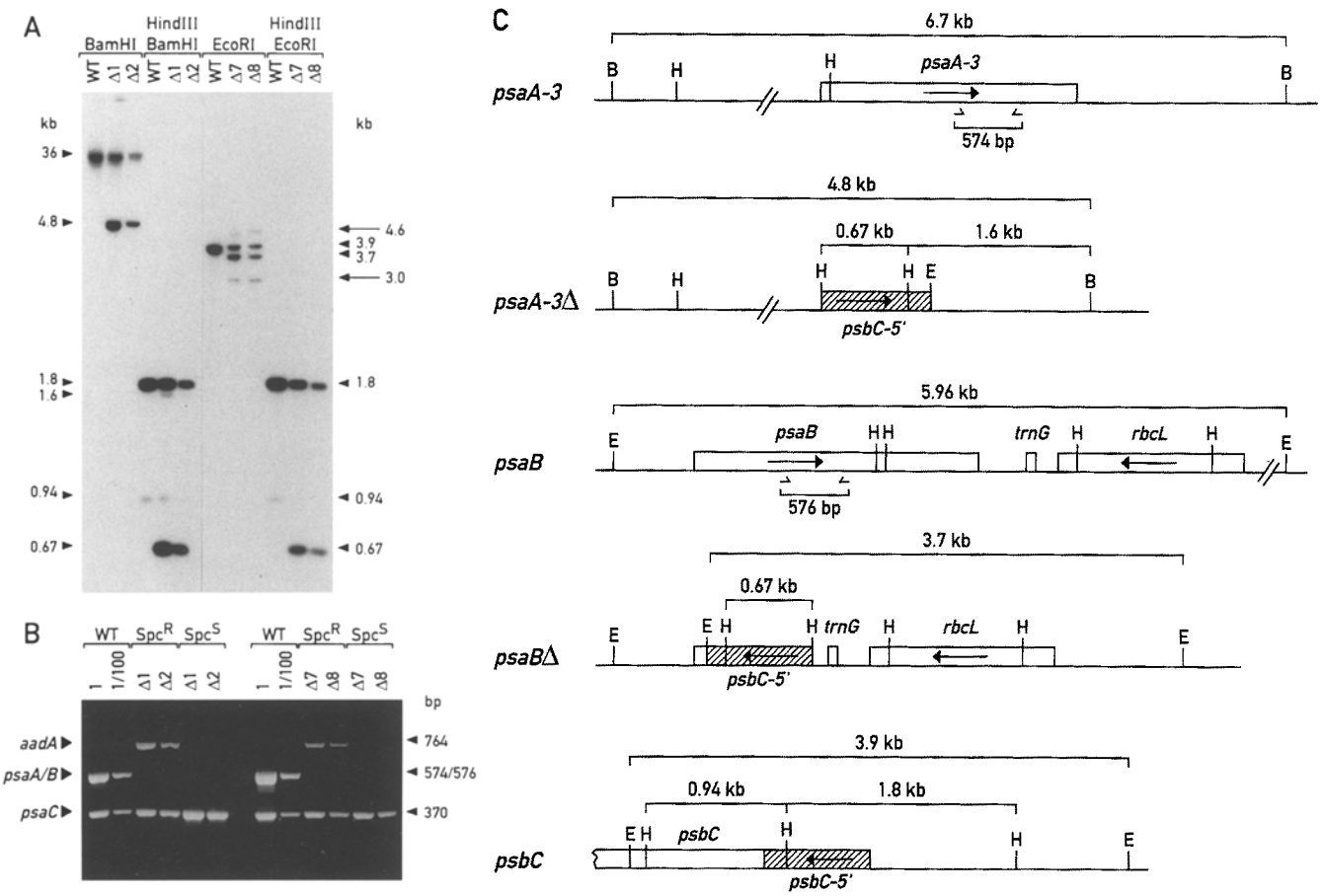

Fig. 3A–C Deletion of the *psaA* and *psaB* genes. **A** Southern analysis performed on total genomic DNA from wild-type cells and two independent transformants carrying a deletion of either *psaA-3* (Δ1 and Δ2) or *psaB* (Δ7 and Δ8) after they had become Spc^S. The DNA was digested with *Bam*HI (for *psa*AΔ) or *Eco*RI (for *psaB*Δ), with or without *Hin*dIII. Blotting was performed as in legend to Fig. 2, except that the 832-bp *psbC* promoter-5′ UTR sequence was used as a probe. The sizes of the bands are indicated on each side. **B** Ethidium bromide-stained agarose gels of PCR amplifications performed on total genomic DNA with two pairs of primers for *psaC* and *aadA* plus a pair of primers specific for either *psaA-3* or *psaB*. The DNAs used were the same as those in **A**, except that the "Spc^R" DNAs orginated from the same transformants after they had lost the respective PSI gene but before they had evicted *aadA*. The 1/100 lanes contain wild-type DNA diluted 100-fold to demonstrate that even very small amounts (less than 1 copy per cell, on average) of either gene could be easily detected. The primers for *psaC*, which serves as a positive internal PCR control, and *aadA* are the same as in Fig. 2. The primers for *psaA-3* and *psaB* amplify products of 574 or 576 bp, respectively. **C** Predicted structure of the chloroplast genome around the *psaA-3*, *psaB*, and *psbC* genes before and after the deletion events have taken place. The probe used for Southern hybridization (psbC-5) and expected DNA fragment sizes after digestion or PCR amplification are indicated. Restriction enzymes abbreviations are as in Fig. 2, except for H (*Hin*dIII)

clones (Fig. 5A, B). The *aadA*-specific PCR product was lost in the Spc^S clones (Fig. 5A), and the 3.2-kb *Eco*RI fragment characteristic of the pORF472::*aadA* allele was not detected by Southern analysis (Fig. 5B). As expected, the Spc^S clones retained the band characteristic of the *psaB* deletion.

Transformants which had lost the *aadA* cassette using either the direct repeat method or the essential ORF strategy could be easily retransformed with the *aadA* expression cassette (data not shown).

Discussion

Two different strategies have been used to recycle the chloroplast selectable marker *aadA*. One takes advantage of homologous recombination between two direct repeats, which leads to the excision and loss of the DNA fragment located in between. Different repeat lengths have been tested; repeats up to 230 bp did not recombine at a sufficient frequency to allow the recovery of Spc^S cells. Ceruti et al. (1995) were readily able to observe recombination between direct repeats of 216 bp. However, in this case, the recombination event resulted in the restoration of *chlL*, a gene required for light-independent chlorophyll synthesis. The consequent appearance of green sectors in dark-grown colonies did not require that the recombination product become homoplasmic. Here, in contrast, all copies of the *aadA* cassette must be lost to abolish

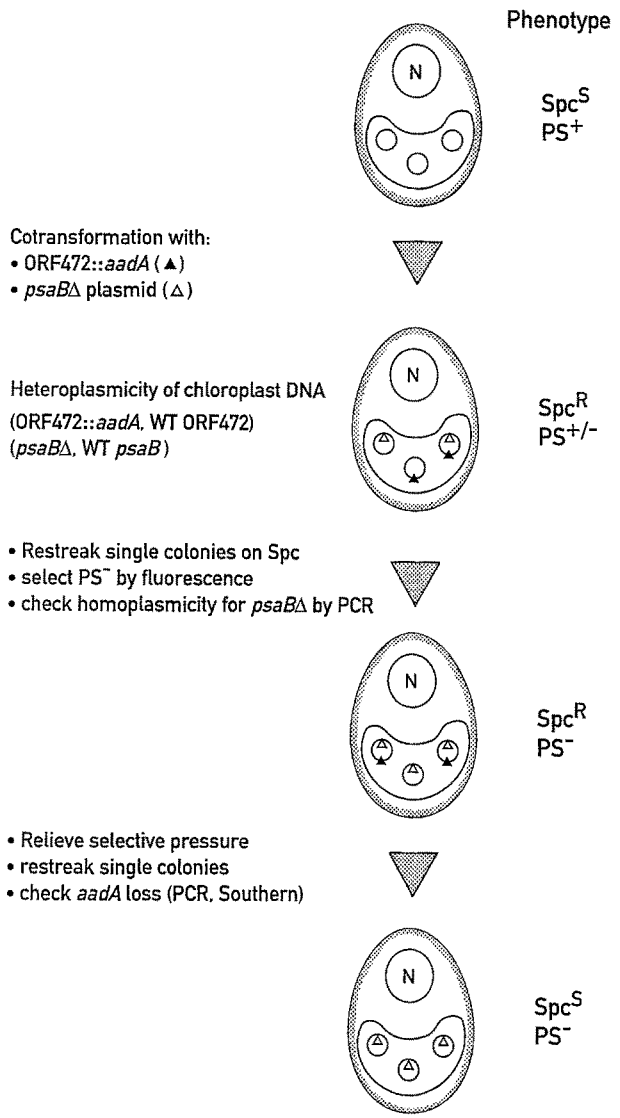

Fig. 4 Strategy for creating chloroplast DNA deletions by co-transformation with ORF472:*aadA*. Oval-shaped *Chlamydomonas* cells are drawn with nucleus (N) and chloroplast with its genome (*circles*). *Filled and open triangles* represent ORF472::*aadA* and *psaB*Δ in the chloroplast genome, respectively

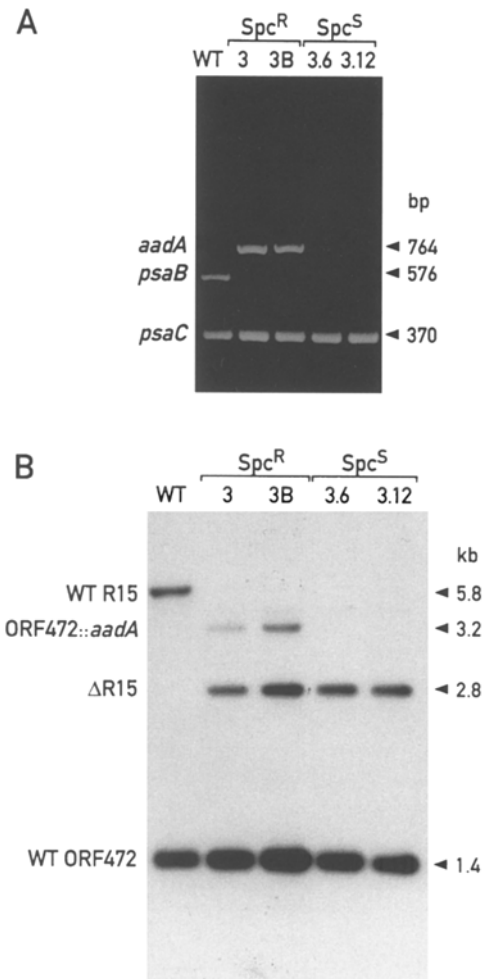

Fig. 5A, B Deletion of the *psaB* gene by co-transformation with ORF472:*aadA*. **A** Ethidium bromide-stained agarose gels of PCR amplifications performed on total genomic DNA from wild-type cells and two independent transformants carrying deleted for *psaB* and either still Spc^R, or Spc^S after the loss of the *aadA* cassette. Lane WT contains wild-type DNA mixed in a proportion of 1:100 with DNA from one of the *psaB*Δ strains, to indicate that the reaction is able to detect even a single wild-type copy in an otherwise mutant background. All the PCR reactions were performed with a mixture of three primer pairs specific for *psaC* (as in Fig. 2), *aadA* (as in Fig. 2), and *psaB* (as in Fig. 3). **B** Southern analysis performed on total genomic DNA digested with *Eco*RI from a wild-type strain, two independent Spc^R clones and the same clones after loss of the *aadA* cassette. The probe used is a gel-purified restriction fragment containing *aadA*, the 3′ UTR of *rbcL* and a part of ORF472. This allows one to detect simultaneously the wild-type ORF472, the ORF472::*aadA* disruption, and the wild-type and partially deleted R15 fragments

spectinomycin resistance. The 483-bp and 832-bp repeats allowed rapid excision of the selectable marker. Thus, there appears to exist a threshold length between 230 bp and 480 bp that allows rapid excision of *aadA* and efficient production of Spc^S cells. The fact that no Spc^S colonies appeared even after growing the cells transformed with the 230-bp *atpA* construct for several weeks on TAP plates, suggests that the relationship between efficiency of recombination and size of the repeats is not a linear one. However, since the longer and shorter repeats had different sequences, we cannot rule out some dependence of the efficiency of the excision/recombination on the nucleotide sequence.

Our results obtained with the transformants containing the 832-bp repeat from the *psbC* promoter-5′UTR suggest that recombination occurred between this repeat and the endogenous copy of the *psbC* gene located on the other side of the genome (>60 kb away). Such a recombination event would either fragment the chromosome or convert the large inverted repeats of the chloroplast genome into direct repeats, which could then lead to cleavage of the genome by recombination between them. This might explain why the

recombination products were present in such low amounts (Fig. 3A). Svab and Maliga (1993) have also observed the deletion of a 26 kb region of the chloroplast genome in tobacco transformants, mediated by a 0.4 kb repeated sequence from the *psbA* 3′ UTR. One advantage of the 483-bp repeats from the pACYC184 plasmid is that this DNA fragment cannot recombine with any endogenous DNA, preventing the generation of undesired deletions or inversions.

The second strategy we employed is based on the presence of essential ORFs in the chloroplast genome. Inactivation of such genes with the *aadA* selectable marker does not produce a chloroplast genome homoplasmic for the deletion, but rather a mixture of inactivated and wild-type copies of the essential ORF. A co-transformation strategy that involves two different plasmids (one containing the inactivated ORF, the other containing the deletion of the gene of interest) allows one to select for transformants and later provides an easy way to get rid of the selectable marker. Since it has also been shown in higher plants that co-tranformation with a selectable marker and an unselectable, unlinked gene is feasible at reasonable frequency (Carrer and Maliga 1995), this strategy could also be applied to higher plant plastids. It is not immediately clear why this strategy was capable of producing a population of chloroplast DNA molecules homoplasmic for the deletion of *psaB*. The initial co-transformation event presumably produces a heterogeneous population of DNA molecules comprising a mixture of wild-type, ORF472::*aadA*, *psaB*Δ, and ORF472::*aadA*–*psaB*Δ genomes (see Fig. 4 for a schematic view). Through several single-colony purification steps on antibiotic-containing medium, we were able to obtain a subset of clones that had completely lost the *psaB* ORF, i.e. in which the chloroplast molecules containing the deletion had become homoplasmic. As expected from previous work (Goldschmidt-Clermont 1991), the ORF472::*aadA*-containing genomes remained heteroplasmic in these clones (see Fig. 5B), implying that the fraction of the chloroplast DNA molecules that initially contained both ORF472::*aadA* and the *psaB* deletion did not simply become homoplasmic. As there must exist two populations of chloroplast genomes as long as selection is maintained (i.e., one with ORF472 and one with ORF472::*aadA*), the population with ORF472 must eventually become homoplasmic with respect to the state of *psaB*. This process probably occurs by the same mechanisms that enable a homoplasmic state to be reached following conventional chloroplast transformation (Boynton et al. 1992), and may involve a combination of intergenomic recombination and random segregation of chloroplast genomes. Once the selective pressure is released, it is the ORF472 population of genomes that becomes homoplasmic.

These two strategies represent two complementary methods for the removal of introduced DNA sequences from the chloroplast of *C. reinhardtii*. The direct repeat method has the advantage of allowing for straightforward selection for the integration event by exploiting the *aadA* cassette. This achieves homoplasmicity of the integration faster and more reliably than in the essential ORF method, which relies upon an indirect selection. Also, in the latter method it is necessary to analyze a larger number of transformants in the initial stage of the screening. On the other hand, the essential ORF method allows for the production of a "clean" deletion event, without any foreign sequences left at the deletion site. This allows for the serial modification of several loci without undesirable rearrangements caused by carry-over of repeat sequences. Several plasmids containing essential ORFs inactivated with the *aadA* cassette have been constructed in different laboratories (Table 1), and could be readily used for co-transformation, although we have not tested them.

Once the desired mutation is homoplasmic, both approaches allow rapid loss of the *aadA* marker in the absence of selection. The loss is dependent on the size of the repeats in the case of the direct repeat method, and the presence of short direct repeats does not lead to loss of *aadA*. It is interesting to compare the efficiency of the homologous recombination event in the chloroplast of *C. reinhardtii* to the same process in the nucleus of *S. cerevisiae* (Alani et al. 1987): the reuse of the *URA3* marker is well established in the latter system, and it occurs at a frequency of around 10^{-4} with a size of the repeats around 1.2 kb. Although a direct comparison is not possible, since the chloroplast genome is a polyploid genome (with a copy number of about 80), we found around 40% of homoplasmic deletion events after only three passages on non-selective medium with a repeat size of only 483 bp. We were also able to observe the product of the recombination between the *psbC* direct repeats by Southern analysis even before the selective pressure had been released (data not shown). This suggests that homologous recombination is an extremely efficient process in the chloroplast of *C. reinhardtii*.

The *aadA* cassette has been shown to be a useful tool for disrupting genes and introducing site-directed mutations anywhere in the chloroplast genome of *C. reinhardtii*. The ability to recycle this marker allows the stepwise introduction of different mutations into the same strain. It is also possible to generate strains carrying a disruption of a particular gene that can then serve as recipient strains for the introduction of site-directed or randomly mutated versions of the same

Table 1 Essential chloroplast genes

Gene	Reference
ORF472	Goldschmidt-Clermont (1991)
ClpP	Huang et al. (1994)
Rpo B1, Rpo B2, Rpo C2	Rochaix J-D, unpublished results

gene. In such strains only the mutated gene would be expressed, eliminating the need for verification of homoplasmicity of the chloroplast genome. The *aadA* cassette had already opened the door for chloroplast reverse genetics, a powerful way of studying chloroplast gene function and expression in *C. reinhardtii* and higher plants. Recycling and re-use of this cassette allows much faster generation and analysis of mutants as well as multiple gene disruptions, making this tool even more versatile. It is also possible to imagine other, more general uses for these methods to transiently introduce desirable DNA sequences into the chloroplast genome and induce their loss once their usefulness is exhausted.

Acknowledgments The authors wish to thank W. Zerges and M. Goldschmidt-Clermont for supplying plasmids and for advice, N. Rolland (CNRS, Grenoble) for initially suggesting the essential ORF method and N. Roggli for preparing the figures. This work was supported by grant 31.34014.92 from the Swiss National Fund to J.-D. Rochaix. K. Redding received support from the Human Frontier Science Program and from an NSF Plant Biology Postdoctoral Fellowship.

References

Alani E, Cao L, Kleckner N (1987) A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics 116:541–545

Bennoun P, Girard J, Chua N-H (1977) A uniparental mutant of *Chlamydomonas reinhardtii* deficient in the chlorophyll-protein complex CP1. Mol Gen Genet 153:343–348

Blowers AD, Bogorad L, Shark KB, Sanford JC (1989) Studies on *Chlamydomonas* chloroplast transformation: foreign DNA can be stably maintained in the chromosome. The Plant Cell 1:123–132

Boynton JE, Gilham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark KB, Sanford JC (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240:1534–1538

Boynton JE, Gilham NW, Harris EH, Newman SM, Randolph-Anderson BL, Johnson AM, Jones AR (1990) Manipulating the chloroplast genome of *Chlamydomonas*: molecular genetics and transformation. Curr Res Photosynth 3:509–516

Boynton JE, Gillham NW, Newman SN, Harris EH (1992) Organelle genetics and transformation of *Chlamydomonas*. In: Herrmann RC (ed) Cell organelles (Advances in Plant Gene Research, vol. 6). Springer-Verlag, Vienna

Carrer H, Maliga P (1995) Targeted insertion of foreign genes into the tobacco plastid genome without physical linkage to the selectable marker gene. BioTechnology 13:791–794

Cerutti H, Johnson AM, Boynton JE, Gilham NW (1995) Inhibition of chloroplast DNA recombination and repair by dominant negative mutants of *Escherichia coli* RecA. Mol Cell Biol 15:3003–3011

Chua N-H, Matlin K, Bennoun P (1975) A chlorophyll-protein complex lacking in photosystem I mutants of *Chlamydomonas reinhardtii*. J Cell Biol 67:361–377

Finer JJ, Vain P, Jones MW, McMullen MD (1992) Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep 11:323–328

Goldschmidt-Clermont M (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of *Chlamydomonas*. Nucleic Acid Res 19:4083–4089

Harris EH (1989) The *Chlamydomonas* sourcebook. Academic Press, San Diego

Huang C, Wang S, Chen L, Lemieux C, Otis C, Turmel M, Liu XQ (1994) The *Chlamydomonas* chloroplast *clpP* gene contains translated large insertion sequences and is essential for cell growth. Mol Gen Genet 244:151–159

Kindle KL, Richards KL, Stern DB (1991) Engineering the chloroplast genome: techniques and capabilities for chloroplast transformation in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 88:1721–1725

Künstner P, Guardiola A, Takahashi Y, Rochaix J-D (1995) A mutant strain of *Chlamydomonas reinhartii* lacking the chloroplast photosystem II *psbI* gene grows photoautotrophically. J Biol Chem 270:9651–9654

Newman SM, Boynton JE, Gilham NW, Randolph-Anderson BL, Johnson AM, Harris EH (1990) Transformation of chloroplast ribosomal RNA genes in *Chlamydomonas*: molecular and genetic characterization of integration events. Genetics 126:875–888

Newman SM, Gilham NW, Harris EH, Johnson AM, Boynton JE (1991) Targeted disruption of chloroplast genes in *Chlamydomonas reinhardtii*. Mol Gen Genet 230:65–74

Rochaix J-D (1978) Restriction endonuclease map of the chloroplast DNA of *Chlamydomonas reinhardtii*. J Mol Biol 126:597–617

Rochaix J-D, Mayfield S, Goldschmidt-Clermont M, Erickson J (1988) Molecular biology of *Chlamydomonas*. In: Shaw CH (ed) Plant molecular biology: a practical approach. IRL Press, Oxford, pp 253–275

Rodday SM, Webber AN, Bingham SE, Biggins J (1995) Evidence that the Fx domain in photosystem I interacts with the subunit PsaC: site-directed changes in PsaB destabilize the subunit interaction in *Chlamydomonas reinhardtii*. Biochemistry 34:6328–6334

Roffey R, Sayre R (1990) Isolation of site-specific chloroplast *psbA* mutations in *Chlamydomonas* having null phenotypes via cotransformation. Plant Physiol 93 (Suppl):22

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Svab Z, Maliga P (1993) High efficiency plastid transformation in tobacco by selection for chimeric *aadA* gene. Proc Natl Acad Sci USA 90:913–917

Takahashi Y, Goldschmidt-Clermont M, Soen S-Y, Franzen L-G, Rochaix J-D (1991) Directed chloroplast transformation in *Chlamydomonas reinhardtii*: insertional inactivation of the *psaC* gene encoding the iron sulfur protein destabilizes photosystem I. EMBO J 10:2033–2040

Zerges W, Rochaix J-D (1994) The 5′ leader of a chloroplast mRNA mediates the translational requirements for two nucleus-encoded functions in *Chlamydomonas reinhardtii*. MCB 14:5268–5277