Mol Gen Genet (2000) 263: 404–410
© Springer-Verlag 2000

ORIGINAL PAPER

J. M. Bateman · S. Purton

Tools for chloroplast transformation in *Chlamydomonas*: expression vectors and a new dominant selectable marker

Received: 26 October 1999 / Accepted: 28 December 1999

Abstract Reverse-genetic studies of chloroplast genes in the green alga *Chlamydomonas reinhardtii* have been hampered by the paucity of suitable selectable markers for chloroplast transformation. We have constructed a series of vectors for the targeted insertion and expression of foreign genes in the *Chlamydomonas* chloroplast genome. Using these vectors we have developed a novel selectable marker based on the bacterial gene *aphA-6*, which encodes an aminoglycoside phosphotransferase. The *aphA-6* marker allows direct selection for transformants on medium containing either kanamycin or amikacin. The marker can be used to inactivate or modify specific chloroplast genes, and can be used as a reporter of gene expression. The availability of this marker now makes possible the serial transformation of the chloroplast genome of *Chlamydomonas*.

Key words *Chlamydomonas* · Chloroplast transformation · *aphA-6* · Selectable marker

Introduction

Since the first demonstration of chloroplast transformation (Boynton et al. 1988), the field of chloroplast genetic engineering has developed rapidly. Transformation involves the high-velocity bombardment of cells with DNA-coated microprojectiles, and has been successfully applied to the green unicellular alga *Chlamydomonas reinhardtii* and to several higher plant species (reviewed by Erickson 1996). Early selection methods for *Chlamydomonas* transformants were based on the rescue of chloroplast deletion mutants using wild-type DNA fragments, or the introduction of endogenous genes carrying point mutations that confer resistance to antibiotics or herbicides (see Boynton and Gillham 1993). A major breakthrough came with the development of a portable dominant marker based on the bacterial gene *aadA*, which encodes an aminoglycoside adenyltransferase. The *aadA* coding region was fused to the promoter and untranslated regions (UTRs) of endogenous chloroplast genes and shown to confer resistance to spectinomycin and streptomycin when introduced into the organellar genome of *Chlamydomonas* (Goldschmidt-Clermont 1991) and higher plants (Svab and Maliga 1993; Sikdar et al. 1998; Sidorov et al. 1999). This "aadA cassette" has been used extensively for the disruption or modification of numerous chloroplast genes, and as a reporter of gene expression (Rochaix 1997; Goldschmidt-Clermont 1998). Additional selectable markers have been developed for plastid transformation in higher plants. These include a kanamycin-resistance marker (Carrer et al. 1993), a negative selection marker based on the gene for cytosine deaminase (Serino and Maliga 1997), and a dual marker comprising *aadA* fused to the gene for green fluorescent protein (Khan and Maliga 1999). Unfortunately, similar progress in the development of new dominant markers for transformation of *Chlamydomonas* has not been forthcoming, and the *aadA* cassette remains the only available marker. This has complicated attempts to manipulate different regions of the chloroplast genome by serial transformation. To circumvent this problem, Fischer et al. (1996) devised an elegant, but elaborate, technique for recycling the *aadA* cassette, in which the removal of antibiotic selection after recovery of transformants results in loss of the cassette from the genome. This approach has proved successful for the generation of novel deletion mutants (Redding et al. 1998) but cannot be applied to existing *aadA* transformants. There is therefore a need for additional dominant markers

Communicated by R. G. Herrmann

J. M. Bateman[1] · S. Purton (✉)
Department of Biology, University College London,
Gower Street, London, WC1E 6BT, UK
Tel.: +44-171-5042675; Fax: +44-171-380709
E-mail: s.purton@ucl.ac.uk

Present address:
[1] Department of Molecular Biology,
University of Texas, Southwestern Medical Center,
Dallas, TX 75235-9148, USA

similar to *aadA*, that confer drug resistance and are portable to any site on the chloroplast genome.

In this paper we describe the construction of a set of integration/expression vectors that allow the targeting of foreign genes to an intergenic site within the *Chlamydomonas* chloroplast genome, and their expression from three different endogenous promoters. Using these vectors we have successfully introduced into the genome the bacterial gene *aphA-6*, which codes for an aminoglycoside phosphotransferase, and shown that the gene confers kanamycin and amikacin resistance on the transformants. We demonstrate that the *aphA-6* gene can function as a selectable marker for transformation and can be used as a portable cassette for gene disruption.

Materials and methods

Strains and media

All recombinant DNA work was carried out in the *Escherichia coli* strain DH5α (Sambrook et al. 1989). The wild-type *C. reinhardtii* strain CC-1021 mt+ is derived from isolate 137c (Harris 1989), and was obtained from the Chlamydomonas Genetics Center at Duke University (Chapel Hill, N.C.). The *psbH*::*aadA* disruption strain, Bst-opp, has been described previously (O'Connor et al. 1998). Cells were grown on TRIS-acetate phosphate (TAP) medium or TRIS-minimal (Tris-min) medium (Rochaix et al. 1988), supplemented with kanamycin monosulphate, amikacin or spectinomycin where required. All antibiotics were purchased from Sigma. Cultures were incubated in the light (45 μE/m^2/s PAR) at 25 °C.

Construction of chloroplast transformation vectors

Plasmid p72B was obtained from the Chlamydomonas Genetics Center and contains a 4.5-kb *Eco*RI fragment (Eco19) of the *C. reinhardtii* chloroplast genome inserted in pUC8. To eliminate unwanted restriction sites in the insert, p72B was cut with *Sph*I and *Hin*dIII, the overhangs were filled using Klenow enzyme and the plasmid was religated to give p72B-SH, which retains the 3.2-kb E*co*RI-*Sph*I region of Eco19 (Fig. 1). The *rbcL* 5′/3′ expression cassette was amplified by PCR from the plasmid rbcX (Goldschmidt-Clermont 1991), using mismatch primers, RBCX5 and RBCX3, that incorporate an *Mlu*I site at the 5′ end of the cassette and a *Bss*HII site at the 3′ end. The PCR product was digested with these two enzymes and ligated into the unique *Mlu*I site downstream of *psbH* in p72B-SH, selecting for recombinants that carry the cassette in the opposite orientation to *psbH*. Finally, the unique *Hin*dIII site between the 5′ and 3′ *rbcL* elements was destroyed by filling in the overhangs using Klenow enzyme and religating, to give the expression vector p72-rbcL (Fig. 1). Two other expression vectors, p72-atpB and p72-psbA, were made by replacing the *Mlu*I-*Nco*I region of p72-rbcL with the corresponding 5′ region from *atpB* and *psbA*, respectively. For p72-atpB, a 397-bp fragment from *atpB* (Woessner et al. 1986) containing upstream sequence, the 5′-untranslated leader and the first five codons, was amplified by PCR from total wild-type DNA using primers ATPB5 and ATPB3. For p72-psbA, a similar 267-bp fragment from *psbA* (Erickson et al. 1984) was amplified using primers PSBA5 and PSBA3. In both cases, an *Mlu*I site was introduced at the 5′ end of the fragment and an *Nco*I site at the 3′ end. The coding sequence from *aphA-6* was amplified by PCR from the plasmid pAT235 (Martin et al. 1988) using primers APHA5 and APHA3. Using the *Nco*I and *Pst*I sites incorporated into APHA5 and APHA3, respectively, the amplified gene was ligated into the multiple cloning region of each of the three expression vectors to give p72-rbcL-aphA6, p72-atpB-aphA6 and p72-psbA-aphA6. The chimeric 5′psbA::aphA-6::3′rbcL cassette in p72-psbA-aphA6 was amplified by PCR using primers MLU5 and MLU3, and cloned into the *Eco*RV site of pBluescript SK+ (Stratagene) to create pSK.KmR. In order to create additional sites flanking the cassette, *Sma*I and *Bam*HI sequences were introduced into MLU3 (see Fig. 4A). The *aphA-6* cassette was used to disrupt the *psbH* gene on p72B-SH by excising the cassette from pSK.KmR using *Sma*I, and inserting it, in the opposite orientation, into the unique *Bst*XI site (Klenow-filled) within the *psbH* coding region (Fig. 4B). All *E. coli* transformants containing *aphA-6* were selected on LB agar containing 50 μg/ml ampicillin and 15 μg/ml kanamycin, and grown in LB liquid medium containing either 50 μg/ml ampicillin or 10 μg/ml kanamycin.

Full details of the primer sequences used in the construction of the plasmids, together with the complete sequence of pSK.KmR. can be found at http://www.ucl.ac.uk/biology/prg.htm.

Chloroplast transformation

Chloroplast transformation using the *psbH* marker was carried out as described by Erickson (1996), using the PDS-1000/He Biolistic particle delivery system (Biorad). Transformations using the *aphA-6* constructs as the selectable marker were carried out as follows.

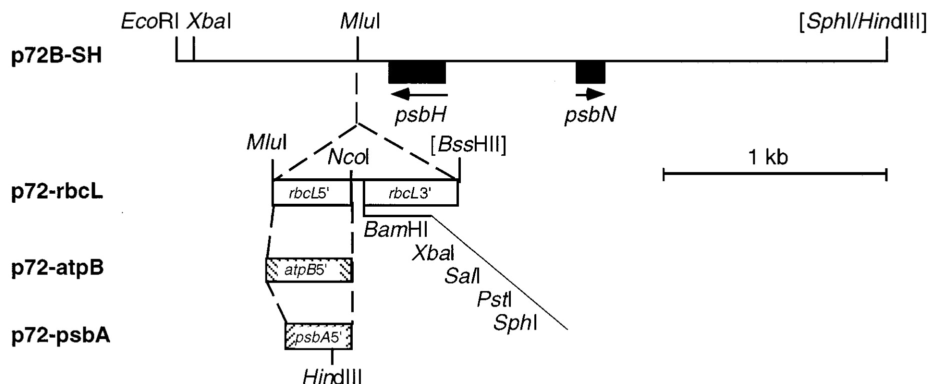

Fig. 1 Structure of the three expression vectors. The 3.17-kb *Eco*RI-*Sph*I fragment of chloroplast DNA carrying the two PSII genes, *psbH* and *psbN* (filled boxes), in p72B-SH is shown schematically at the *top*. The *rbcL* cassette was introduced into the *Mlu*I site, in the orientation shown, as a PCR product generated from plasmid rbcX (Goldschmidt-Clermont 1991). Elimination of the *Hin*dIII site within the polylinker region yielded the p72-rbcL vector. Two other vectors (p72-atpB and p72-psbA) were made by excising the 5′ *rbcL* element as a *Mlu*I-*Nco*I fragment and replacing it with the corresponding 5′ regions amplified from *atpB* and *psbA*, respectively (*hatched boxes*). All restriction sites are unique, with the exception of *Xba*I. Sites destroyed during ligation are shown in *brackets*

Wild-type cells were grown to a density of 1×10^6 cells/ml in TAP medium. The cells in a 10-ml aliquot of the culture were pelleted by centrifugation, resuspended in 0.5 ml of TAP medium and spread on to sterile nylon filters (Hybond-N; Amersham) laid on TAP plates containing 2% Bactoagar (Difco). The plates were bombarded with 1-μm gold particles (Biorad) coated with the appropriate plasmid using an 1100 psi rupture-disc (Biorad). After transformation, the plates were incubated at 21 °C in dim light (2 $\mu E/m^2/s$) for 24 h. The cell-covered filters were then transferred to fresh TAP plates containing either 65 μg/ml kanamycin or 150 μg/ml amikacin, and incubated at 25 °C in moderate light (45 $\mu E/m^2/s$) for 1–2 weeks. Colonies were transferred to TAP plates supplemented with 100 μg/ml kanamycin. Primary transformants were restreaked three or four times on TAP plates containing 100 μg/ml kanamycin or 150 μg/ml amikacin. For liquid culture, *aphA-6* transformants were grown in TAP medium with 2 μg/ml kanamycin.

Nucleic acid techniques

Total genomic DNA was prepared from *C. reinhardtii* as described (Rochaix et al. 1988). Southern blotting was carried out according to standard protocols (Sambrook et al. 1989). Radiolabelled DNA probes were prepared according to the 'Prime-It II' kit (Stratagene). Hybridisation was at 65 °C overnight in 6 × SSC, 0.01 M EDTA, 5 × Denhardt's solution, 0.5 × SDS, 100 μg/ml denatured salmon sperm DNA. Washing of membranes was at 65 °C in 2 × SSC/0.1 × SDS for 1 h then 0.1 × SSC/0.1 × SDS for 1 h.

Electron paramagnetic resonance (EPR) analysis

EPR analysis was carried out as described previously (Evans et al. 1995). A 0.3-ml aliquot of packed cells was placed in a quartz EPR tube (3-mm diameter), dark adapted for 30 min and frozen in liquid nitrogen in the dark. EPR analysis was performed at 8°K using a Jeol REIX spectrometer with a liquid helium cryostat (Oxford Instruments).

Results

Chloroplast integration/expression vectors

DNA introduced into the *Chlamydomonas* chloroplast integrates into the genome via homologous recombination (Boynton et al. 1988). Foreign DNA can therefore be targeted to any site in the genome by flanking the DNA of interest with the appropriate endogenous sequences (Goldschmidt-Clermont 1998). We have shown previously that insertion of the *aadA* cassette immediately downstream of the photosystem II (PSII) gene *psbH* does not generate any mutant phenotype (O'Connor et al. 1998). In contrast, disruption of *psbH* with *aadA* prevents phototrophic growth. It is therefore possible to target foreign DNA to the neutral site downstream of *psbH*, using the *psbH* gene itself as a selectable marker to rescue the *psbH* disruption mutant to phototrophic growth. Expression of a foreign gene is achieved by fusing the coding sequence to upstream and downstream sequences from *Chlamydomonas* chloroplast genes (Goldschmidt-Clermont 1998).

We therefore created three insertion/expression vectors that utilise the *psbH* selection strategy (Fig. 1). Each vector comprises an expression cassette cloned into a 3.2-kb fragment of the *Chlamydomonas* chloroplast genome containing *psbN* and *psbH* (Johnson and Schmidt 1993). The cassettes are derived from the rbcX vector made by Goldschmidt-Clermont (1991), and each contains: the promoter region and 5′ UTR from *rbcL*, *atpB* or *psbA*; a polylinker region allowing translational fusions at the ATG within the *Nco*I site, and the 3′ UTR from *rbcL*. Foreign genes cloned downstream of the different promoters can be introduced into the *Chlamydomonas* chloroplast by transformation rescue of the *psbH*::*aadA* mutant Bst-opp (O'Connor et al. 1998).

aphA-6 as a selectable marker

The following criteria were used to identify a suitable foreign gene for development as a new chloroplast marker: (1) the gene should confer resistance to a commercially available antibiotic (or preferably several different antibiotics) that specifically affects chloroplast gene expression; (2) the gene should be relatively small and have a similar A + T content to that found in chloroplast genes of *C. reinhardtii* (A + T content = 70%); (3) to aid cloning, the gene should also confer resistance when expressed in *E. coli*. A search of the literature identified *aphA-6* as a possible candidate. This gene from *Acinetobacter baumannii* encodes the aminoglycoside phosphotransferase enzyme APH(3′)-VI and confers resistance to kanamycin and other related aminoglycoside antibiotics including amikacin (Shaw et al. 1993). The coding region of *aphA-6* is 777 bp long, and has an A + T content of 67%. The gene has been expressed in *E. coli* and shown to confer resistance to kanamycin (Martin et al. 1988).

The *aphA-6* coding sequence was inserted into each of the three expression vectors to generate the plasmids p72-rbcL-aphA6, p72-atpB-aphA6 and p72-psbA-aphA6. Previous studies have shown that chloroplast promoters are often functional in *E. coli* (e.g. Hanley-Bowdoin and Chua 1987). This has proved useful when creating constructs in *E. coli* carrying transformation markers such as the *aadA* cassette (Goldschmidt-Clermont 1991). We therefore tested whether the *aphA-6* plasmids conferred kanamycin resistance in *E. coli*. Each plasmid conferred resistance on solid medium, with the p72-psbA-aphA6 construct giving the highest level (20 μg/ml), followed by the p72-atpB-aphA6 construct (10 μg/ml) and p72-rbcL-aphA6 (2 μg/ml).

Introduction of *aphA-6* into the chloroplast

All three *aphA-6* plasmids were used to transform the *psbH*::*aadA* mutant to phototrophic growth. Restoration of *psbH* by homologous replacement of the disrupted gene was confirmed by the observed loss of the spectinomycin resistance phenotype conferred by the *aadA* marker (data not shown). Transformant colonies were then scored for APH(3′)-VI activity by assessing

Table 1 Levels of kanamycin and amikacin resistance in *psbH* transformants carrying *aphA-6* under the control of various promoter elements

Promoter[a]	Kanamycin concentration (μg/ml)					Amikacin concentration (μg/ml)				
	0	50	100	150	200	0	50	100	150	200
WT	+	–	–	–	–	+	+	+/–	–	–
atpB	+	+/–	–	–	–	+	+	+	+	+/–
rbcL	+	+	+	+	+	+	+	+	+	+
psbA	+	+	+	+	+	+	+	+	+	+

[a] The *aphA-6* gene was placed under the control of promoter elements isolated from the genes *atpB*, *rbcL* and *psbA*. WT, wild-type strain CC-1021. +, growth comparable to that of controls grown in the absence of antibiotic; +/–, some residual growth; –, no growth

their ability to grow as single colonies on solid medium containing increasing concentrations of kanamycin or amikacin. As shown in Table 1, all three classes of transformant showed increased levels of antibiotic resistance as compared to the wild-type strain (i.e. Bst-opp rescued to a wild-type phenotype using the p72B-SH plasmid). The transformants bearing p72-rbcL-aphA6 and p72-psbA-aphA6 were viable on the highest kanamycin concentration tested (200 μg/ml), whereas p72-atpB-aphA6 transformants were killed at levels above 50 μg/ml. When tested on amikacin, all three transformants were resistant to 150 μg/ml of the antibiotic, whereas growth of the wild-type strain was inhibited. At 200 μg/ml, growth of the p72-atpB-aphA6 transformants was partially inhibited, whereas the other two transformant classes remained viable (Table 1). Based on these results, we decided to use p72-psbA-aphA6 in attempts to select directly for antibiotic-resistant transformants.

Direct selection for *aphA-6* transformants

Bombardment of the wild-type strain CC-1021 with p72-psbA-aphA6, followed by selection on TAP medium containing either kanamycin at 65 μg/ml or amikacin at 150 μg/ml, gave rise to between 5 and 100 colonies per plate. Four randomly chosen clones isolated on medium containing 65 μg/ml kanamycin were able to grow on 100 μg/ml kanamycin and 150 μg/ml amikacin (data not shown). PCR analysis of these four strains confirmed that they all contained the *aphA-6* coding sequence (Fig. 2). The integration of the 1.5-kb *aphA-6* cassette downstream of *psbH* in the chloroplast DNA was confirmed by Southern analysis. Digestion of total DNA with *Eco*RI should yield two bands of 4.7 and 1.3 kb when blots are probed with the *aphA-6* DNA. This is the case for all four transformants (Fig. 3), confirming that *aphA-6* has integrated into the chloroplast genome at the correct site. An additional minor band seen in transformant 3 may be result from recombination between the *psbA* or *rbcL* elements of the marker and the corresponding endogenous genes, thereby generating a minor population of fragmented genomes, as discussed by Goldschmidt-Clermont (1998).

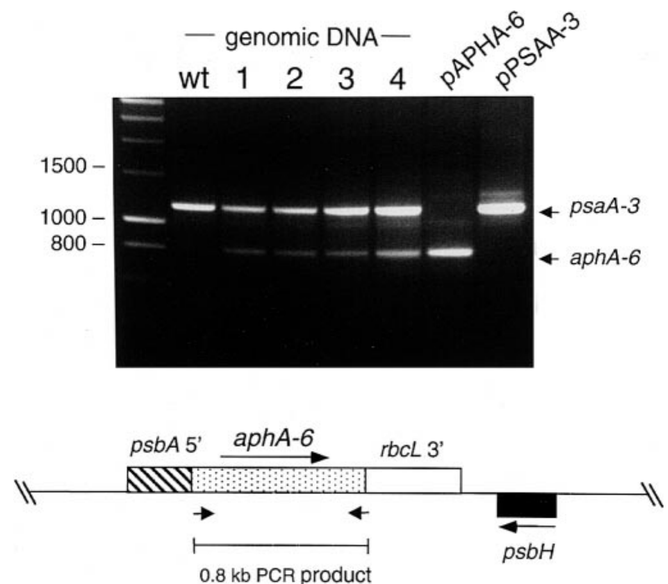

Fig. 2 PCR analysis of four directly selected transformants. PCR products were generated from total genomic DNA using a primer pair specific for the *aphA-6* coding region, together with a primer pair for an endogenous chloroplast gene (*psaA-3*) as a control. Products were analysed on an ethidium bromide-stained agarose gel. Products of the expected sizes for *aphA-6* (0.8 kb) and *psaA-3* (1.2 kb) are seen in all four transformants, whereas only the *psaA-3* product is seen in the wild type (wt). Plasmids pAPHA-6 and pPSAA-3 were used as template controls for each primer set

Insertional mutagenesis using *aphA-6*

The *aadA* cassette has been used extensively as an insertional mutagen to disrupt target chloroplast genes (Rochaix 1997). We therefore tested whether the chimeric *aphA-6* gene in p72-psbA-aphA6 could be similarly used to create chloroplast mutants. The 5′ *psbA*::*aphA-6*::3′*rbcL* region was amplified by PCR and inserted into pBluescript SK(+) to make plasmid pSK.KmR. Additional restriction sites were introduced into the ends of the PCR product to facilitate the excision of the cassette using *Bam*HI, *Sma*I or a combination of two different enzymes (Fig. 4A).

The *aphA-6* cassette was used to disrupt the *psbH* gene by isolating the cassette as a *Sma*I fragment from pSK.KmR and cloning it into a unique *Bst*XI site within the coding region of *psbH* (Fig. 4B). Bombardment of

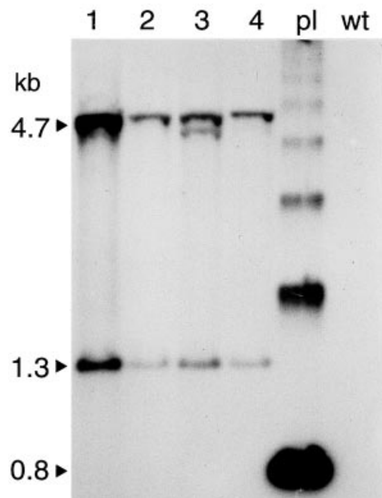

Fig. 3 Southern analysis of the four transformants. Total genomic DNA was digested with *Eco*RI, blotted to Hybond-N membrane and hybridised to a radiolabelled probe corresponding to the *aphA-6* coding sequence. Lanes 1–4 contained DNA from each of the four p72-psbA-aphA6 transformants. In lane pl, the transforming plasmid was digested with *Nco*I and *Pst*I to release the 0.8-kb *aphA-6* coding region. Lane wt was loaded with wild-type genomic DNA digested with *Eco*RI

wild-type cells with the construct yielded kanamycin-resistant colonies as before. Selected clones were checked for the presence of the *aphA-6* gene by PCR (data not shown) and then taken through four rounds of streaking to single colonies on selective medium to obtain homoplasmic transformant lines (O'Connor et al. 1998). The phototrophic competence of three of these transformants was then examined by comparing their growth in the light on medium with and without acetate as a reduced carbon source. Unlike the untransformed strain, all three transformants were unable to grow in the absence of acetate, as expected for a *psbH* disruption mutant (O'Connor et al 1998). The loss of photosystem II function in the *aphA-6* transformants was confirmed by low-temperature EPR analysis of whole cells (Evans et al. 1995). As shown in Fig. 4C, the transformants lack the dark-stable EPR signal arising from the tyrosine radical Y_D of the PSII reaction centre, confirming that the acetate-dependent phenotype of the transformants is due to a requirement for the PsbH protein in PSII assembly or function.

Discussion

We have developed a series of chloroplast expression vectors that allow the insertion and expression of foreign genes in the chloroplast genome of *Chlamydomonas*. The foreign DNA is targeted to an intergenic region downstream of *psbH*, and selection for transformants is based on the rescue of a *psbH*::*aadA* disruption mutant to phototrophic growth. Since the expression cassette in each vector is flanked on both sides by chloroplast DNA

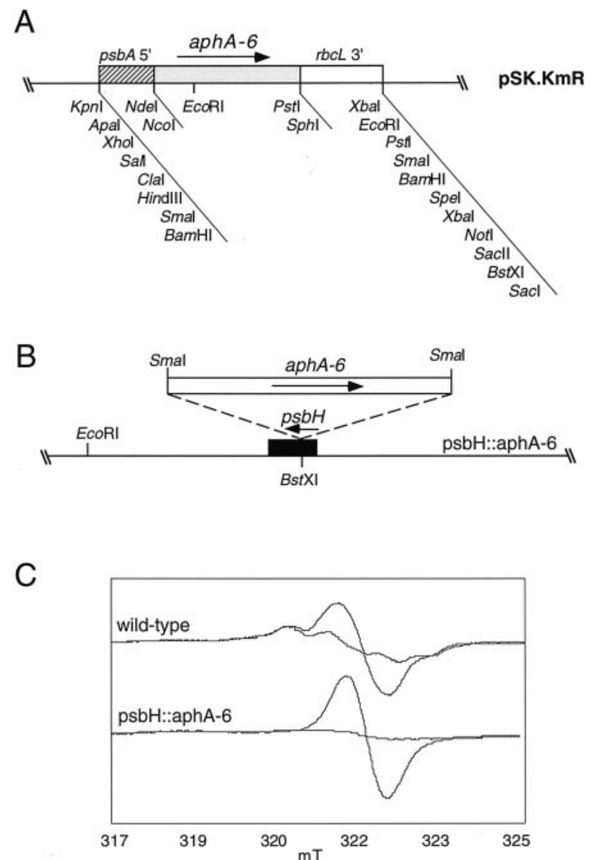

Fig. 4A–C A portable *aphA-6* marker and its use as an insertional mutagen. **A** PCR amplification of the *aphA-6* marker from p72-psbA-aphA6 generated a 1.55 kb product that was blunt-end ligated into the *Eco*RV site of pBluescript SK(+) to create pSK.KmR. Major restriction sites are shown. **B** The marker was excised from pSK.KmR as a *Sma*I fragment and cloned into the *Bst*XI site within the *psbH* coding region on plasmid p72B-SH, to create psbH: : aphA-6. **C** Low-temperature electron paramagnetic resonance (EPR) analysis of whole cells from the wild-type strain CC-1021 and one of the CC-1021 transformants generated using psbH: : aphA-6. In dark-adapted wild-type cells the spectrum of the PSII tyrosine radical Y_D is seen (*inner trace*). Illumination generates the P700+ signal from the photosystem I reaction centre (*outer trace*). In the *psbH*: : *aphA-6* transformant, the Y_D signal is absent demonstrating that the transformant lacks a functional PSII complex

sequence, and the cassette and the *psbH* marker are tightly linked (the *psbH* disruption site and the cassette are only 350 bp apart), integration of the foreign gene downstream of *psbH* occurs readily and involves a simple double-recombination event. In these respects, our expression vectors are superior to the vectors originally developed by Goldschmidt-Clermont (1991). These have flanking DNA on only one side of the expression cassette with the selectable marker (*tscA*) lying more than 1 kb from the expression cassette. Consequently, transformation with these earlier vectors yields a complex integration pattern (Goldschmidt-Clermont 1991). Furthermore, the greater distance between marker and cassette in these vectors suggests that separation of the two elements by recombination is more likely to occur, resulting in a failure of the foreign gene to integrate.

The choice of *aphA-6* as a candidate for a dominant marker was based on its small size, A + T content and the activity of the gene product against a range of aminoglycoside antibiotics. One drawback of using such antibiotics to select chloroplast transformants is the relatively high frequency with which spontaneous resistance mutants arise due to mutations in chloroplast genes encoding components of the 70S ribosome (Harris 1989). However, we find a frequency of spontaneous resistance mutants of only ≈10%, when *aphA-6* transformants are selected on medium containing kanamycin at 65 μg/ml. Furthermore, we have found that many of these mutants, but not true transformants, die when transferred to medium supplemented with 100 μg/ml kanamycin (data not shown).

Of the three chloroplast promoter elements tested, the *psbA* promoter gave the highest levels of expression of *aphA-6* in the *Chlamydomonas* chloroplast and in *E. coli*, as judged by the level of antibiotic resistance of transformants. The chimeric construct psbA5′::aphA-6::rbcL3′ was therefore chosen for development as a portable selectable marker and cloned into a pBluescript vector as a cassette that can be excised using various restriction enzymes.

The availability of the different markers, *aphA-6* and *aadA* (Goldschmidt-Clermont 1991), will extend the possibilities for reverse-genetic studies of chloroplast genes and their expression in *Chlamydomonas*. Previously, transformants generated using the *aadA* marker could not be modified further unless an elaborate technique of "*aadA* recycling" was used, such that transformants subsequently lost the *aadA* marker when antibiotic selection was removed (Fischer et al. 1996). Not only does the availability of two markers allow the creation of double mutants with modifications to two separate genes on the chloroplast genome, but null mutants can be created by gene deletion using one marker and then modified versions of the gene re-introduced using the second marker for selection. Using a null mutant avoids the problems of competition and copy-correction of the modification by the wild-type version of the gene in the initially heteroplasmic transformant cell (Redding et al. 1998). Furthermore, the availability of the second antibiotic marker circumvents the need for the modified gene itself to confer a selectable phenotype. For example, in the study of photosynthetic genes, non-functional variants can be introduced into the genome since selection for phototrophic growth is not necessary.

The *aadA* marker has also been used as a reporter gene to investigate the interaction of specific chloroplast transcripts with nucleus-encoded factors required for their stability (Nickelsen 1998). It appears that such factors bind to the 5′ untranslated region of the mRNA since chloroplast transformants containing the *aadA* marker fused to the 5′ UTR of the gene are spectinomycin resistant in a wild-type nuclear background, but not in a mutant background lacking the stability factor (see, for example, Stampacchia et al. 1997). The three classes of *aphA-6* transformant we have made, in which *aphA-6* is fused to the 5′ UTR of *rbcL*, *atpB* or *psbA*, could be similarly used to investigate nucleus-encoded factors required for the transcription of these genes or the stability of their mRNA products. Nuclear mutants for such factors have already been described for *rbcL* (mutant *76–5EN*; Hong and Spreitzer 1994) and *atpB* (mutant *thm24*: Drapier et al. 1992) and could be crossed to the transformants to assess the expression of *aphA-6* in these mutant backgrounds.

Acknowledgements We thank P. Courvalin and M. Goldschmidt-Clermont for their gifts of the plasmids pAT235 and rbcX, respectively. This work was supported by the Biotechnology and Biological Sciences Research Council.

References

Boynton JE, Gillham NW (1993) Chloroplast transformation in *Chlamydomonas*. Methods Enzymol 217: 510–536

Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark K, Sanford JC (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240: 1534–1537

Carrer H, Hockenberry TN, Maliga P (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco. Mol Gen Genet 241: 49–56

Drapier D, Girard-Bascou J, Wollman F-A (1992) Evidence for nuclear control of the expression of the *atpA* and *atpB* chloroplast genes in *Chlamydomonas*. Plant Cell 4: 283–295

Erickson JM (1996) Chloroplast transformation: current results and future prospects. In: Ort DR, Yocum CF (eds) Oxygenic photosynthesis: the light reactions. Kluwer Academic, Dordrecht, pp 589–619

Erickson JM, Rahire M, Rochaix JD (1984) *Chlamydomonas reinhardtii* gene for 32000 mol wt protein of photosystem II contains four large introns and is located entirely within the chloroplast inverted repeat. EMBO J 3: 2753–2762

Evans MCW, Hallahan BJ, Hanley JA, Heathcote P, Gumpel NJ, Purton S (1995) Isolation and characterization of photosynthetic reaction centres from eukaryotic organisms. In: Brown GC, Cooper CE (eds) Bioenergetics: a practical approach. IRL Press, Oxford, pp 183–217

Fischer N, Stampacchia O, Redding K, Rochaix JD (1996) Selectable marker recycling in the chloroplast. Mol Gen Genet 251: 373–380

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

Goldschmidt-Clermont M (1998) Chloroplast transformation and reverse genetics. In: Rochaix JD, Goldschmidt-Clermont M, Merchant S (eds) The molecular biology of chloroplasts and mitochondria in *Chlamydomonas* (Advances in Photosynthesis, vol 7). Kluwer Academic, Dordrecht, pp 139–149

Hanley-Bowdoin L, Chua N-H (1987) Chloroplast promoters. Trends Biochem Sci 12: 67–70

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

Hong S, Spreitzer RJ (1994) Nuclear mutation inhibits expression of the chloroplast gene that encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol 106: 673–678

Johnson CH, Schmidt GW (1993) The *psbB* gene cluster of the *Chlamydomonas reinhardtii* chloroplast: sequence and transcriptional analysis of *psbN* and *psbH*. Plant Mol Biol 22: 645–658

Khan MS, Maliga P (1999) Fluorescent antibiotic resistance marker for tracking plastid transformation in higher plants. Nature Biotechnol 17: 910–915

Martin P, Jullien E, Courvalin P (1988) Nucleotide sequence of *Acinetobacter baumannii aphA-6* gene: evolutionary and functional implications of sequence homologies with nucleotide-binding proteins, kinases and other aminoglycoside-modifying enzymes. Mol Microbiol 2: 615–625

Nickelsen J (1998) Chloroplast RNA stability. In: Rochaix JD, Goldschmidt-Clermont M, Merchant S (eds) The molecular biology of chloroplasts and mitochondria in *Chlamydomonas* (Advances in Photosynthesis, vol 7). Kluwer Academic, Dordrecht, pp 151–163

O'Connor HE, Ruffle SV, Cain AJ, Deak Z, Vass I, Nugent JHA, Purton S (1998) The 9-kDa phosphoprotein of photosystem II. Generation and characterisation of *Chlamydomonas* mutants lacking PSII-H and a site-directed mutant lacking the phosphorylation site. Biophys Biochim Acta 1364: 63–72

Redding K, MacMillan F, Leibl W, Brettel K, Hanley J, Rutherford AW, Breton J, Rochaix JD (1998) A systematic survey of conserved histidines in the core subunits of Photosystem I by site-directed mutagenesis reveals the likely axial ligands of P700. EMBO J 17: 50–60

Rochaix JD (1997) Chloroplast reverse genetics: new insights into the function of plastid genes. Trends Plant Science 2: 419–425

Rochaix JD, 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

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

Serino G, Maliga P (1997) A negative selection scheme based on the expression of cytosine deaminase in plastids. Plant J 12: 697–701

Shaw KJ, Rather PN, Hare RS, Miller GH (1993) Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol Rev 57: 138–163

Sidorov VA, Kasten D, Pang S-Z, Hajdukiewicz PTJ, Staub JM, Nehra NS (1999) Stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. Plant J 19: 209–216

Sikdar SR, Serino G, Chaudhuri S, Maliga P (1998) Plastid transformation in *Arabidopsis thaliana*. Plant Cell Rep 18: 20–24

Stampacchia O, Girard-Bascou J, Zanasco J-L, Zerges W, Bennoun P, Rochaix JD (1997) A nuclear-encoded function essential for translation of the chloroplast *psaB* mRNA in Chlamydomonas. Plant Cell 9: 773–782

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

Woessner JP, Gillham NW, Boynton JE (1986) The sequence of the chloroplast *atpB* gene and its flanking regions in *Chlamydomonas reinhardtii*. Gene 44: 17–28