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Article

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ABSTRACT

Expression vectors for *Chlamydomonas reinhardtii* chloroplast transformation have been constructed with transcription and translation signals from chloroplast genes. The bacterial *aadA* sequence, coding for aminoglycoside 3″ adenyl transferase, was inserted in these vectors and introduced into the *C. reinhardtii* chloroplast by particle gun transformation. The stable transgenic expression of this foreign protein in the chloroplast confers spectinomycin and streptomycin resistance to the transformed cells. This new marker can be used as a reporter of gene expression, and as a portable selectable cassette for chloroplast reverse genetics. Targetted gene disruption mutants of loci required for photosynthesis, *tscA* and *psaC*, were thus obtained. A gene disruption of an unidentified open reading frame, ORF472, remained heteroplasmic, suggesting that it has a vital function.

INTRODUCTION

The development of efficient chloroplast transformation in *C. reinhardtii* using microprojectile bombardment has opened the way for a systematic analysis of chloroplast gene function and expression. The initial report of chloroplast transformation with a particle gun made use of a photosynthetic marker for the selection of transformed cells (1). A deletion mutant of the chloroplast *atpB* gene was used as a host, and was bombarded with a fragment of wild-type DNA spanning the deletion. This provides a powerful selection, but has limitations for experiments where photosynthetic genes are being analyzed because only phototrophic cells can be selected. Similar strategies were used with other chloroplast photosynthetic genes such as *rbcL* or *psbA* (2). Another approach has been to use the mutations in the psbA gene which confer resistance to certain herbicides (3), or mutations in the ribosomal RNA genes which confer resistance to antibiotics such as spectinomycin, streptomycin or erythromycin (2). Co-transformation with such an rDNA drug resistance marker and a separate mutated *atpB* gene was further shown to allow the recovery of mutant strains bearing both markers (4). Transformation of the tobacco chloroplast has also been achieved using an analogous rDNA marker confering resistance to spectinomycin (5).

Here I describe transformation vectors that allow the expression of foreign genes in the *C. reinhardtii* chloroplast, as illustrated with the coding sequence of *aadA*, a bacterial gene that confers resistance to aminoglycosides such as spectinomycin and streptomycin. This is the first example of stable transgenic expression of a foreign protein in a chloroplast. The chimeric *aadA* gene provides a portable, dominant selectable marker which can be used for site-directed mutagenesis independently of photosynthetic function. The *aadA* sequence can also be used as a reporter to study chloroplast gene expression.

MATERIALS AND METHODS

Strains and media

C. reinhardtii 137c and the chloroplast mutant H13 (6) were grown as described by Harris (7). Where necessary the media (Tris acetate phosphate medium (TAP) or high salt minimal medium (HSM)) were solidified with 2% Bacto agar (Difco) and supplemented with spectinomycin (Sigma) or streptomycin (Calbiochem), taking into account the fact that these antibiotic preparations were only partially pure. For genetic crosses (7) the agar was washed with distilled water and dried before use.

DNA constructs

Procedures for the preparation of recombinant DNA plasmids are described by Sambrook et al. (8). The bacterial hosts were *E. coli* C600 or JM101 (when a screen for white versus blue colonies on X-gal (lacZ complementation) was applicable).

The HindIII and XbaI sites in the plasmid vector pBluescript KS− (Stratagene) were sequentially destroyed by cutting with the enzyme, filling-in with Klenow polymerase I fragment, religating and treating with the restriction enzymes again prior to transformation to obtain plasmid KSHX. A Sau3A−PstI 450 bp fragment from R15 containing the 3′end of *rbcL* (9) was inserted in BamHI PstI cut KSHX to obtain plasmid KSX-3′LS. A 1.0 kb EcoRI−NlaIV fragment containing the 5′end of *rbcL* was cloned in EcoRI SmaI digested pUC18 to yield pLS1. The 1.0 kb EcoRI−HindIII fragment from pLS1 was inserted into KSX-3′LS cut with the same enzymes (the HindIII site is within the 3′rbcL segment) to obtain plasmid LSX. The plasmid pR12-3 contains the chloroplast EcoRI fragment R12 cloned in pBluescript KS− (Choquet, Girard-Bascou and Rochaix, in

preparation). A 3.1 kb KpnI−ClaI fragment from pR12-3 (the ClaI site is in the polylinker) was introduced into LSX cut with KpnI and ClaI to provide the *tscA* marker in plasmid LSX-R12. To improve the translational fusion of inserted coding sequences, the 5′rbcL sequences in LSX were then replaced with the 5′rbcL or 5′atpA segments prepared by the polymerase chain reaction (PCR) as follows. Plasmid R15.0 (9) was linearized with EcoRI and HindIII and used as a template for 5 cycles of amplification as described previously (10), either with oligos LS3 and LS5 for 5′rbcL or oligos atpA3 and atpA5 for 5′atpA (table 1). The 5′rbcL fragment was first subcloned in pBluescript KS− at the SmaI site, from which it was excised with ClaI and BamHI. The 5′atpA fragment was obtained by treating the products of the PCR reaction directly with the same enzymes. The 0.39 kb 5′rbcL and the 0.68 kb 5′atpA ClaI−BamHI fragments were introduced into ClaI BamHI digested LSX-R12 to obtain the plasmid vectors rbcX and atpX (figure 1). The sequence of the expression site polylinker was verified.

The coding sequence of *aadA* was prepared by PCR as above using oligos AAD5 and AAD3 (table 1) and the 2.0 kb EcoRI fragment of pHP45-Omega (11) as a template. This coding segment starts at the Met codon at position 402 in the *aadA* sequence (12), which is probably the true start site (13). The *aadA* segment was subcloned at the SmaI site of pBluescript KS−, and the 0.81 kb NcoI−PstI fragment was excised and introduced into NcoI PstI digested rbcX or atpX to obtain plasmids rbcX-AAD and atpX-AAD (figure 1).

The 1.9 kb EcoRI−SacI fragment of atpX-AAD was further subcloned into EcoRI SacI digested pUC 18 to obtain the plasmid pUC-atpX-AAD (figure 1). The construction of the psaC::AAD gene disruption is described elsewhere (14). The tscA::AAD disruption was obtained by partially digesting the plasmid pR12.3 (see above) with ScaI, treating with Klenow DNA polymerase I fragment to generate blunt ends, isolating the full length linear molecules and introducing the EcoRV−SmaI aadA cassette from pUC-atpX-AAD. The *E.coli* transformants were selected on LA containing 50 μg/ml ampicillin and 25ug/ml spectinomycin.

The ORF472::AAD disruption was derived from pCM3 (15) by digesting with NsiI, treating with T4 DNA polymerase to obtain blunt ends and introducing the EcoRV−SmaI cassette as above.

Transformation

Transformation with a particle gun was as described previously (16, 10). For spectinomycin selection the cells were carefully resuspended and split into three aliquots which were plated in 0.6% top agar on TAP medium containing 100 μg/ml spectinomycin, and grown in dim light (approx 300 lux). After 2−3 weeks the colonies were restreaked on TAP + spectinomycin and grown for approximately one week. The cells were then resuspended and grown for 1 day in liquid TAP medium containing 20 μg/ml spectinomycin, diluted and plated on TAP + spectinomycin and on HSM minimal medium to obtain single colonies and to measure plating efficiency. These steps of restreaking and replating were repeated several times sequentially to obtain pure and when possible homoplasmic lines. Representative transformants were grown for DNA and RNA extraction and analysis (17).

Aminoglycoside adenyl transferase assay

Cells were grown to a density of approximately 10^7/ml in TAP medium, harvested by centrifugation, washed in distilled water and finally resuspended in 1/40 th volume AAD-RB (25 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 100 mM NH4Cl, 0.5 mM DTT). The cells were broken by sonication, and the debris were removed by centrifugation for 30 min at 20000g. (The clear supernatants can be stored frozen with only a small loss in activity.).

AAD catalyses the transfer of an adenyl moiety from ATP to streptomycin; activity can be measured using radiolabelled ATP. The assay is based on the binding of streptomycin adenylate, but not ATP, to negatively charged phosphocellulose paper (18). The reactions were in 30 μl AAD-RB containing 15 μl crude sonic extract, 100 μM rATP, 60 μg/ml streptomycin and 0.1 μCi alpha^{32}P-rATP. Blank reactions lacking either the crude extract or streptomycin were treated in parallel. After 30 min at 35°C, duplicate 10 μl aliquots were spotted on phosphocellulose paper filters (1.5×1.5 cm^2, Whatmann P81) and allowed to adsorb for 30 seconds. The filters were immediately washed in water, first at 75°C, then 4 times at room temperature. The filters were dried and counted in toluene based scintillation fluid to measure the streptomycin adenylate formed. Total protein was measured using the Bio-Rad protein assay.

RESULTS

Expression vectors

Two vectors for the expression of polypeptide coding sequences in the chloroplast of *C. reinhardtii* have been constructed (figure 1). Both contain the promoter, the untranslated leader and the translation initiation ATG codon of *C. reinhardtii* chloroplast genes. Any coding sequence of interest can thus be placed under the control of homologous chloroplast transcription and translation signals. The first vector, rbcX, contains a 363 base pair (bp) fragment from the *rbcL* gene: 268 bp of upstream sequence, the 92 bp untranslated 5′-leader and the ATG (9). The second one, atpX, contains a 653 bp fragment form the *atpA* gene: 221 bp of upstream sequence, the 429 bp 5′-leader and the ATG. Just

Table 1. Oligonucleotides used in this work

Oligo:	Sequence	Linker	Position
LS5:	gcacatcgATGGGTTTATAGGTATTTTGAGACCAG	ClaI	831−857
LS3:	cgcaggatccatggaCATTTATATAAATAAATGTAAC	BamHI NcoI	1193−1171
ATPA5:	gtactcatcgatGACTTTATTAGAGGCAGTG	ClaI	911−893
ATPA3:	gtcatcggatccatggaCATTTTCACTTCTGGAGTG	BamHI NcoI	259−277
AAD5:	cgcaccatggctcgtGAAGCGGTtATCGCCGAAG	NcoI	408−426
AAD3:	gcacctgcagTTATTTGCCaACTACCTTaGTGATC	PstI	1193−1172

The oligonucleotides used as primers for the PCR are listed in 5′-3′ orientation with the bases matching the template in uppercase, and the linkers or site specific changes to improve codon usage in lower case. Numbering is as in the references for *rbcL* and *atpA* (9) and for *aadA* (12).

downstream of the ATG in these vectors, there is a set of cloning sites, followed by a 437 bp fragment containing the 3'-end of the *rbcL* gene (figure 1a). Both vectors also contain the *tscA* gene as a selectable marker that can restore the photosynthetic growth of *tscA* deletion mutants such as H13 (10; Choquet, Girard-Bascou and Rochaix, in preparation).

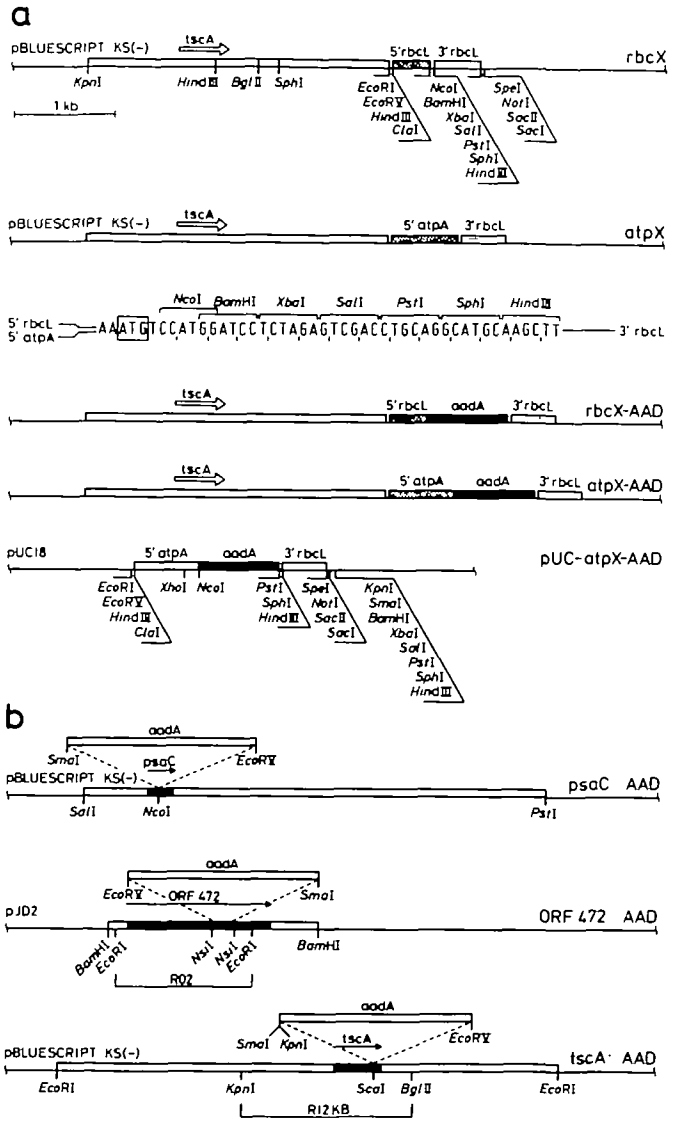

Fig. 1. Structure of the plasmids used for transformation. The bars represent segments of DNA that have been inserted into plasmid vectors (thin lines) as indicated on the left (the plasmid DNA is only partly shown and linearized at arbitrary positions in these maps). **Panel a**: transformation vectors. Open bar: fragment of R12 (for chloroplast DNA fragments, see ref. 32) carrying the *tscA* gene; hatched bars: fragments of R15 from the 5' part of the *rbcL* and *atpA* genes respectively; stippled bar: fragment of R15 with the 3'part of *rbcL*; black bar: coding sequence of the *E.coli aadA* gene. The sequence of the multiple cloning sites in the expression vectors is shown with the translation initiation ATG codon boxed. **Panel b**: gene inactivations. For the *psaC* gene disruption, the *aadA* cassette was inserted into a 4.6 kb SalI PstI fragment of R23 at a unique NcoI site (14). For the ORF472 disruption, a 232 bp NsiI fragment was replaced by the *aadA* cassette in pCM3, a 2.2kb MboI fragment from Ba7 cloned in the vector pJD2 (15). For the *tscA* disruption, the *aadA* marker was inserted into one of the ScaI sites of R12. The hybridization probes used in figure 3 are indicated by brackets: R02 (EcoRI fragment) and R12KB (KpnI-BglII fragment).

Expression of the aadA gene

The *E.coli aadA* gene encodes the enzyme aminoglycoside 3''-adenyltransferase (AAD), and confers resistance to streptomycin and spectinomycin. Its coding sequence (12, 13) was inserted in the two expression vectors to obtain the plasmids rbcX-AAD and atpX-AAD (figure 1). The plasmids were introduced into the *C. reinhardtii* host H13 by bombardment with a particle gun, and transformants were selected for photosynthetic growth by rescue of *tscA*. The transformants were tested for growth on media containing spectinomycin or streptomycin. All eight atpX-AAD transformants tested and seven out of eight rbcX-AAD transformants showed resistance to spectinomycin (150 μg/ml). One rbcX-AAD transformed strain (5A) was not resistant. All the atpX-AAD transformants were also resistant to streptomycin (50 μg/ml) but the rbcX-AAD transformants were not. These levels of resistance are well above those of the untransformed host H13, which is only resistant to approximately 50 μg/ml spectinomycin and 20 μg/ml streptomycin. The transformed strains are thus apparently expressing the foreign DNA. Drug resistance, particularly to streptomycin, is higher with the atpX vector than with rbcX.

Seven strains were checked for the presence of the *aadA* sequences by Southern analysis (data not shown). Strain 5A, which was not resistant, did not have detectable *aadA* sequences. Three drug resistant rbcX-AAD transformants and three atpX-AAD transformants had incorporated the foreign DNA. These transformants had a complex pattern of integrated fragments suggesting multiple recombination events. This is probably because there are only short segments of homology between the host and the transforming DNA, because these segments are found at three different loci (*tscA*, *rbcL* and *atpA*) or because there are also regions of repetitive DNA in the sequences adjacent

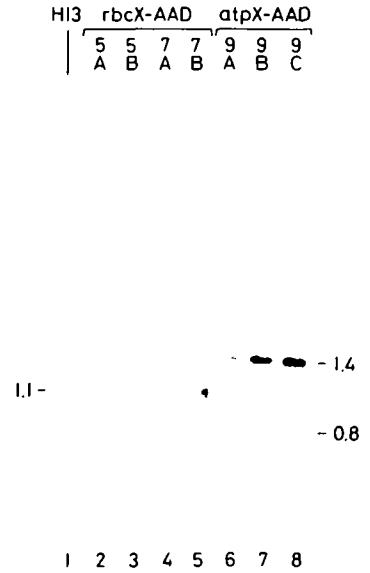

Fig. 2. Northern analysis of chimeric aadA transcripts. RNA from the untransformed host H13 and from seven independent transformants was subjected to agarose gel electrophoresis, blotted to nitrocellulose and hybridized with a radioactive probe containing the *E.coli aadA* gene. Strains 5A, 5B, 7A,and 7B are rbcX-AAD transformants; 9A, 9B and 9C are atpX-AAD transformants. Transformant 5A is phototrophic but is drug sensitive and lacks any detectable *aadA* sequence by Southern analysis. Approximate sizes in kb are indicated alongside.

to *tscA* present in these vectors (Choquet, Girard-Bascou and Rochaix, in preparation).

To confirm these results, the AAD enzyme activity was assayed directly in crude extracts of the transformed cells (table 2). The transfer of the ^{32}P-labelled adenyl group from ATP to streptomycin or spectinomycin can be measured in a simple filter binding assay. The transformed strains did have AAD activity, confirming that the resistance to the drugs is due to the expression in *Chlamydomonas* of the foreign *aadA* DNA. The specific activity of AAD was on the average higher in the atpX-AAD than in the rbcX-AAD transformants, in rough agreement with their different degree of resistance to streptomycin. There were large variations in the levels of AAD activity in different transformants, possibly because in these strains there is a heterogeneous set of integration events. In the case of a simple integration at a single locus (psaC::AAD and ORF472::AAD, see below), the distribution of activities in different isolates seemed more narrow.

Total RNA was extracted from transformed cells and subjected to Northern analysis using the E. coli *aadA* gene as a probe (figure 2). A single band of approximately 1.1 kb hybridizes in the rbcX-AAD transformants, the size expected for a mRNA beginning at the same site in the *rbcL* promoter as the normal transcripts, extending through the *aadA* sequence and ending at the position of the *rbcL* 3′end (9). In the atpX-AAD transformants, two bands are observed: a 1.4 kb RNA, as expected for the *atpA* promoter and *rbcL* 3′end, and a smaller RNA (0.8 kb, the exact nature of this transcript and the reason for its appearance were not investigated). No *aadA* homologous transcripts are detected in the untransformed host H13 or in the transformant lacking *aadA* sequences (5A). The 1.1 kb and 1.4 kb mRNAs accumulate to similar levels in the rbcX-AAD and atpX-AAD transformants, suggesting that the difference in AAD expression and streptomycin resistance in the two sets of transformants are due to differences in the translation efficiency of the *rbcL* and *atpA* 5′-leader sequences. These differences could be intrinsic to the 5′-leader sequences, or be due to the compatibility between the 5′-leaders and the *aadA* coding region of these chimeric genes.

Table 2. Aminoglycoside adenyl transferase activity in transformed strains

STRAINS:	AAD ACTIVITY	AVERAGE ± SD.
Hosts:		
WT	2	
H13	0	–
rbcX-AAD:		
6A	9	
6B	26	24 ± 21
7A	57	
7B	4	
atpX-AAD:		
10A	30	
10B	67	169 ± 134
10C	210	
10D	370	
psaC::AAD:		
1B2	460	390 ± 70
1D2	320	
ORF472::AAD:		
21A	36	
23A	26	32 ± 9
24A	44	
25A	22	

Extracts from the untransformed hosts (H13 and wild-type (WT)) and from a number of transformants were assayed in duplicate for AAD activity and for total protein content as described in Materials and Methods. The activity is expressed as cpm incorporated/μg protein.

Uniparental inheritance of the resistance marker

Are the *aadA* sequences expressed in the chloroplast?: this is likely because the selection of the transformants is based on a chloroplast marker (*tscA*), because the chimeric *aadA* gene is under the control of chloroplast transcription and translation signals, and because the size of the transcripts suggests that these signals are used normally. However particle gun transformation can also deliver DNA to the nucleus (19, 20), so that co-transformation of the chloroplast (*tscA* rescue) and of the nucleus (*aadA* expression) remains a possibility. In genetic crosses of *Chlamydomonas reinhardtii*, nuclear markers segregate in tetrads according to Mendelian genetics (2:2 in a backcross), but chloroplast genes are inherited uniparentally from the mt(+) parent in the majority of tetrads and segregate 4:0. Two atpX-AAD transformants (spcR, mt+) were crossed to the wild-type (spcS, mt−). In all of the 34 tetrads tested the segregation was 4:0 (spcR:spcS), indicating that the spectinomycin resistance is indeed inherited as a chloroplast marker (11 tetrads from a cross with transformant 10A and 23 tetrads from a cross with 10B). In a reciprocal cross (spcS, mt+×spcR, mt−), all 13 tetrads examined showed 0:4 segregation as expected.

Site-directed chloroplast gene inactivations

Because the atpX-AAD chimeric gene shows higher resistance to the antibiotics, it was chosen as a selectable marker cassette. The chimeric *aadA* gene was transferred from atpX-AAD to the plasmid vector pUC18 from which it can be conveniently excised, in particular to yield a blunt-ended fragment (figure 1). The marker can thus be inserted into any target sequence; cloning is facilitated by the fact that the chimeric *aadA* gene is also expressed in *E. coli* and confers spectinomycin and streptomycin resistance to the bacteria.

The chimeric atpX-AAD gene was inserted into three chloroplast genes: *psaC* (14), *tscA*, and ORF472 (figure 1). The *psaC* gene encodes a 9 kD subunit of photosystem I with the F_A and F_B iron-sulfur centers. The *tscA* gene is required for

Table 3. Plating efficiency of gene disruption strains on minimal medium

STRAINS.	PLATING EFFICIENCY 1st round	3d round.
psaC::AAD		
1B2	.05	$<10^{-4}$
1D2	.03	$<10^{-4}$
tscA::AAD		
61A	.002	$<10^{-5}$
62A	.03	$<10^{-5}$
ORF472::AAD		
21A	3.5	1.7
22A	2.4	2.6
24A	3.5	1.0
25A	2.8	2.5

The initial transformed colonies were first restreaked on acetate spectinomycin medium, then grown for one day in acetate spectinomycin medium, and finally plated on minimal medium and on acetate spectinomycin medium. The plating efficiency after this first round of segregation is expressed as the ratio of the number of colonies growing on the minimal medium versus colonies growing on the acetate medium. The plating efficiency after two further rounds of restreaking, liquid culture and plating is shown in the column labelled 3d round.

trans-splicing of the *psaA* mRNA (21, 6, 10), and thus for the expression of one of the chl-a apoproteins of photosystem I. The function of ORF472 in Chlamydomonas is not known, it does not have similarity to any gene from plant chloroplasts (15, 22).

The plasmid DNAs were bombarded into wild-type *C. reinhardtii*, and transformants were selected for resistance to spectinomycin. The plates contained acetate as a source of reduced carbon and were placed in dim light because photosynthetic mutants are often light-sensitive. The initial transformed colonies were streaked on spectinomycin acetate medium and tested in two ways: fluorescence induction kinetics and plating efficiency on minimal medium (phototrophic growth).

As expected, all of the *tscA::aadA* and most of the *psaC::aadA* transformants tested had the high fluorescence yield and fluorescence induction curves typical of photosystem I deficient mutants (not shown). The plating efficiency on minimal medium was low, but still approximately 10^{-2} (table 3), suggesting that at least some of the cells still contained wild-type copies of the gene (each *C. reinhardtii* cell contains a single chloroplast with approximately 70 copies of the chloroplast genome). After two additional rounds of re-plating under selective conditions on spectinomycin acetate medium, the plating efficiency on minimal medium had dropped to less than 10^{-5}. Thus the initial transformants were probably heteroplasmic and contained both wild-type and mutant (spectinomycin resistant) copies of the chloroplast genome that segregated during growth on selective medium, finally giving rise to strict acetate-requiring photosynthetic mutants. Southern analysis of the *tscA::aadA* mutants (figure 3) showed the presence of the interrupted genes (2.3 kb), but wild-type copies (1.7 kb) were not detectable. A similar analysis of the *psaC::aadA* mutants also showed that only interrupted mutant copies were present, but not wild-type ones (14).

One of the *psaC::aadA* transformants (1B2; mt +) was crossed with the wild-type, and the progeny were scored for acetate requirement, for mutant fluorescence induction kinetics and for spectinomycin resistance. Segregation was again 4:0 in all 14 tetrads examined, as expected for a chloroplast mutation.

The nature of the photosystem I deficiency in the *tscA::aadA* mutants was further characterized by Northern analysis of *psaA* mRNA maturation (figure 4). The *tscA* gene is required for *trans*-splicing of exons 1 and 2 (21, 6). Its product is a small chloroplast RNA which probably base pairs with the two separate exon precursors and forms part of the catalytic core for the trans-splicing of exons 1 and 2 (10; figure 5). Chloroplast and nuclear mutants defective in this step belong to class C (23, 6); they accumulate a *trans*-splicing intermediate containing exons 2 and 3. The *tscA::aadA* mutants failed to produce wild-type *psaA* mRNA, but accumulated this intermediate as expected (figure 4).

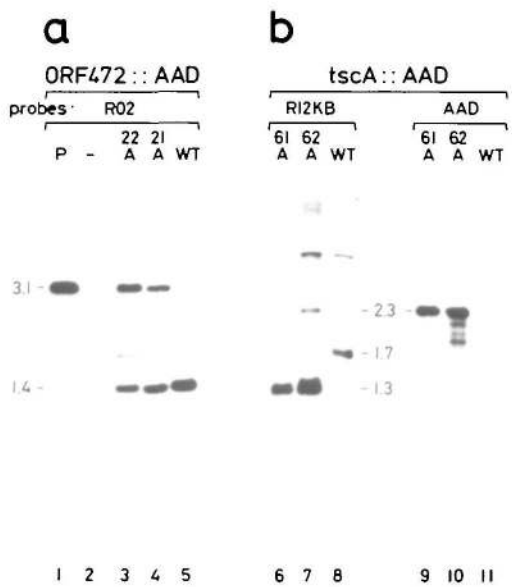

Fig. 3. Southern analysis of ORF472::AAD and tscA::AAD transformants. **Panel a**: DNA from the untransformed wild-type (WT), from two ORF472::AAD transformants (22A, 21A) and from the plasmid used for transformation (P) were digested with EcoRI, subjected to agarose gel electrophoresis, blotted to a nylon membrane and hybridized with the radioactive EcoRI fragment R02 as a probe (see figure 1b). Both the wild-type 1.4 kb fragment and the 3.1 kb insertion derivative are observed in the transformants. (The nature of the minor band of intermediate size was not determined.) **Panel b**: DNA from the untransformed wild-type (WT) and from two tscA::AAD transformants (61A, 62A) was digested with KpnI and BglII, subjected to agarose gel electrophoresis, blotted to a nylon membrane and hybridized with the radioactive R12KB fragment (lanes 6–8; see figure 1b). The wild-type 1.7 kb fragment is not detected in the transformants (even after extended exposure), but is replaced by two fragments of 1.3kb and 2.3kb. The latter contains the *aadA* sequence and also hybridizes to the AAD probe (lanes 9,10). (The R12KB probe contains repetitive sequences which also hybridize to larger fragments in the wild-type and in the transformants.)

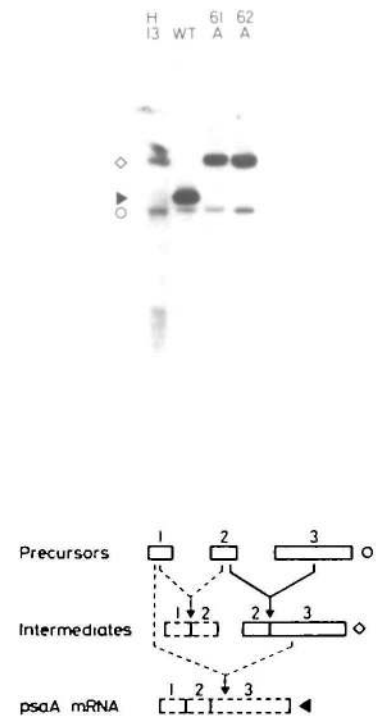

Fig. 4. Northern analysis of psaA mRNA trans-splicing in tscA::AAD transformants. RNA from the wild-type (WT), from a *tscA* deletion mutant (H13), and from two tscA::AAD transformants was subjected to agarose gel electrophoresis, blotted to a nylon membrane and hybridized with a probe specific for exon 3 of *psaA*. As diagrammed at the bottom, psaA mRNA is assembled from three separate precursors in two steps of *trans*-splicing, starting either with exons 1 and 2 or with exons 2 and 3 (23, 6). The product of the *tscA* gene is a small RNA which is thought to assemble with exons 1 and 2 and is required for their *trans*-splicing (10). The tscA::AAD mutants, like the *tscA* deletion (H13), accumulate a 3.8 kb *trans*-splicing intermediate (◇) containing exons 2 and 3 (and *psbD*, which is co-transcribed with exon 2) but fail to *trans*-splice exons 1 and 2, and thus lack the 2.7 kb wild-type mRNA (◀). A precursor containing only exon 3 (○) is also present in the wild-type and in the mutants.

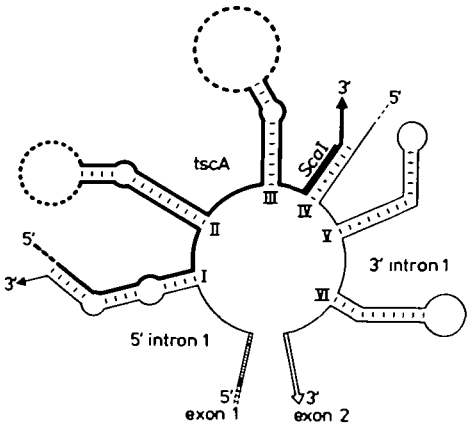

Fig. 5. Secondary structure model for the split intron 1 of *psaA*. In this model (10), the conserved core structure of group II introns (33) is composed of three separate transcripts : the precursor of exon 1 (with the 5′ part of intron 1), the precursor of exon 2 (with the 3′ part of intron 1), and the tscA RNA (thick line). The sequence corresponding to the ScaI site in the *tscA* gene (black bar) base-pairs with the 3′ part of intron 1 to form helix IV. The large loops in domains II and III (dotted) are not drawn to scale.

The ORF472::aadA transformants had normal fluorescence induction kinetics, and had a high plating efficiency on minimal medium, even after two rounds of re-plating on spectinomycin medium (table 3). This could be interpreted in two ways: one possibility is that ORF472 is dispensable for photosynthetic growth, so that the mutant does not have an apparent phenotype under laboratory conditions. The other is that ORF472 is essential even on acetate medium and that a heteroplasmic mixture of wild-type and mutant genomes is maintained by balanced selection for viability and for spectinomycin resistance. To distinguish between these two possibilities, DNA from the transformants was subjected to Southern analysis (figure 3). Both wild-type and interrupted copies of the gene were observed. Thus ORF472 is apparently essential for cell viability on acetate medium. The AAD enzyme activity was lower than in the *psaC::aadA* mutants (table 2), which might be explained by the presence of a lower number of copies of the marker in these heteroplasmic strains.

DISCUSSION

The chimeric aadA gene as a reporter and a selection marker for *C. reinhardtii* chloroplast transformation

The two expression vectors, rbcX and atpX, allow the expression of coding sequences driven by homologous transcription and translation signals in the *C. reinhardtii* chloroplast. A coding sequence can be fused to the inititator ATG with just three additional intervening codons. The *tscA* marker on these vectors provides a selection, in a *tscA* deletion host, for transformants which can then be tested for expression. Using this approach I have obtained the expression of the *E.coli aadA* coding sequence in the *C. reinhardtii* chloroplast. The cells contain chimeric *aadA* mRNA, have aminoglycoside adenyl transferase activity, are resistant to spectinomycin, and with the atpX vector, also to streptomycin. The resistance is inherited uniparentally from the mt+ parent as expected for a chloroplast gene. To my knowledge this is the first example of the stable expression of a foreign protein in a transgenic chloroplast. Transcripts encoding glucuronidase (GUS) and neomycin phosphotransferase (NPTII) have been obtained with chimeric constructs, but expression of the polypeptides or enzyme activity were not detected (24, 25). Transient expression of chloramphenicol acetyl transferase and GUS in the chloroplasts of tobacco cultured cells were observed after particle gun transformation with chimeric constructs (26, 27).

The chimeric *aadA* gene can serve both as a reporter and as a portable selectable marker for chloroplast transformation. The use of *aadA* as a reporter can be at the mRNA level (figure 2) or at the protein level since a very simple filter binding assay for AAD activity has been adapted to *C. reinhardtii* (table 2). It will probably prove important to use constructs that give identical transcripts for comparisons of AAD activity, because there is evidence that the structure of the transcript can influence its stability (25) and probably also its translation efficiency: although atpX-AAD and rbcX-AAD transcripts accumulate to similar levels in transformed cells, only atpX-AAD transformants are resistant to streptomycin, and AAD activity is on the average higher with the atpX-AAD construct.

Towards chloroplast reverse genetics

The chimeric *aadA* gene can be inserted into chloroplast DNA *in vitro* and cloned in *E. coli* where the spectinomycin and streptomycin resistance are also expressed. This is consistent with previous findings that chloroplast promoters are often active in bacterial systems (28, 29). The *aadA* construct is then introduced into the *C. reinhardtii* chloroplast by particle gun transformation. A process of homologous recombination can give rise to substitutions where the endogenous DNA is replaced by the *aadA* construct in some of the multiple chloroplast genomes. Provided that cells bearing the construct are viable under appropriate conditions, further growth under spectinomycin selection will give rise to homoplasmic strains. Using this approach we have generated site-directed gene disruptions of two chloroplast genes that are required for photosynthesis: *psaC* and *tscA*. The mutants are unable to grow phototrophically on minimal medium and are acetate-requiring. They have the expected fluorescence induction patterns for photosystem I deficiency. A more detailed analysis of the *psaC::aadA* mutants will appear elsewhere (14).

The *tscA* gene is required *in trans* for the *trans*-splicing of psaA mRNA, and is thought to interact with the separate exon 1 and exon 2 transcripts to form the characteristic core structure of group II introns (figure 5). In previous studies of *tscA* function, we have shown that insertions at certain sites in the *tscA* gene still allowed some degree of function, so that the *tscA* deletion host could be rescued by transformation and selection for photosynthetic growth (10). In our model, these insertions are in the periphery of the intron structure, in the loops of domains II and III. However one insertion (at a ScaI site) failed to give rise to transformed colonies, and was assumed to inactivate *tscA* activity more completely. This insertion is in one of the core helices of the proposed structure and could prevent the interaction of the tscA RNA with the 3′part of intron 1 in the exon 2 precursor (figure 5, ScaI site in helix IV). The *aadA* insertion described here is at the same site, and because the selection for spectinomycin resistance is independent of photosynthetic growth, the mutant strain can now be recovered. Its photosystem I deficiency and *psaA* trans-splicing defect confirm our previous inference.

The function of ORF472 in Chlamydomonas is not known. In contrast to the situation with the other gene disruptions, where the wild-type copy of the gene could be replaced by the mutant one, intact copies of ORF472 were always maintained. The

persistance of a heteroplasmic mixture of wild-type and interrupted copies of this ORF in the transformed strains suggests that both may be under selective pressure: for cell viability on the one hand, and for spectinomycin resistance on the other. Such a balanced mixture of two genotypes in the chloroplast has been described for unstable suppressors of *rbcL* nonsense mutations. It was proposed that the suppressor alleles which restored *rbcL* function were also deleterious to the chloroplast, so that wild-type and suppressor copies were maintained by selection (30, 31). Although it appears that ORF472 is essential for cell viability, the nature of this requirement is unclear. It is unlikely that ORF472 is directly involved in photosynthesis, because other photosynthetic mutants could be generated and maintained on medium containing acetate. The chloroplast probably ensures many other vital functions (e.g. in amino acid or lipid metabolism) in which ORF472 could participate either directly, for example by encoding an enzyme subunit, or indirectly by encoding a component generally required for gene expression (transcription, translation) or for DNA replication.

The tools described here should prove useful to study many aspects of chloroplast function. The *aadA* gene as a reporter with an easy assay could be used to study different aspects of gene expression at the transcriptional, post-transcriptional and translational levels. The chimeric *aadA* gene as a portable cassette and dominant selectable marker is a convenient tool for chloroplast reverse genetics. It could be used to interrupt and inactivate any gene whose function is dispensible under appropriate conditions. It should also be possible to introduce a large variety of mutations into the chloroplast genome by placing the selectable marker adjacent to cloned genes which would be subjected to more refined forms of site-directed mutagenesis. Transformation and selection for the *aadA* marker should give rise to a proportion of strains also carrying the adjacent, unselected mutation.

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