Construction of a novel vector for the nuclear transformation of the unicellular green alga *Chlamydomonas reinhardtii* and its stable expression

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ABSTRACT

Plasmid pBI221aadAGUS which carried both of GUS (β-glucuronidase) and aada (aminoglycoside transferase) genes besides of the 35S cauliflower mosaic virus promoter was constructed and used for stable nuclear transformation of *Chlamydomonas reinhardtii*. The vector was transformed into the alga by particle gun bombardment and two positive colonies were selected on spectinomycin-containing medium. The restriction analysis of the DNA of the positive colonies showed that aada was inserted in two orientations. The presence of introduced genes in the transformed colonies was confirmed by (PCR) using primers specific to GUS and aada genes. The expression of aada and GUS genes was revealed in all colonies that were grown on spectinomycin in liquid culture for 3–4 generations. The usefulness of this vector, differing in the orientation of the aada cassette, was manifested by transforming *C. reinhardtii* to spectinomycin resistance in the stable expression. This constructed plasmid-based expression vector system would help to unravel the functions of various genes in the green alga.

1. Introduction

Unlike bacteria, the transformation of algae is not easily accomplished and poses various technical problems [1]. The major problem is the design of vectors (i.e. plasmids or viruses) that could be successfully incorporated into, accepted by and expressed in the algal cells. The green alga *Chlamydomonas reinhardtii* and some other algal species have been used as model systems to standardise transformation protocols in algae [2–7].

Techniques used in the transformation of *Chlamydomonas* cells include bombardment of cells with DNA-coated gold particles [8–10], electroporation [6,11–14], agitation of algal protoplasts with glass beads [15,16], agitation of the intact cells in the presence of plasmid DNA and silicon carbide (SiC) whiskers [17] and transformation with *Agrobacterium tumefaciens* [18–20]. In brown algae and *Chlorella*, virus infection [21] and electroporation [22] have been successfully used, respectively.

Homologous promoters are usually preferred in *Chlamydomonas* [23,24], since heterologous promoters sometimes do not drive the expression of the transformed genes in an efficient way as reported in dinoflagellates [25]. However, Walker et al. [26] indicated that the heterologous *Dunaliella tertialecta* RbcS (ribulose-1,5-bisphosphate carboxylase/oxygenase) promoter and 3’ untranslated could be used to mediate the expression of bleomycin resistance gene (ble) in *C. reinhardtii*. Hallmann and Wodniok [27] also showed that a construction of aminoglycoside 3’ phosphotransferase (aphVIII)-based gene with 3’ and 5’ untranslated flanking sequences (including promoters) from the green alga *Volvox* worked in *Chlamydomonas*.

*Chlamydomonas* proved to be a model system for genetic and biochemical studies of various cellular processes [7,28–32]. In the current study, we succeeded in constructing a plasmid-based expression vector system for the stable nuclear transformation of the unicellular green alga *Chlamydomonas reinhardtii* using microprojectile bombardment. This system would help to unravel the functions of various genes in the green alga.

2. Materials and methods

2.1. Alga strain and cultivation conditions

*Chlamydomonas reinhardtii* strain (137c±) was cultivated in a tris-acetate-phosphate (TAP) medium as reported by Harris [28]. The medium was solidified with 2% bacto-agar (Merck) and provided with 150 mg.l⁻¹ spectinomycin sulphate (Sigma, St. Louise, USA) for the selection of transformants. The cells were cultivated in a liquid medium on a shaking incubator at 25°C under...
continuous illumination by fluorescence tubes giving a light intensity of 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \).

2.2. Isolation of total DNA from Chlamydomonas cells

Total cellular DNA was isolated according to a modified method of Harris [28] by El-Sheekh [9]. Cells were gathered in the logarithmic growth phase at a cell density of 3 \( \times 10^6 \) cells/ml. After centrifugation, the pellet was resuspended and washed in TEN buffer (10 mM Tris-HCl, pH 8.0; 10 mM EDTA; 150 mM NaCl). TEN buffer was used to resuspend the cells again to a total volume of 50 ml in a centrifuge tube. Five milliliters of 20% SDS, 5 ml of 20% sarkosyl, and 4 ml of protease (proteinase K) solution were slowly added. The tubes were rotated for 24 h at 4°C. A mixture of phenol:chloroform:isoamyl alcohol (25:24:1, respectively) was added to the tubes, followed by rotation at RT for 30–60 min. The mixture was centrifuged at 30,000 \( \times \) g for 15 min. The upper aqueous phase was taken and transferred to a clean tube. In order to avoid any contamination of the interphase materials, DNA was re-extracted with phenol:chloroform:isoamyl alcohol for 30 min. After centrifugation, the upper phase from the second extraction was mixed with the previous one. DNA was precipitated by adding 2 volumes of 95% ethanol and kept at \(-20^\circ\)C in a freezer overnight, then centrifuged at 15,000 \( \times \) g for 20 min. The precipitated DNA was then washed with 70% ethanol. DNA was dried and then dissolved in water.

2.2.1. Polymerase chain reaction (PCR)

PCR amplification of GUS and aadA fragments was carried out using Vent DNA polymerase for 30 cycles as follows: 94°C (1 min), 42°C (1 min) and 72°C (2 min), for denaturation, annealing and primer extension, respectively.

2.2.2. Plasmid construction

The expression vector pBI221aadAGUS was constructed using polyA signal and 35S promoter [29] from pRT101 and aadA from Puc-atpx-aadA. The various steps involved in the construction were as follows:

2.2.3. Cloning of the aadA gene to a vector containing the GUS gene

(a) Preparation of Puc-atpx-aadA plasmid vector

Plasmid puc-atpx-aadA (Figure 1(A)) was cut by restriction enzymes Ncol-PstI and the 800 bp fragment was purified using QIAquick Spin Column (Qiagen catalog no. 28115) (Qiagen Inc., Valencia, Calif.). The sticky end of the aadA fragment was filled and purified again using the Qiagen column.

(b) Digestion of the plasmid pRT101 with Sma I

Plasmid pRT101 was cut with SmaI (Figure 1(B)) and purified with phenol-chloroform extraction and ethanol precipitation. The purified plasmid was finally dissolved in sterile water.

(c) Ligation of aadA fragment to the linearized pRT101 vector

The purified aadA fragment was ligated to the linearized pRT101 vector using T4 ligase (Figure 1(C)). [30]. The aadA pRT101 was cloned to competent E. coli DH5\( \alpha \) on LB agar medium supplemented with 100 µg.ml\(^{-1}\) ampicillin and 25 µg.ml\(^{-1}\) spectinomycin. aadA pRT101 vector was isolated from the transformed colonies by alkaline lysis.

(d) Digestion of pRT101-aadA and pRT101 without insert

The plasmid pRT101-aadA and pRT101 were digested by restriction enzymes Hind III and EcoRI and the results are shown in Figure 2.

2.2.4. Digestion of pRT101-aadA and pBI221

The plasmid pRT101-aadA was cut with Hind III in order to obtain a fragment of about 1.5 kb containing 35S promoter, poly A signal and aadA genes (Figure 3(A)). The plasmid pBI221 was also cut by Hind III in order to linearize the DNA molecule (Figure 3(B)). The 1.5 kb fragment obtained above was then ligated to the linearized pBI221 (Hind III restricted) using T4 ligase and the resulting pBI221-aadA was used to transform E. coli (DH5\( \alpha \)). Transformants were grown on LB medium containing 100 µg.ml\(^{-1}\) ampicillin and 25 µg.ml\(^{-1}\) spectinomycin.
The plasmid was extracted from two transformed colonies by alkaline lysis for further experiments.

2.3. Nuclear transformation of C. reinhardtii

Stable nuclear transformation of Chlamydomonas cells with intact cell walls was performed using a particle gun as described previously [33,34]. The cells of Chlamydomonas were grown in a liquid medium for three days (logarithmic growth phase). Cell suspension (10 mL) was centrifuged and resuspended in 1 mL of the medium to give a cell concentration ~ 4 × 10^7 cell/mL. Thereafter, 100 µL was plated as a thin layer on TAP plate having 150 µg/ml of spectinomycin. Cells were bombarded using gold microprojectiles coated with the suitable DNA using a Biolistic PSD-100/He particle gun (Bio-rad USA). The bombarded cells were kept at 25°C for 10 days. Green colonies were cultured 3 times on TAP medium containing 150 µg/ml spectinomycin to obtain homotransplastosomes.

2.4. Amino glycoside transferase (aadA) assay

Cells of Chlamydomonas (2 × 10^9/ml) were washed in H2O and resuspended in 1/40 volume of AAD-BR (25 mM Tris-HCl pH 8.0, 5 mM MgCl2, 100 mM NH4Cl, 0.5 mM DTT). Cells were then sonicated by exposing them to pulses of 15 min of sonication interspersed with cooling on ice. AAD regulates the transfer of an acetyl phosphate moiety from ATP to streptomycin producing streptomycin adenylation and therefore the activity can be measured using radiolabelled ATP [35]. The volume of reaction mixture was 30 µl and contained 15 µl of crude sonic algal extract, 100 µM rATP, 60 µg/ml of streptomycin and alpha-32P-rATP with 370 kBq radioactivity. The control lacked the crude extract and/or streptomycin. After 30 min incubation at 35°C a 10 µl aliquot was spotted on phosphocellulose filter (1.5 × 1.5 cm^2, Whatmann p81) in duplicate and left to be adsorbed for 30 s. The filter was washed with H2O twice rapidly at 75°C, and then for 4 min at room temperature. The filter was dried and 32P radioactivity of the streptomycin p-adenyl formed in toluene based scintillation fluid was determined using a liquid scintillation counter (Packard, USA).

2.5. GUS assay

The fluorometric GUS activity assay was carried out as described by Jefferson et al. [36] using a DNA fluorometer (Hsi Hoefer Scientific Instruments DNA Fluorometer Model TKO 100). Cells were then disrupted in buffer using a sonicator (Ultrasonic Homogenizer, Model 150VT, 115 V/60HZ) with 2 times intervals, 15 min each, followed by cooling step. In control, non-bombarded cells were used for GUS assay.

2.6. Protein estimation

Protein was estimated by the method of Bradford [37] using serum albumin as a standard.

3. Results

3.1. Orientation of the insertion of aadA into pBI221

Digestion of DNA from the two transformed colonies 1 and 2 with different restriction enzymes such as Pst

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**Figure 2.** The plasmid pRT101-aadA from E.coli transformants digested with restriction enzymes Hind III + EcoR I, which yielded a band of 2.3 kb in both the transformants (see lanes A and B), pRT101 uncut without insert did not give any DNA fragment (C), DNA from colony 1 cut by BamH I + EcoR I (D), DNA from colony 2 cut with BamH I + EcoR I (E), uncut DNA plasmid (F).

**Figure 3.** (A) Restriction of pRT101-aadA with Hind III and run on agarose gel. Two bands appeared, the lower is about 1.5 kb and the upper is the rest of the plasmid. (B) Restriction map of the plasmid pBI221 restricted by Hind III to linearize DNA molecule.
I, Sph I, BamH I, Xba I, and EcoR I, was carried out in order to confirm the orientation of the insertion of \( \text{aadA} \) into pBII21 (Figures 4 and 5). Two DNA fragments, with sizes 5.8 and 1.5 kb, were recovered from digestion of insert of colony 1, using Pst I, Sph I, respectively. BamH I resulted in 2 bands, 6.3 kb and 1050 bp. Xba I gave 2 bands of the same size, 6.3 kb and 1050 bp. EcoR I gave 2 bands, 3.8 and 3.5 kb. In colony 2 (Figure 5) Pst I and Sph I gave 6050 and 1250 bp, respectively. BamH I and Xba I gave 2 bands, 5.0 and 2.3 kb, respectively. EcoR I gave a band, about 3.7 kb.

Figures 4 and 5 show that colony 1 is different from colony 2 in the orientation of the insert. The diagrammatic representation of the 2 plasmids with the 2 insertions is shown in Figure 6 (A and B).

### 3.2. Stable nuclear transformation of *Chlamydomonas* cells by the vector pBII21-\( \text{aadA-GUS} \)

In the present study, transformants were observed when cells were bombarded with gold particles coated with the transformation vector pBII21-\( \text{aadA-GUS} \) and subcultured on TAP liquid medium containing 150 mg/l spectinomycin. In order to confirm stable nuclear transformation in the transformants, the DNA was isolated and used to amplify the \( \text{GUS} \) and \( \text{aadA} \) genes (Figure 7). The results indicated that the transformed DNA contained \( \text{aadA} \) genes of 800 bp (A), and \( \text{GUS} \) gene of 1.8 kb (B).

### 3.3. Expression of \( \text{aadA} \) and \( \text{GUS} \)

Expression of both \( \text{aadA} \) and \( \text{GUS} \) was verified by the measurements of the enzyme activities (Table 1). These
mid pBI221aadAGUS, which carries the background [5,38]. In this work, the constructed plasmid pBI221aadAGUS was transformed into C. reinhardtii cells by bombardment with a particle gun, and the resultant transformants were chosen on spectinomycin-containing medium because of the high resistance of transformants to spectinomycin (150 μg/ml) in comparison with the untransformed cells, which resist only to 40 μg/ml spectinomycin and 20 μg/ml streptomycin [1]. The function of aadA was confirmed by the quantitative assay of the aadA expression. Expression of the reporter gene uidA was confirmed by detection of β-glucuronidase (GUS) activity in extracts from transformed cells as reported previously [9,41,42]. Therefore, both genes could be utilized as gene expression reporters for analysis of transcription [41,43]. Ishikura et al., [43] showed that the activity of GUS introduced into the chloroplast of Chlamydomonas was 130 U protein under photoautotrophic conditions. In this work, the GUS activity was also low (170 nmol/h/mg protein) as compared with that of Ishikura et al., [43]. The difference might be attributed to the utilization of different growth conditions and chimeric genes. The main differences between the present work and the previous work carried out by El-Sheekh [9] is that the earlier work used the same selection and reporter genes but with different promoters and terminators and the vector contained the pea origin 4.1 kb D-Loop containing sequence which is integrated into the chloroplast DNA due to homology. On the other hand, the present vector was designed for nuclear transformation of C. reinhardtii. The present work is also different from Ishikura et al. [43] (1999) that reported on chloroplast transformation with β-glucuronidase (GUS), uidA genes but with different promoters, rbcl, psbA and atpA. The culture conditions were heterotrophic and it makes difference in the expression level of the introduced genes from photoautotrophic cultivation. Zaslavskaiia et al. [44] used GUS gene to transform Phaeodactylum tricornutum, and the GUS expression reached 15 μg.g.g−1 of total soluble protein in some transformants. Liu et al. [45] also transformed Chlorella ellipsosidea using plasmid pSP-Ubi-GUS carrying zeocin resistance gene and GUS. Transformants were obtained with a transformation efficiency of 2.25 × 103 transformants/μg of plasmid DNA.

To date, stable transformants in Chlamydomonas have been successfully obtained when the introduced DNA that was homologous with the recipient was recombined with the chloroplast genome [46]. The aforementioned work also stated that introducing chimeric constructs generates duplication of those segments, already present in the host genome. Recombination between the introduced copy and the endogenous one may cause rearrangement or deletions. As described before, the nuclear transformation of Chlamydomonas reinhardtii requires facile genetic

Figure 7. PCR amplification of aadA and GUS using genomic DNA of Chlamydomonas reinhardtii transformed with pBI221-aadA-GUS (A) aadA primer (B) GUS primer.

Table 1. Aminoglycoside adenyltransferase activity (expressed as MBq incorporated per mg protein) and GUS expression expressed as nmol.(4MU)mg−1(protein) h−1 in transformed cells and of Chlamydomonas reinhardtii.

| Colony number | aadA activity (MBq/ g protein)* | GUS activity (nmol/ mg protein/h)* |
|---------------|---------------------------------|----------------------------------|
| Control (untransformed) | 0.0 | 4.60 ± 0.2 |
| 1 | 8.3 ± 1.0 | 146.7 ± 3.9 |
| 1A | 7.8 ± 1.1 | 166.8 ± 5.3 |
| 2 | 7.6 ± 1.05 | 170.4 ± 4.1 |
| 2A | 8.1 ± 2.1 | 165.6 ± 3.8 |

*Results are expressed as mean ± standard error of three replicates.

results show that both aadA and GUS activities were detected in the stable transformed colonies of Chlamydomonas reinhardtii.

4. Discussion

Expression of eubacterial genes that confer antibiotic resistance could be used as dominant selectable markers in Chlamydomonas reinhardtii under various genetic background [5,38]. In this work, the constructed plasmid pBI221aadAGUS, which carries the GUS gene as a reporter gene and aadA as selectable gene was successfully transformed into Chlamydomonas cells. Introduction of antibiotic resistance into Chlamydomonas cells was achieved by the transformation of the cells with the bacterial aadA gene to induce expression of aminoglycoside 3′-adenyl transferase (AAD) that gives the transformants resistance to spectinomycin and streptomycin [39]. The predicted protein product of a commonly used atpX-AAD construct is a fusion of AAD to the first 25N-terminal amino acids of atpA [40].

In this work, the plasmid pBI221aadAGUS was transformed into C. reinhardtii cells by bombardment with a particle gun, and the resultant transformants were chosen on spectinomycin-containing medium because of the high resistance of transformants to spectinomycin (150 μg/ml) in comparison with the untransformed cells, which resist only to 40 μg/ml spectinomycin and 20 μg/ml streptomycin [1]. The function of aadA was confirmed by the quantitative assay of the aadA expression. Expression of the reporter gene uidA was confirmed by detection of β-glucuronidase (GUS) activity in extracts from transformed cells as reported previously [9,41,42]. Therefore, both genes could be utilized as gene expression reporters for analysis of transcription [41,43]. Ishikura et al., [43] showed that the activity of GUS introduced into the chloroplast of Chlamydomonas was 130 U protein under photoautotrophic conditions. In this work, the GUS activity was also low (170 nmol/h/mg protein) as compared with that of Ishikura et al., [43]. The difference might be attributed to the utilization of different growth conditions and chimeric genes. The main differences between the present work and the previous work carried out by El-Sheekh [9] is that the earlier work used the same selection and reporter genes but with different promoters and terminators and the vector contained the pea origin 4.1 kb D-Loop containing sequence which is integrated into the chloroplast DNA due to homology. On the other hand, the present vector was designed for nuclear transformation of C. reinhardtii. The present work is also different from Ishikura et al. [43] (1999) that reported on chloroplast transformation with β-glucuronidase (GUS), uidA genes but with different promoters, rbcl, psbA and atpA. The culture conditions were heterotrophic and it makes difference in the expression level of the introduced genes from photoautotrophic cultivation. Zaslavskaiia et al. [44] used GUS gene to transform Phaeodactylum tricornutum, and the GUS expression reached 15 μg.g.g−1 of total soluble protein in some transformants. Liu et al. [45] also transformed Chlorella ellipsosidea using plasmid pSP-Ubi-GUS carrying zeocin resistance gene and GUS. Transformants were obtained with a transformation efficiency of 2.25 × 103 transformants/μg of plasmid DNA.

To date, stable transformants in Chlamydomonas have been successfully obtained when the introduced DNA that was homologous with the recipient was recombined with the chloroplast genome [46]. The aforementioned work also stated that introducing chimeric constructs generates duplication of those segments, already present in the host genome. Recombination between the introduced copy and the endogenous one may cause rearrangement or deletions. As described before, the nuclear transformation of Chlamydomonas reinhardtii requires facile genetic
transformation protocol as well as efficient and suitable vector involving promoters and terminators. In the present work, we showed that the constructed plasmid pBI221aadAGUS with its promoters and terminators could stably be integrated with the native DNA of *Chlamydomonas reinhardtii* by biolistic bombardment with heterologous genes. Interestingly, the level of expression was higher than that of previous reports, which have used other vectors.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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