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Stable transformation of the intact cells of *Chlorella kessleri* with high velocity microprojectiles

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Abstract

A transgenic expression system of *Chlorella kessleri* using the gene for β-glucuronidase (GUS) was developed. Cells of this unicellular green alga were bombarded with the plasmid pBI 121, which bears β-glucuronidase under the control of CaMV 35S promoter and the kanamycin resistant gene. Maximum GUS activity was obtained after 48 h of bombardment using a helium pressure of 900 kPa; GUS activity was then assayed for many generations. The stable transformants were able to grow on kanamycin containing medium after repeated passages between selective and nonselective medium and exhibited GUS activity comparable to that of control cells. Stable transformed cells were confirmed by polymerase chain reaction (PCR) and Southern hybridization of GUS probe with the genomic DNA of *C. kessleri*.

Additional key words: algae transformation, bacterial genes, gene expression.

Introduction

The unicellular green alga *Chlorella* has long been a favorite experimental organism for studying physiology, biochemistry, metabolic activity, biomass production and molecular biology of a number of cellular processes. A little has been done on *Chlorella* transformation by foreign genes (Nagels *et al.* 1989, Jarvis and Brown 1991). In algae, gene transfer has proceeded at a slower rate as compared with bacteria, only recently have reliable methods been developed for the efficient transformation of the green alga *Chlamydomonas reinhardtii* (Rochaix 1996, Lardans *et al.* 1997). Microalgae combine many of the advantages of plant systems with the ability to apply conventional microbiological techniques. In order to achieve transient or stable transformed cells, DNA must be taken by the cells and a marker gene must be expressed. A number of reporter genes have been used successfully in plant system, including chloramphenicol acetyl transferase, β-glucuronidase, neomycin

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phosphotransferase and firefly luciferase (Weising *et al.* 1988, Jarvis and Brown 1991) and ARG7 gene of *Chlamydomonas reinhardtii* encoding the enzyme argininosuccinate lyase (Purton and Rochaix 1995). The bacterial β-glucuronidase gene, commonly referred to as the GUS gene, combined with the increasing number of plant species accessible to molecular transformation, has become the major reporter gene used as a tool for the analysis of plant gene expression (Willmitzer 1988, Walden and Schell 1990). A number of techniques are available for introducing DNA into plant cells. These include direct transfer methods, such as protoplasting, electroporation, particle bombardment and *Agrobacterium*-mediated methods for higher plants (Ma *et al.* 1992). Successful transformation has been achieved in *Chlamydomonas* using particle bombardment (Boynton *et al.* 1988, Debuchy *et al.* 1989, Kindle *et al.* 1989, Mayfield and Kindle 1990, Goldschmidt-Clermont 1991), or by agitation of cell wall-deficient cells in the presence of DNA and glass beads (Kindle 1990, Sizova *et al.* 1997). This technique has been used to transform the organelles of lower eukaryotic cells, *e.g.*, the chloroplasts of *Chlamydomonas* (Blowers *et al.* 1989) and the mitochondria of yeast (Johnston *et al.* 1988, Fox *et al.* 1988). *Chlorella* belongs to eukaryotic organisms that undergo a simple cell division cycle, in most cases without a sexual type stage, enabling them to complete their cell cycle within a few hours and making genetic selection and strain screening relatively quick and easy. This also allows much more rapid development and demonstration of production processes than with other agriculture crops. It is also likely that genetic manipulation will be applied to algae in order to increase production of valuable primary or secondary metabolites, pharmaceuticals and other biologically active compounds.

The main objectives of this study is *1*) to prove that DNA can be introduced into intact *Chlorella* cells using DNA-coated tungsten microprojectiles accelerated by particle gun, and *2*) optimization of stable transformation in cells of *Chlorella kessleri*.

Materials and methods

***Chlorella* strain and culture conditions:** *Chlorella kessleri* 211-11h (Algal Culture Collection, Plant Physiology Institute, Göttingen University, Germany) was used in this study. The cells were grown without bubbling in YEG medium (1 % yeast extract and 1 % glucose without any additions). The cultures were maintained at 28 °C while horizontally shaken with a reciprocal motion at a frequency of 90 cycles min^{-1}

Plasmid DNA: The plasmid DNA pBI 121 (*Clontech*), containing the cauliflower mosaic virus CaMV 35S promoter cloned upstream the GUS gene and genticin resistant gene (NPT II) was used (Jefferson *et al.* 1987). Plasmid isolation was according to the alkaline lysis method of Birnboim and Doly (1979).

***Chlorella* transformation:** Particle gun (model *PSD-100/He Biolistic Particle Delivery System*, Hercules, USA) was used for *C. kessleri* transformation. The cells of the alga were grown to a density of 2.4×10^6 cell cm^{-3}. Cells from 4 cm^3 were spread on a *Millipore* membrane (*Nalgene*) under vacuum, the membrane was then transferred to 60 mm Petri plates containing semisolid medium. The tungsten or gold microprojectiles (0.7 μm diameter) were prepared and delivered using particle gun mentioned above as described by Zumbrunn *et al.* (1989) under different pressures. After transformation, the plates were incubated for 24 - 48 h in light (50 μmol m^{-2} s^{-1}). For selection of the transformed cells, *Millipore* membranes were then lifted from plates to a new ones containing 15 μg cm^{-3} kanamycin and incubated for another 48 h. The transformed colonies were subcultured repeatedly on semisolid medium containing the antibiotic kanamycin.

GUS assay was performed according to Jefferson *et al.* (1987) using DNA Fluorometer (*Model 100, Hoefer Scientific Instruments*, San Francisco, USA). The cells were sonicated in extraction buffer (*Labsonic U.B. Braun 2000u*) for two intervals, each 15 min, followed by cooling on ice. After centrifugation (6 000 *g* for 5 min), GUS activity was assayed in the supernatant. Values represent average of 3 independent experiments. Control (not bombarded cells) was also assayed for GUS activity and its value was subtracted from that of bombarded cells.

Protein was estimated by the method of Bradford (1976) using serum albumin as a standard.

Isolation of total genomic DNA: The method of Grant *et al.* (1980) modified as follows was used to isolate total genomic DNA from *Chlorella* cells. The cells from 300 cm^3 culture were harvested by centrifugation at 20 000 *g* for 10 min. The cells were resuspended in 40 cm^3 TEN buffer (10 mM Tris-HCl, pH 8.0, 10 mM Na_2EDTA, 150 mM NaCl) in 45 cm^3 plastic tube and then centrifuged for 5 min at 20 000 *g*. The cells resuspended in 5 cm^3 TEN buffer and 0.5 cm^3 20 % SDS, 0.5 cm^3 20 % sarkosyl and 50 mm^3 protease (200 μg cm^{-3}) were added slowly while stirring the mixture. The vials were incubated at 4 °C on a rotator for 24 h. Thirteen cm^3 of mixture of phenol + chloroform + isoamyl alcohol was added to each sample and shaken on rotator for 30 min at room temperature. The mixture was transferred to plastic tube and centrifuged for 15 min at 30 000 *g*. The DNA was precipitated by adding 0.1 volume of 3 M ammonium acetate, 2 volumes of cold (-20 °C) ethanol. DNA was taken up to Eppendorf tube containing 1 cm^3 of 70 % ethanol and washed twice by 70 % ethanol, dried and dissolved in water.

DNA gel electrophoresis and blotting: DNA isolated from *C. kessleri* cells was digested with restriction endonucleases under the conditions recommended by the supplier (*Promega*, Madison, USA), and resolved by electrophoresis on a 0.8 % agarose gel in 1X (TBE) buffer (40 mM Tris borate, pH 8.3, 0.2 mM EDTA) and then transferred to a gene screen plus membrane hybond N+ as described by Southern (1975). The 1.9 kb *BamH I-Sst I* restriction fragment from the plasmid

pBI 221 (*Clontech*) containing the entire coding region of the bacterial GUS gene was used as GUS probe. The probe was labeled by non-radioactive ECL (*Enhanced chemoluminescence*, Amersham, Great Britain) kit *in vitro* (*ECL-random prime system*). After hybridization and washing, membranes were analyzed by autoradiography using *X-Omat AR5* film (*Kodak*, Rochester, USA) at room temperature.

Polymerase chain reaction: PCR amplification of GUS fragment were done with *Vent* DNA polymerase (*New England Biolabs*, Massachusetts, USA) 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. Concentrations of template DNA and primers were as recommended for *Vent* DNA polymerase.

Results and discussion

The particle bombardment device was found to deliver DNA efficiently into *Chlorella* cells. The transformed colonies were selected on a kanamycin containing semisolid medium (Fig. 1). Several factors were found to affect DNA delivery and thus gene expression, including bombardment pressure, the distance of sample from adding screen, topology and vector sequence and amount of plasmid DNA. The intermediate pressure (900 kPa) was more efficient than high (1 100, 1 500 kPa) and low pressure (450 kPa) (Fig. 2*A*). However, a high pressure (1 000 - 1 200 kPa) was beneficial for DNA delivery in alfalfa suspensions (Brown *et al.* 1994). This difference may be due to the use of *Chlorella* cells as a thin film on the *Nalgene* membrane on the surface of a semisolid medium, which provides a support

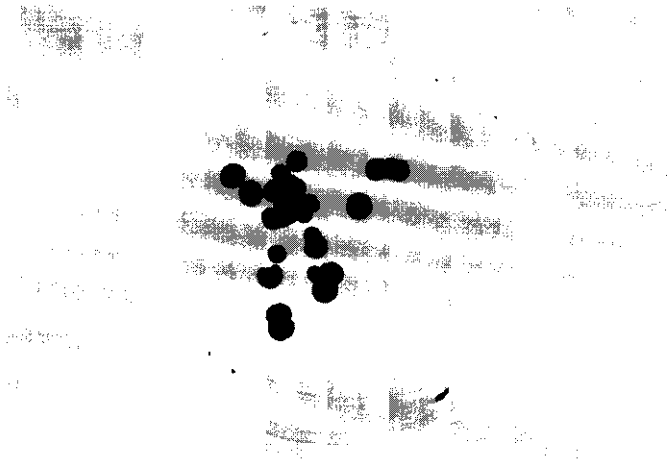

Fig. 1. *Chlorella kessleri* colonies on bombarded plates. Approximately 3×10^6 cells were spread on the whole area of Petri plate containing YEG agar medium supplemented with 15 μg cm^{-3} kanamycin. Cells were bombarded with pBI 121 DNA-coated tungsten particles.

background for the cells, and hence the high pressure may have induced injury to the algal cells. On the other hand, low pressure was not efficient due to the rigidity of the *Chlorella* cell wall, which makes the delivery of DNA difficult.

Linearization of pBI 121 DNA plasmid with Hind III exposed the 5′-end of the CaMV 35S promoter, digestion with EcoR I exposed the 3-end of polyadenylation signal sequences and double digestion led to excision of the gene with 5′- and 3′-regulatory sequences from the vector. Linearization of supercoiled plasmid at either 5′- or 3′-end of the gene did not influence the level of GUS gene expression

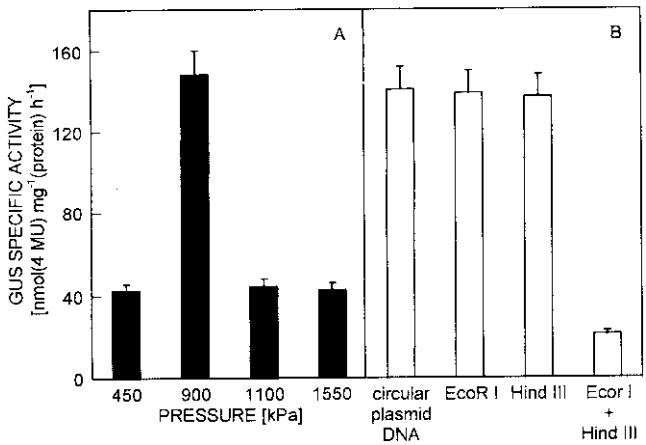

Fig. 2. *A* - Effect of variable pressure on GUS expression as reflected by GUS activity in transformed cells of *Chlorella kessleri* after 4 d of bombardment (MU-methyl umbelliferone). *B* - Effect of DNA topology and vector sequence on GUS expression in transformed cells after 4 d of incubation. Plasmid DNA was digested with the restriction enzymes EcoR I and Hind III, alone or in combination to linearize plasmid or to isolate the chimeric gene cassette, respectively. *Bars* represent SE of the mean ($n = 3$).

(Fig. 2*B*). On the other hand, double digestion of pBI 121 by Hind III and EcoR I decreased the expression of GUS level by about 85 % as compared with the control (circular DNA). Most of the work on genetic transformation using particle gun involved use of circular DNA. Iida *et al.* (1990) has shown that linearization of plasmid DNA did not appreciably influence the transient expression of the GUS gene. Similar result was also obtained by Klein *et al.* (1989) using neophospho-transferase II (NPT II) gene. Meanwhile, the use of linearized forms of template in transient expression has been shown to cause variable expression in jack pine, black spruce and tobacco protoplasts (Tautorus *et al.* 1989, Harkins *et al.* 1990, Chaudhury *et al.* 1993). For pBI 121 the topology of the vector did not influence gene expression in *Nicotiana plumbaginifolia* protoplasts (Ainley and Key 1990).

In order to confirm the stable transformation, independent transformant colonies were analyzed by Southern hybridization using GUS probe. The hybridizations were carried out at high stringency (65 °C and washing in 0.1 × SSC, 1 % SDS) (Fig. 3). The total genomic DNA of *C. kessleri* was isolated and restricted with BamH I-Sst I endonuclease that gave a known size (1.9 kb) fragment. Hybridization of GUS probe

with the genomic DNA from the transformed colonies showed the stable transformation of pBI 121 vector (Fig. 3, *lanes* 1,2,3). The untransformed colonies did not show any detectable hybridization (Fig. 3, *lane* 4). The polymerase chain reaction (PCR) technique was used to localize the GUS gene in the transformed cells (Fig. 4). The amplification of the GUS gene resulted in a fragment of approximately 1.9 kb that was obtained from DNA of colonies which were transformed and grown on the antibiotic containing medium (Fig. 4, *lanes* 1 - 8), meanwhile no amplification product was obtained from DNA of non-transformed cells (Fig. 4, *lane* 9). The transformed colonies were subcultured for many generations with repeated passage between selective and nonselective medium and the results of Southern hybridization and PCR were positive for GUS gene.

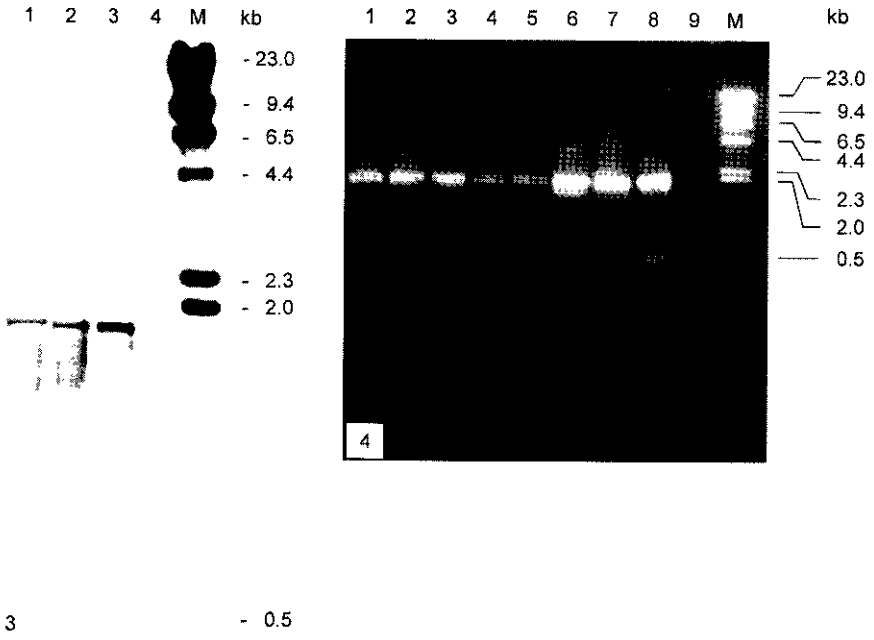

Fig. 3. Autoradiogram of a Southern blot of cellular DNA from transformed colonies of *Chlorella kessleri* digested with BamH I and Sst I, separated on a 0.8 % agarose gel, and probed with GUS gene using ECL kit. *Lane* M shows a size marker. *Lane* 4, control (non-transformed cells). *Lanes* 1,2 and 3 show transformed colonies (*left*).

Fig. 4. Polymerase chain reaction (PCR) products derived from different colonies of *Chlorella kessleri* DNA transformed by pBI 121 (*lanes* 1 - 8), *lane* 9 represents non-transformed DNA. *Lane* M shows a size marker (*right*).

In conclusion, this work characterize the ability of *Chlorella kessleri* to express foreign genes with an eventual goal of establishing an eukaryotic protein expression in green algae. This work also represents an important step in the development of a transformation system for *Chlorella* for possible biotechnological exploitation.

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