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K.-C. Chow · W. L. Tung

Electrotransformation of *Chlorella vulgaris*

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Abstract Using hygromycin B resistance as a marker for selection, we have established the conditions required for the transformation of *Chlorella vulgaris*. The exponentially grown *C. vulgaris* cells were transformed by electroporation with plasmid pIG121-Hm, and transformants were selected with hygromycin B at a concentration of 50 μg/ml. Cell extracts prepared from the late-log cultures of the transformants exhibited glucuronidase activities as conferred by the *gus* gene on pIG121-Hm. The maintenance of plasmid in the algal cells seemed to be transient as many cultures derived from the hygromycin B-resistant colonies gradually lost the hygromycin resistance upon prolonged growth. The result of Southern blotting of the genomic DNAs prepared from transformant cultures exhibiting persistent hygromycin resistance showed that integration of part of the plasmid DNA into the host chromosome had taken place.

Key words *Chlorella vulgaris* · Electrotransformation · Electroporation · Hygromycin B resistance

Introduction

Because it is unicellular, of a small genome size, easy to culture and able to serve as a food supplement, the alga *Chlorella* should be an attractive plant system for both genetic and molecular studies. However, interest in *Chlorella* has been superseded by that in another unicellular alga, *Chlamydomonas*, even though *Chlorella* has a faster growth rate and can be cryopreserved with better recovery (Johnson and Dutcher 1993; Chow and Chow 1997). One of the reasons that makes *Chlorella* less desirable for genetic and molecular studies is that a method for the direct genetic transformation for this microalga has not been established and, consequently, it is relatively difficult to identify and characterize the genes of interest derived from this organism.

Very recently, successful transformation of *Chlorella* by using microprojectile bombardment has been reported, and transformants were selected by gene conversion of mutants (Dawson et al. 1997). We reason that since it is possible to transform *Chlamydomonas reinhardtii* through electroporation (Brown et al. 1991), it should be possible to adopt such an approach to the transformation of *Chlorella* as well. Transformation by electroporation has the advantages that the operation is more straightforward as foreign DNA for transformation does not need special treatment and an electroporator is far less expensive to use than a gene gun.

Since *Chlorella* is insensitive to chloramphenicol and kanamycin, plamids like pCaMVCAT (Brown et al. 1991) and pBI121 (Clontech) that confer either chloramphenicol or kanamycin resistance to *Chlamydomonas* for selection are not compatible for *Chlorella* selection. Recent reports showing that hygromycin B is an effective antibiotic in a number of plant culture selections (Ortiz et al. 1996; Lin et al. 1996) prompted us to use the hygromycin B resistance marker-carrying plasmid pIG121-Hm (Akama et al. 1992) to explore the feasibility of transforming *Chlorella* by electroporation. In this communication, the conditions established for the successful transformation are reported.

Materials and methods

Chlorella vulgaris (North Carolina Biological Supply Company) was used as our host strain for transformation. The algal culture was grown in YA medium (Harris, 1989) at room temperature under natural light illumination and constant shaking.

The plasmid pIG121-Hm used for transformation was propagated in *E. coli* DH5α. This plasmid was purified by cesium chloride gradient ultracentrifugation as described by Sambrook et al. (1989).

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K.-C. Chow (✉) · W. L. Tung
Department of Biochemistry,
The Hong Kong University of Science and Technology,
Clear Water Bay, Kowloon, Hong Kong
Fax: 852-2358-1552
e-mail: bckchow@usthk.ust.hk

Table 1 Transformation efficiency of *Chlorella* at different electric field strengths [a]

Field strength (V/cm)	Number of colonies resistant to hygromycin B [b]
2000	500±18
1800	549±27
1500	370±40
1250	281±31
1000	206±11
800	125±18
600	46±9

[a] All samples were resuspended in sterilized distilled water and mixed with 5 μg pIG121-Hm. The cells were then electroporated with the Bio-Rad Gene Pulser at 25 μF, 200 ohm and the specified field strength. Samples electroporated in the absence of pIG121-Hm did not give any resistant colonies

[b] Mean±SD (n = 3)

Electrotransformation was performed using a Gene Pulser (Bio-Rad). An algal culture grown to a cell density of 10^6 cells/ml was harvested by centrifugation at 450 *g* at room temperature and was washed once with HS medium (Harris 1989). Washed cells were resuspended in 1/100 volume of sterilized distilled water. Eighty microliters of the resuspended cells was mixed with 5 μg plasmid pIG121-Hm and transferred to a sterile electroporation cuvette with aluminium electrodes spaced 0.2 cm apart. Electrotransformation was conducted at 25 μF and 200 ohm at a field strength ranging between 600 to 2000 V/cm. Electroporated cells were incubated on ice for 5 min. A 5-ml aliquot of YA medium was added, and the cells were allowed to grow in the dark at room temperature for 24 h. The efficiency of the transformation was determined by plating the culture on YA agar plates supplemented with 50 μg/ml hygromycin B. After about 10 days of incubation at 25 C, colonies were scored.

β-glucuronidase (GUS) activity was determined by fluorometric assay as described by Jefferson et al. (1986). Protein concentration of the cell-extract samples was determined by Bradford's method (1976) using bovine serum albumin as the standard. Equal amounts of extract protein were used in each enzyme assay.

Chromosomal DNA samples derived from transformant cultures were prepared according to procedures described by Harris (1989). About 10 μg of *Hin*dIII-cleaved total DNA from each sample was electrophoresed on a 0.7% agarose gel and transferred to a Gene Screen Plus membrane. Hybridization was carried out according to the protocol described by the manufacturer. The hybridization probe was prepared by cleaving pIG121-Hm with *Hin*dIII and then labelled with *α*-[^{32}P]-dCTP by random priming (Stratagene Kit).

Results and discussion

Table 1 shows the conditions attempted for the transformation of *C. vulgaris*. Similar to the electrotransformation of *Chlamydomonas*, bacteria and fungi where a high electric field strength is more desirable (Shimogawara et al. 1998; Dower et al. 1992; Becker and Guarente 1992), a better efficiency of transformation of *C. vulgaris* was also attained at a relatively high field strength, i.e. 1800 V/cm. This characteristic is not shared by the plant protoplasts (Saunders and Bates 1992). At a higher field strength, i.e. 2000 V/cm, the efficiency leveled off, possibly the killing effect associated with higher field strength outweighed the efficiency of introducing foreign DNA into the cells.

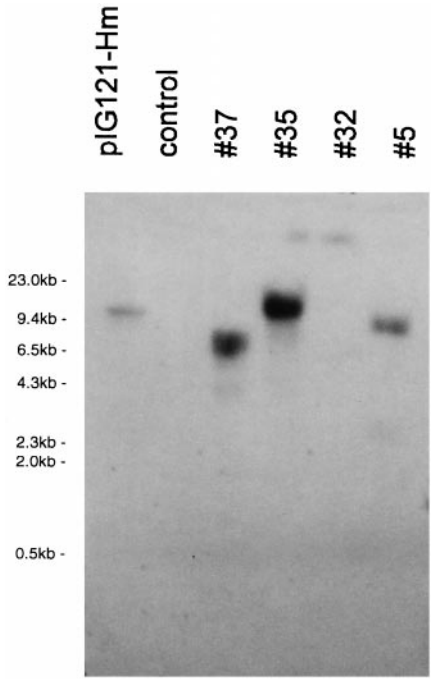

Fig. 1 Southern blot of *Hin*dIII-cleaved DNA derived from *Chlorella* transformant cultures. *Lane 1* 0.1 ng pIG121-Hm, *lane 2* DNA digest derived from an untransformed culture, *lanes 3–6* DNA samples derived from *Chlorella* cultures grown from hygromycin-resistant colonies. The DNA samples were probed with the [P^{32}]-labelled linearized pIG121-Hm

Altogether 45 hygromycin resistant colonies were used for further subculturing; upon prolonged propagation, most of them gradually lost their ability to grow in the medium supplemented with hygromycin. We believe this to be caused from the plasmid not being stably maintained in the algal cell. Eventually, we produced 4 clones that could persistently grow well in the presence of selective hygromycin B. When DNA samples were prepared from these algal clones and probed with the [P^{32}]-labelled pIG121-Hm, 3 of the clones, namely nos. 5, 35 and 37, gave positive signals on their respective *Hin*dIII-cleaved DNA profile while clone no. 32 and the control culture gave no signal (Fig. 1). The positive signals so detected correspond to DNA bands of different sizes and none of these sizes matches that of the linearized pIG121-Hm. These are good indications that the plasmid integrated into different positions on the host chromosomes. Since pIG121-Hm carries a *Hin*dIII site upstream of the 35S promoter that controls the expression of the *gus* gene, in the T-DNA, the presence of only one signal band for clone no. 35 suggests that the DNA sequence upstream of the *gus* and *hyromycin resistance* (*hpt*) genes was lost during integration. The phenomenon that the part close to the B_L end seemed to be better preserved in this transformant can be accounted for by the fact that the *hpt* gene is located close to the B_L end and that selection for the phenotype of hygromycin resistance inevitably favoured the preservation of the B_L end. The absence of a detectable sequence for clone no. 32 suggests

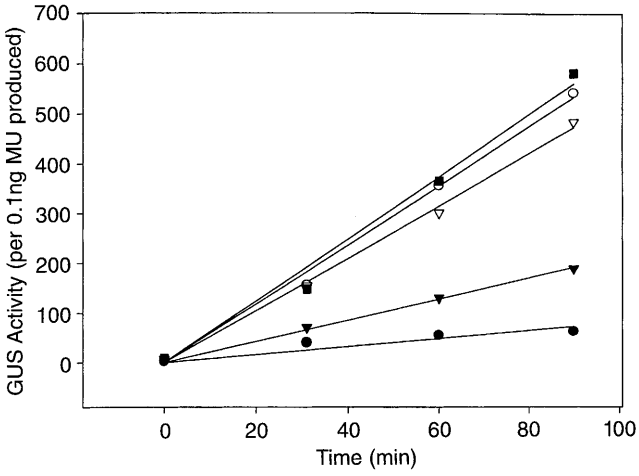

Fig. 2 GUS assay of the transformed *Chlorella* cultures. Cultures were grown in 50 ml YA medium and were harvested in the late log phase. Pellets were ruptured by freeze-thaw cycles, and 0.5 ml extraction buffer was added. Cell debris was removed by microcentrifugation at 4 °C, and 50 µl of each sample was used for enzyme assay. Protein concentration was calibrated with Bradford's method. *Solid circle* Control, *open circle* Clone no. 5, *solid triangle* no. 32, *open triangle* no. 35, *square* no. 37

that this clone was unlikely to be a transformant. Possibly, it had acquired its hygromycin resistance through mutation.

When the *C. vulgaris* transformants were assayed for GUS, the transformants showed more than a tenfold increase in activity over the control (Fig. 2). The improvement can be accounted for as the promoter for *gus* is 35S, a reasonably strong plant promoter. Clone no. 32 gave a result only slightly higher than that of the control. This result, together with the absence of a Southern blot signal, convincingly indicates that this clone was derived from mutation and had never been transformed by the plasmid pIG121-Hm.

The genetic study of *Chlorella* has long been hampered by the absence of a means for direct genetic transformation. With a proper combination of antibiotic and marker gene selection as well as the technology of electroporation, we have now shown that it is possible to transform *Chlorella*. This development provides a convenient approach for the analysis of the genetics and molecular biology of *Chlorella*. As a matter of fact, the integration of foreign DNA into the algal chromosome at different positions suggests that it may be possible to conduct gene-tagging on *Chlorella*. Such a technique is valuable for generating insertional mutants of *Chlorella* and will make gene isolation easier. The feasibility of integrating a foreign DNA sequence into the chromosome gives *Chlorella* special value in biotechnology. That is, foreign genes transmitted into the algal cell will not get lost upon propagation and, therefore, through proper design, it should be possible to develop *Chlorella* as an alternative system for the overexpression of heterologous genes that are not compatible with the commonly used hosts such as *E. coli* or yeast.

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