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Review. Methodologies for transferring DNA into eukaryotic microalgae

J. M. Coll

Dept. Biotechnology. SGIT-INIA. Ctra. de La Coruña, km 7. 28040 Madrid. Spain

Abstract

Agitation with glass beads, electroporation and microparticle bombardment are all used to transfer exogenous genes into unicellular eukaryotic algae (microalgae). For nuclear transformation most researchers use glass beads techniques or, to a lesser extent, electroporation, while for chloroplast transformation bombardment is often used. Glass bead agitation and electroporation require the removal of the cell wall while bombardment can be performed with intact microalgae. *Chlamydomonas reinhardtii* has been the microalga most commonly transformed, but success has also been reported with *Volvox carteri, Chlorella* spp., *Dunaliella salina, Haematococcus pluvialis*, *Euglena gracilis,* diatoms (*Phaeodactylum tricornutum*, *Navicula saprophila, Cyclotella cryptica* and *Thalassiosira weissflogii*), dinoflagellates (*Amphidinium klebsii* and *Symbiodinium microadriaticum*) and red algae (*Porphyridium* spp and *Cyanidioschyzon merolae*). The first *C. reinhardtii* transformants produced by making use of *Agrobacterium tumefaciens* have also been reported. A comparison of the above methods might help provide the information necessary to successfully transform further microalgal species.

Additional key words: electroporation, glass beads, particle bombardment, transformation of microalgae.

Resumen

Revisión. Metodología para la transferencia de ADN a microalgas: estudio comparativo

Se utilizan tres métodos para transferir y expresar genes exógenos en algas unicelulares eucarióticas (microalgas): la agitación con microesferas de vidrio, la electroporación y el bombardeo con micropartículas. La mayoría de los investigadores usan microesferas de vidrio y, en menor frecuencia, la electroporación para transformaciones nucleares, mientras que el bombardeo se usa casi siempre para transformaciones de cloroplastos. Mientras que tanto las microesferas de vidrio como la electroporación requieren la ausencia de paredes celulares, el bombardeo se puede efectuar en microalgas intactas. Se han descrito transformantes principalmente en *Chlamydomonas reinhardtii*, pero también en *Volvox carteri, Chlorella* spp*., Dunaliella salina, Haematococcus pluvialis*, *Euglena gracilis,* diatomeas (*Phaeodactylum tricornutum*, *Navicula saprophila, Cyclotella cryptica* y *Thalassiosira weissflogii*), dinoflagelados (*Amphidinium klebsii* y *Symbiodinium microadriaticum*) y algas rojas (*Porphyridium* spp. y *Cyanidioschyzon merolae*). Además, se han obtenido los primeros transformantes de *C. reinhardtii* usando *Agrobacterium tumefaciens*. El estudio comparativo de las principales variables de los tres métodos puede ayudar a la transformación de nuevas especies de microalgas.

Palabras clave adicionales: bombardeo con partículas, electroporación, esferas de vidrio, transformación de microalgas.

Introduction

Unicellular eukaryotic algae (referred to as microalgae in this review) stably expressing an exogenous gene have been obtained for basic research and for certain practical applications (Table 1). The majority of DNA transfer methods used to introduce these genes can be classified under the headings of agitation with glass beads, electroporation, or microparticle bombardment.

To select the few transformants produced among the many transfected microalgae obtained by these methods, two procedures are possible: 1) the rescue of auxotrophic mutants (which involves the use of homologous wild type nuclear or chloroplast genes), or 2) selection by resistance to antibiotics/herbicides (which involves the

* Corresponding author: juliocoll@inia.es
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Table 1. Possible uses and applications of transformed eukaryotic microalgae

Use	Examples[1]	Microalgae	References
Insertional mutagenesis	Random knock out	*Chlamydomonas reinhardtii* *Dunaliella salina*	Tam and Lefevre, 1993 Jin *et al.,* 2001
Homologous recombination	Knock out of genes	*Chlamydomonas reinhardtii*	Sodeinde and Kindle, 1993
Genetic mapping	Rescue with YAC	*Chlamydomonas reinhardtii*	Vashishtha *et al.,* 1996
Antiviral agents	Anti-VHSV	*Porphyridium cruentum* *Dunaliella tertiolecta*	Fabregas *et al.,* 1999
Feed production	Proteins	*Chlorella* sp.	Stevens and Purton, 1997
Chemical production	ß carotene	*Dunaliella salina*	Ben-Amotz, 1999
Gas production	Hydrogen	*Chlamydomonas reinhardtii*	US patent 20030162273
Bioremediation	Heavy metals	*Chlamydomonas reinhardtii*	Cai *et al.,* 1999; Siriporna-dulsil *et al.,* 2002
Aquaculture	Larval feeding	*Chlamydomonas reinhardtii* *Phaeodactylum tricornutum* *Chlorella* sp. *Thalassiosira* sp. *Dunaliella* sp.	Coll, 1983
Biotechnological products	HGH FGH RD RNP-1 Antibodies Insecticides Bacterial vaccines Viral vaccines hBAg	*Chlorella vulgaris* *Chlorella ellipsoidea* *Chlorella ellipsoidea* *Chlorella ellipsoidea* *Chlamydomonas reinhardtii* *Chlamydomonas reinhardtii* *Chlamydomonas reinhardtii* *Chlamydomonas reinhardtii* *Dunaliella salina*	Hawkins and Nakamura, 1999 Kim *et al.,* 2002 Yiqin *et al.,* 2001 Chen *et al.,* 2001 Mayfield *et al.,* 2003 DeCosa *et al.,* 2001 US patent 20030022359 Sun *et al.,* 2003 Geng *et al.,* 2003

[1] FGH: fish growth hormone. hBAg: hepatitis B surface antigen. HGH: human growth hormone. RD: rabbit defensin. RNP-1: rabbit neutrophyl peptide 1. VHSV: viral haemorrhagic septicaemia virus, a rhabdovirus of salmonids. YAC: yeast artificial chromosome.

use of heterologous genes). The stability of the transformant clones obtained is then examined by confirming the presence of the exogenous DNA in the microalgal genome (to rule out reversion mutants or the presence of episomal DNA) using Southern (DNA), Northern (RNA) or Western (protein) blotting. The maintenance of the acquired transformation phenotype is achieved by long-term culture under non-selection conditions.

To obtain large quantities of true transformants, their numbers must be greater than the rate of spontaneous mutation. Only in a few cases has this rate been reported lower than the transformation rate. For instance, in the haploid *Chlamydomonas reinhardtii*, one single mutant in ~ 0.1 per million microalgae spontaneously reverts to the wild type (Rochaix and VanDillewijn, 1982), while for double mutants this figure is one in ~ 0.01 per million (Boynton *et al.*, 1988). In the haploid *Volvox carteri*, one mutant is estimated to spontaneously revert in every 0.003 million (Schiedlmeier *et al.*, 1994; Hallmann *et al.*, 1997). In *Euglena gracilis*, which shows high spontaneous mutation rates, a double antibiotic (spectinomycin and streptomycin) selection method needs to be used (Doetsch *et al.*, 2001).

As pointed out recently by Walker *et al.* (2005): «The difficulty of producing stable transformants has meant that the field of transgenic microalgae is still in its infancy». However, now that the initial problems encountered with the expression of heterologous genes have been overcome, and given the present availability of many homologous and heterologous promoters and selectable genes, the number of eukaryotic microalgal species that could be reproducibly and stably modified could be increased (Table 2).

This work does not discuss the constructs used in transformation, nor nuclear versus chloroplast transformation, marker genes, or integration/large deletion issues, etc., since these have been the subject of other,

Table 2. Characteristics of the eukaryotic microalgae that have been transformed

Class	Species[1]	Water	Ploidy[2]	Motile	Shape[3]	Size[4] (~ µm)	Cell-wall
Chloroficeae (green algae)	*Chlamydomonas reinhardtii*	Fresh	Haploid	Yes	Ovoid	10 × 5	Elastic thin
	Volvox carteri	Fresh	Haploid	Yes	Round	8	Elastic thin
	Chlorella ellipsoidea	Brackish	Diploid?	No	Ovoid	4	Rigid polysaccharides
	Chlorella saccharofila	Brackish	Diploid?	No	Round	4	Rigid polysaccharides
	Chlorella vulgaris	Fresh	Diploid?	No	Round	4	Rigid polysaccharides
	Chlorella sorokiniana	Brackish	Diploid?	No	Round	4	Rigid polysaccharides
	Chlorella kessleri	Brackish	Diploid?	No	Round	4	Rigid polysaccharides
	Dunaliella salina	Marine	Diploid	Yes	Ovoid	18 × 9	Elastic thin
	Haematococcus pluvialis	Fresh	Diploid	Yes	Round	25	Mucilaginous
Bacillariophyceae (diatoms)	*Phaeodactylum tricornutum*	Marine	Diploid	No	Long	16 × 3	Hard siliceous
	Navicula saprophila	Fresh	Diploid	Yes	Long	3 × 0.5	Hard siliceous
	Cyclotella cryptica	Fresh	Diploid	No	Round	5	Hard siliceous
	Thalassiosira weissflogii	Marine	Diploid	No	Round	3	Hard no siliceous
Dinophyceae (dinoflagellates)	*Amphidinium klebsii*	Marine	Haploid	Yes	Round	15	Rigid cellulose theca
	Symbiodinium microadriaticum	Marine	Haploid	Yes	Round	15	Rigid cellulose theca
Euglenophyceae	*Euglena gracilis*	Fresh	Haploid	Yes	Ovoid	50 × 20	Elastic thin
Rodophyceae (red algae)	*Porphyridium* sp.	Marine	Diploid	No	Round	18	Sulphated polysaccharides
	Cyanidioschyzon merolae	Hot water	Haploid	No	Ovoid	2	Polysaccharides

[1] Most of the species are unicellular, except *Volvox carteri,* which is pluricellular but with few cells. The names of the different *Chlorella* spp. are those given by the authors of the corresponding reports (according to the UTEX catalogue; however, some of these might be synonymous). *Cyanidioschyzon merolae* is an extremophile that can survive at pH < 1, 45ºC and 100% CO_2; it has one of the smallest genomes of all eukaryotic cells (16 Mbp). [2] Ploidy in some of the species is difficult to define because of the alternation between haploid and diploid stages. [3] Shapes have been classified as round, ovoid and long. [4] The size offered is an approximate average of the figures given by different authors.

excellent reviews (Leon-Bañares *et al.*, 2004; Walker *et al.*, 2005). Instead it focuses on certain details of the methodology of transformation and compares certain variables associated with the glass bead, electroporation and bombardment techniques used to transfer exogenous DNA into eukaryotic microalgae. Although the first reliable report of chloroplast transformation was published in 1988 (Boynton *et al.*, 1988), and the first to report nuclear transformation appeared in 1989 (Debuchy *et al.*, 1989; Kindle *et al.*, 1989), a few reports prior to these dates are discussed.

Biological characteristics of successfully transformed eukaryotic microalgae

Most of the eukaryotic microalgae successfully transformed belong to the green algae or Chloroficeae class (*Chlamydomonas, Volvox, Chlorella, Dunaliella* and *Haematococcus*). The transformation of *C. reinhardtii* has been extensively reviewed (Stevens and Purton, 1997; Kindle, 1998; Lumbreras and Purton, 1998; Fuhrmann, 2002; Franklin and Mayfield, 2004). Other recent reviews have focused on the use of transformed microalgae in the manipulation of lipid production (Dunahay, 1996), the remediation of heavy metal contamination (Siripornadulsil *et al.*, 2002), and the production of commercially important proteins (Borowitzka, 1995; He, 2004; León-Bañares *et al.*, 2004; Walker *et al.*, 2005). Yet others have focused on the use of promoters from viruses that infect microalgae (Henry and Meints, 1994). The other eukaryotic microalgal classes that have been transformed belong to the diatoms or Bacillariophyceae (*Phaedodactylum, Navicula, Cyclotella* and *Thalassiosira*), the dinoflagellates or Dinophyceae (*Amphidinium* and *Symbiodinium*), Euglenophyceae (*Euglena*), and the red algae or Rodophyceae (*Porphyridium* and *Cyanidioschyzon*) (Table 2).

All of the eukaryotic microalgae transformed have a cell wall, an organelle that resists efforts to achieve

DNA penetration during transformation experiments. Cell walls vary from the more elastic and thinner type of *Chlamydomonas, Volvox* or *Dunaliella*, to the highly gelatinous type of red algae, the more rigid type of *Chlorella*, and the extremely hard siliceous type possessed by diatoms. The shape and size of microalgal cells are also important variables to take into account during transfection procedures. Extreme transfection conditions are required when the smaller (< 5 µm) *Chlorella* and diatoms are to be transformed, while the larger (10-50 µm) *Chlamydomonas*, *Dunaliella*, *Haematococcus* and *Euglena,* etc. are generally less demanding.

High mobility, such as that shown by *Chlamydomonas, Volvox, Euglena* or *Dunaliella* must also be taken into consideration when transfection and cloning methods are designed; their movement makes transfection more difficult.

Haploidy (as shown by *Chlamydomonas* and *Volvox*) is considered an advantage when trying to obtain transformants since this condition allows the immediate phenotypic expression of non-lethal mutations. Diploidy, in contrast, is a problem that has to be circumvented. However, most of the microalgae that have been successfully transformed are diploid or alternate between haploid and diploid stages during their cell cycles.

Agitation with glass beads

Agitation with glass beads was one of the first methods used to transform *C. reinhardtii* (Kindle, 1990). Since then it has been widely used in many laboratories because of its simplicity and reproducibility. It was then found that the addition of the membrane fusion agent polyethylene glycol (PEG) during agitation increased by 5-12-fold the number of transformants produced (Kindle, 1990). Other early methods (now in disuse given their unreliability), such as the use of poly-L-ornithine (Rochaix and VanDillewijn, 1982) and agitation with silicon carbide whiskers, might also be classed within this group (Dunahay, 1993; Lohuis and Miller, 1998).

Agitation methods usually employ 30-100 million microalgae in a 0.4 ml volume, 1-20 µg of exogenous DNA, 300 mg of glass beads (500 µm of diameter), a 5-13% solution of PEG (molecular weight 4,000-8,000 kDa), and possibly carrier DNA (25-50 µg) (Table 3). By vortexing *C. reinhardtii* with this mixture for 10-15 s, transient permeabilization is obtained which enables the exogenous DNA to enter the microalgae while preserving their viability. Increasing the vortexing time to 60 s reduces viability to 25% (Kindle, 1990). This method, however, is only efficient when using mutants that lack cell walls or with *C. reinhardtii* previously treated with autolysin to remove the cell wall (Kindle, 1990). Extending the vortexing time to several minutes for cell wall-possessing strains does not increase the number of transformants (Kindle, 1990). Exceptionally, walled *C. reinhardtii* transformants can be obtained when previously grown in ammonium-deficient medium to avoid the formation of complete cell walls (Nelson and Lefevre, 1995; Kindle, 1998). Similarly, in *Chlorella vulgaris*, glass bead transformation is only possible when the cell wall is digested with enzymes (Hawkins and Nakamura, 1999). In these conditions transformation can be achieved even without the use of the glass beads (Jarvis and Brown, 1991; Kim *et al.*, 2002). Glass bead transformation has also been also successful with *Dunaliella salina*, probably because this organism is devoid of a true cell wall (Jin *et al.*, 2001), but not with cell wall-lacking strains of *V. carteri* (these are killed in the first seconds of vortexing) (Hallmann and Rappel, 1999).

Because of the diversity and complexity of microalgal cell walls, little success has been achieved in producing viable protoplasts of most strains. Enzymatic removal of the cell wall before successful transfection has been achieved with cellulolysin (Jarvis and Brown, 1991), cellulase, macerase and pectinase in *Chlorella ellipsoidea* (Kim *et al.*, 2002), with cellulase, macerase, pectinase, hemicellulase and diselase in *Ch. vulgaris* and *Ch. sorokiniana* (Hawkins and Nakamura, 1999), and with cellulase and macerozyme in *Ch. saccharofila* (Maruyama *et al.*, 1994). However, these are complex enzyme mixtures that vary in composition and activity between batches. Two highly specific cell-wall lytic enzymes produced by *Chlorella* during sexual reproduction (Sugimoto *et al.*, 2000), as well as a viral chitinase (Hiramatsu *et al.*, 1999) and glucanase (Sun *et al.*, 2000), have been characterised, and it is hoped these will help *Chlorella* spp. protoplasts be produced more easily.

After digestion of the microalgal walls, the enzyme mixtures should be well washed to avoid excessive digestion and/or contamination with DNAses (Bergham and Petterson, 1973; Beldman *et al.*, 1985). Protoplast formation can then be monitored by staining some of the cell wall components (for instance, by fluorescent calcofluor binding to cellulose) (Lee and Tan, 1988)

Table 3. Main variables associated with the glass bead method

Microalgae[2]	Promoter[3]	Gene[4]	Volume (ml)	DNA (µg)	Carrier (µg)	Glass beads		PEG[5]		Cells ×10[6]	Results[6]			References[7]
						µm	mg	Size	%		Col 10^{-6} cells	Sb	St	
*C. reinhardtii**	RbcS2[n]	*nit1*[n]	0.4	2	50	500	300	6,000	5	40	42	Yes	—	Kindle, 1990
*C. reinhardtii**	RbcS2[n]	*ALS*[n]	0.4	2	—	500	300	6,000	5	40	8	Yes	—	Kovar, 2002
*C. reinhardtii**	—	*nit1*[n]	0.4	2	—	500	300	6,000	5	30	10	Yes	—	Sodeinde and Kindle, 1993
*C. reinhardtii**	—	*aph*	NF	20	—	500	300	600	5	50	<1	Yes	Yes	Sizova *et al.,* 1996
*C. reinhardtii**	—	*nit1*[n]	NF	1	—	900	300	8,000	5	40	100	Yes	—	Tam and Lefevre, 1993
*C. reinhardtii**	—	*ARG7*[n]	0.4	2	50	500	300	6,000	5	100	9	Yes	—	Shimogawara *et al.,* 1998
D. salina	—	*ble*	0.4	1	—	500	300	8,000	5	60	83	—	—	Jin *et al.,* 2001
*Ch. vulgaris**	CaMV35S	*hGH*	NF	—	—	<180	50	6,000	—	1,000	<1	No	No	Hawkins and Nakamura, 1999
*Ch. ellipsoidea**	CaMV35S	*ble*	0.4	5	25	—	—	4,000	13	100	—	Yes	Yes	Kim *et al.,* 2002
*Ch. ellipsoidea**	CaMV35S	*luc*	0.4	4	25	—	—	4,000	13	80	—	—	—	Jarvis and Brown, 1991

[1] —: not used, not found or not done. In the case of promoters, the selection was made by complementation of deficient mutants. [2] *: cell wall deficient cells (mutants or digested) were obtained before transfection. [3] [n]: nucleus. CaMV35S: Cauliflower mosaic virus ^{35}S promoter. RbcS2: Rubisco small subunit gene promoter. [4] [n]: nucleus. *ALS:* Acetolactate synthase gene mutant resistant to sulphometuron methyl herbicide. *aph:* Aminoglycoside phosphotransferase type VIII gene from Streptomyces. *ARG7:* Argininosuccinate lyase gene. *ble:* resistance gene to Zeocin. *hGH:* human growth hormone. *luc:* luciferase. *nit1:* Nitrate reductase gene. [5] PEG: polyethylene glycol. [6] Col 10^{-6} cells: colonies of transformants obtained per million microalgae transfected. The number of transformants per million transfected microalgae is the maximum reported and/or calculated from the reference data and approximated to the nearest round value. Sb: Southern blotting confirmation of the presence of inserted exogenous DNA (yes, exogenous sequences were present and no, exogenous sequences were not present). St: stable transformation (yes, growth in the presence of selection was maintained and no, growth in the presence of selection was lost). [7] Of the numerous reports using the glass bead method with *C. reinhardtii,* only those references which detailed the transformation procedures are included in the table.

or by measuring sensitivity to detergent-induced lysis (Fischer *et al.*, 1985). Protoplasts must be kept in isotonic solutions (0.2-0.3 M mannitol or sorbitol) to prevent their rupture and to regenerate the cell-wall (Jarvis and Brown, 1991; Maruyama *et al.*, 1994; Hawkins and Nakamura, 1999).

Transformation by agitation with glass beads is generally performed during logarithmic growth, when the number of cells undergoing mitosis, and therefore the absence of nuclear membranes, is maximal. This aids in the passage of DNA through the cell wall and membranes and, thus entry into the nucleus. After transfection, the selection of the few transformants produced is favoured by axenic cloning for a few weeks in a semisolid medium. In one report, each component of the microalgae culture medium was optimised in order to obtain the maximum growth of *Ch. vulgaris* and *sorokiniana* transformants (Hawkins and Nakamura, 1999).

From ~ 1 to 100 transformants per million transfected microalgae have been obtained with *C. reinhardtii*, *D. salina, Ch. vulgaris* and *Ch. ellipsoidea* using the glass bead method (Table 3). In a few reports, the presence of exogenous DNA in the genomes of *C. reinhardtii* (Rochaix and VanDillewijn, 1982; Sizova *et al.*, 1996) and *Ch. ellipsoidea* (Kim *et al.*, 2002) transformants has been correlated with survival in the absence of selection (Table 3).

Electroporation

In electroporation, electric pulses are varied in intensity, duration and number to control the efficiency of exogenous DNA delivery. The intensity (V cm^{-1}) is inversely proportional to microalgal survival while the duration (ms) of the pulse required is directly proportional to the resistance (Ω) of the medium. The

Ω is inversely proportional to the ionic concentration/volume of the medium and directly proportional to the width of the cuvette used to hold the microalgae.

A preincubation step is generally used to allow the exogenous DNA to come into contact with the microalgal membrane. This contact is enhanced in *C. reinhardtii* by using mutants that lack a cell wall (Brown *et al.*, 1991; Butanaev, 1994; Shimogawara *et al.*, 1998). In *Ch. saccharofila* this is achieved by prior treatment with enzymes that remove this organelle (Maruyama *et al.*, 1994). Preincubation, application of the electric pulse and postincubation are preferentially performed at ~ 4ºC to enhance survival and to allow time for the exogenous DNA to enter the cell before the complete resealing of the electrically-induced holes (Chu *et al.*, 1987). To obtain exogenous DNA inside *C. reinhardtii*, one or two 26 ms pulses are required in cell wall-lacking mutants and wild type strains respectively (Brown *et al.*, 1991).

In *C. reinhardtii*, post-transfection viabilities of cell wall-lacking mutants of 10-50% have been reported (Brown *et al.*, 1991), and either 15-40% (Brown *et al.*, 1991) or 25% (Tang *et al.*, 1995) in wild type strains; these figures provide the highest transformant yields. In *D. salina*, cell viabilities of 30-40% (Sun *et al.*, 2005) provide the maximum transformant yields.

The electric field intensity used to obtain optimal numbers of transformants ranges from 1,000 to 1,800 V cm^{-1} for pulse durations of 2-26 ms (Table 4). In *D. salina* (Degui *et al.*, 2002; Geng *et al.*, 2003) and *Ch. ellipsoidea* (Chen *et al.*, 2001), 6,000 V cm^{-1} and a very high number of pulses (2^{10}) used to be required when using a Baekon commercial system (now discontinued). The cuvettes varied in width from 0.2 to 0.4 cm with volumes ranging from 100 to 800 µl; the cell concentration of microalgae was between 0.2 to 400 million cells ml^{-1}. Carrier lineal DNA (5-50 µg), used to saturate the nucleases present or to block non-specific DNA-binding sites, was generally added to the exogenous DNA in the form of circular plasmids (0.5-10 µg) (Table 4).

The liquid medium used for electroporation, contains 10-30 mM HEPES or Tris (to keep the pH at physiological levels), 5-10 mM $CaCl_2$ (to neutralise the negative DNA charges and therefore increase binding to the microalgal membranes), and 0.04-0.4 M of sucrose, sorbitol and/or mannitol (to maintain osmolarity and thus enhance the survival of the electrically damaged cells). The medium used for electroporation is important since: i) its resistance and osmolarity influence the pulse duration, which in turn influences cell survival, ii) the concentration of divalent ions affects DNA structure and membrane stability, and iii) its general composition affects cell survival since some medium penetrates the electrically permeabilized cells.

In a systematic study using exponentially decaying electric pulses and cell wall-lacking mutants of *C. reinhardtii,* the main variables optimised were voltage-time (1,500 V cm^{-1} at 20 ms, 1800 V cm^{-1} at 6 ms or 3,000 kV cm^{-1} at 2 ms), temperature (10-20ºC), the presence of sucrose in the medium (40 mM), the addition of carrier DNA (> 200 µg ml^{-1}), the concentration of exogenous DNA (1-100 µg ml^{-1}), and the solid medium for maximal cloning efficiency (starch medium). After optimising all these parameters, thousands of transformants per million transfected microalgae were reported (Shimogawara *et al.*, 1998). Taking all the studies in Table 4 into account, the number of transformants that can be obtained by electroporation varies from < 2 to 2,770 per million transfected microalgae. The best transformation efficiencies reported have been obtained when using cell wall-lacking mutants (Brown *et al.*, 1991; Butanaev, 1994; Shimogawara *et al.*, 1998) and/or electric waves (including radio frequencies) (Tang *et al.*, 1995).

The reported 30-fold transformation efficiency variation range for different *C. reinhardtii* strains may reflect differences in the diameter of these cells, the composition of their membranes, and/or cell concentrations or division rates (Shimogawara *et al.*, 1998). In *Ch. saccharofila*, protoplast formation during the different growth phases is related to the maximum transformant yield (40, 50 and 80% in the early, exponential and stationary phases respectively) (Maruyama *et al.*, 1994). In *D. salina*, optimal transformation efficiencies were obtained by growing the cells to 2×10^6 ml^{-1} while saturation densities were reached at 25×10^6 ml^{-1} (Sun *et al.*, 2005; Tan *et al.*, 2005). The highest *D. primolecta* densities (from ~ 10 up to 80×10^6 microalgae ml^{-1}) and the shortest cell cycle durations (12 h) were obtained only when the medium components (trace elements, sugars, amino acids and vitamins) and physical conditions (light ~ 200 µmol photons m^{-2} s^{-1}, 20-30% CO_2, temperature of 28ºC) of the culture were optimised (Santin-Montaya *et al.*, 2006), and/or when 25 mM Na_2HCO_3 were included in the medium as a source of CO_2 (Jin *et al.*, 2001).

The recovery of transformants on semisolid media is dependent on achieving the optimal plating effi-

Table 4. Main variables of electroporation methods used to transform eukaryotic microalgae[1]

Microalgae[2]	Preparation								Electroporation			Results[7]			References[8]
	Promoter[3]	Gene[4]	Temp (°C)	Cuv[5] (cm)	Vol[6] (µl)	DNA (µg)	Carrier (µg)	Cells ×10[6]	V (cm^{-1})	Time (ms)	N.º pulses	Col 10^{-6} cells	Sb	St	
*C. reinhardtii**	CaMV35S	*CAT*	4	0.4	500	5.0	5	10.0	1,000	26	1	<2	Yes	—	Brown *et al.,* 1991++
C. reinhardtii	CaMV35S	*CAT*	4	0.4	800	5.0	5	80.0	1,000	4	2	<2	Yes	—	Brown *et al.,* 1991+++
*C. reinhardtii**	SV40	*nptII*	4	1.0	400	5.0	10	40.0	1,000	2	1	25	Yes	No	Butanaev, 1994
*C. reinhardtii**	—	*ARG7*^{n}	25	0.4	250	2.5	50	60.0	1,800	6	1	1,200	Yes	—	Shimogawara *et al.*, 1998
C. reinhardtii	CaMV35S	*CAT*	25	—	100	2.0	50	0.2	NF	2	15×3	2,770	Yes	—	Tang *et al.,* 1995+++
C. reinhardtii	RbcS2^{n}	*ALS*^{n}	25	0.4	250	2.5	50	100.0	1,800	6	1	250	Yes	—	Kovar, 2002
*Ch. saccharofila**	CaMV35S	*GUS*	25	0.4	1,000	10.0	50	1.0	600	400	1	—	—	—	Maruyama *et al.,* 1994+
Ch. vulgaris	—	*hyg*	4	0.2	80	5.0	—	8.0	1,800	4	1	68	Yes	—	Chow and Tung, 1999
Ch. ellipsoidea	Ubi1^{n}	*NP-1*	4	—	400	4.0	10	40.0	—	50	2^{10}	10	Yes	—	Chen *et al.,* 2001++++
D. salina	Ubi1Ω^{n}	*GUS*	4	0.2	400	4.0	10	0.4	6,000	50	2^{10}	1,900	—	—	Degui *et al.,* 2002++++
D. salina	RbcS2^{n}	*ble*	4	0.4	250	2.5	50	50.0	1,000	10	1	5	Yes	No	Sun *et al.,* 2005
D. salina	Ubi1Ω^{n}	*CAT*	4	—	400	3.0	—	0.4	6,000	5	2^{10}	150	Yes	Yes	Geng *et al.,* 2003++++
Cyanidioschyzon merolae	—	*URA*^{n}	25	—	40	0.5	—	400.0	2,500	1,000	1	<1	Yes	—	Minoda *et al.,* 2004

[1] —: not used, not found or not done. [2] *: cell wall deficient cells (mutants or digested) were obtained before transfection. [3] The selection of promoters was made by complementation of deficient mutants. [n]: nucleus. CaMV35S: Cauliflower mosaic virus ^{35}S promoter. RbcS2: promoter from the Rubisco small subunit gene. SV40: promoter of the animal adenovirus SV40. Ubi1: ubiquitin promoter. Ω: translational enhancer element from *Tobacco mosaic virus*. [4] [n]: nucleus. *ALS:* Acetolactate synthase gene mutant resistant to sulphometuron methyl herbicide. *ARG7:* Argininosuccinate lyase gene. *ble:* resistance gene to Zeocin. *CAT:* Chloramphenicol acetyltransferase gene conferring resistance to chloramphenicol. *GUS:* β-glucuronidase gene. *Hyg:* hygromycin resistance gene. *NP-1:* rabbit neutrophil peptide 1. *nptII:* Neomycin phosphotransferase *E. coli* gene conferring resistance to G418.*URA:* orotidine-5'-phosphoribosyl-transferase. [5] Cuv: width (in cm) of the cuvettes used for electroporation. [6] Vol: volume (in µl) used for electroporation. [7] Col 10^{-6} cells: colonies of transformants obtained per million transfected microalgae. The number of transformants per million transfected microalgae was the maximum reported and/or calculated from the reference data and approximated to the nearest round value. Sb: Southern blotting confirmation of the presence of inserted exogenous DNA (yes, exogenous sequences were present and no, exogenous sequences were not present). St: stable transformation (yes, growth in the presence of selection was maintained and no, growth in the presence of selection was lost). [8] +: Cell porator. ++: Gene pulser. +++: DC-shifted radio frequency at 10 kHz. ++++: Baekon 2000 electroporation system.

ciency of the transfected microalgae. For instance, to obtain maximal numbers of *D. salina* transformants with the bar gene, the electroporated microalgae need to be incubated under low light intensity (3 µmol photons m^{-2} s^{-1}) (Geng *et al.*, 2003) - *Dunaliella* spp. are resistant to phosphinotricine at higher intensities (100-200 µmol photons m^{-2} s^{-1}) (unpublished). *Cyanidioschyzon merolae* provides a further example. Under conventional culture medium conditions (35 µmol photons m^{-2} s^{-1} and bubbling with air) this organism has a doubling time of ~ 32 h, but this can be reduced to 24 h by increasing the concentration of the culture medium. Increasing the illumination to 90 µmol photons m^{-2} s^{-1} and bubbling with 5% CO_2 further shortens the doubling time to 9.2 h, allowing the earlier detection of electroporation transformants (Minoda *et al.*, 2004).

One of the highest transformation efficiencies ever reported (thousands of transformants per million cells electroporated; Table 4) was achieved by improving

the cloning efficiency of cell wall-lacking *C. reinhardtii* - from 0.2-15% when grown on agar to 40-60% when grown on starch (after testing sodium alginate, mineral oil, soft agar and starch as possible alternatives) (Shimogawara *et al.*, 1998). Microscopic examination of cell wall-lacking *C. reinhardtii* spread on the surface of the agar showed most cells had become flattened and lysed as the agar surface dried. Since protoplasts show lower plating efficiencies than non-treated microalgae, a suitable semisolid medium for cloning is required; further research in this area is required to improve on the options available. For example, the clonal growth of *Cyanidioschyzon merolae*, which has never been achieved on agar, becomes possible when gellan gum is used as the semisolid medium and the optimal conditions mentioned above are employed; this allows electroporation transformants to be obtained (Minoda *et al.*, 2004). Similarly, a semisolid culture medium including Phytal (Sigma) improves the cloning efficiency of motile *D. salina* electroporation transformants (Sun *et al.*, 2005). Low melting agar (agar VII from Sigma) or fibrin clots (Coll, 1993) might also be used in the future since, compared to agar, both increase the plating efficiencies of *D. primolecta* (by ~ 300-fold, unpublished).

Transformants of *C. reinhardtii* (Butanaev, 1994) and *D. salina* (Sun *et al.*, 2005) with integrated exogenous DNA lose their phenotype when incubated under selection conditions while *D. salina* transformants retain their phenotype (Geng *et al.*, 2003).

Microparticle bombardment

Microparticle bombardment is the method of choice for chloroplast transformation (Lumbreras and Purton, 1998). The earliest DNA transfections by bombardment were obtained in 1988 with *C. reinhardtii* (Boynton *et al.*, 1988). In 1994 the successful transfection of *V. carteri* was achieved (Schiedlmeier *et al.*, 1994; Hallmann *et al.*, 1997); the same was achieved in 1996 with *P. tricornutum* (Apt *et al.*, 1996; Zaslavskaia *et al.*, 2001).

The bombardment device most often used is the PDS-1000/He apparatus (Biorad, Hercules, CA, USA). Using high-pressure helium, this device accelerates DNA-coated gold or tungsten microparticles to ~ 500 m s^{-1} and shoots them against target monolayers of immobilised microalgae held at subatmospheric pressures (vacuum of 710-740 mmHg) to reduce the friction between the microparticles and the air (Sanford *et al.*, 1993).

The nature of the microparticles (gold or tungsten), their size (from 0.4 to 1.7 μm), their velocity [dependent of the helium pressure (6-106 bars)], and the distance to the immobilised microalgae (3-15 cm) are among the most important variables to be optimised (Sanford *et al.*, 1993; Falciatore *et al.*, 1999) (Table 5). The microparticles most commonly used are made of tungsten because they are cheaper. However, they are more irregular in shape and degrade the transformant DNA more seriously than do gold microparticles. The latter are spherical and inert and therefore give more reproducible results (Sanford *et al.*, 1993). The number of transformants obtained ranges from 0.1 to 100 per million transfected microalgae (Table 5). In *E. gracilis,* increasing the amount of DNA coated on the microparticles and bombarding twice increases the transformation efficiency (Doetsch *et al.*, 2001).

The diatoms are the microalgae with the most resistant cell walls and they require the highest helium pressures (~ 100 bars) if they are to be transformed. Organisms with thinner cell walls, such as *C. reinhardtii*, *V. carteri* and *D. salina*, require lower pressures (6-76 bars). *V. carteri* gonidia have to be transformed at ambient pressure to obtain survivals of ~ 25%; only ~0.01% is achieved under vacuum conditions (Schiedlmeier *et al.*, 1994). The pressure should be as homogeneous as possible otherwise transformants may only appear in a ring of ~ 1 cm surrounding an area of high mortality at the centre of the blast (Kindle *et al.*, 1989; Sanford *et al.*, 1993).

Prior to bombardment, the microalgae need to be spread in monolayers on filters, liquid films or agar (Doetsch *et al.*, 2001). The number of microalgae to be bombarded in order to obtain a sufficient number of transformants varies from 0.2 million in *V. carteri* (Schiedlmeier *et al.*, 1994) to 300 million in the smaller *N. saprophila* (Dunahay *et al.*, 1995).

The addition of carrier DNA in bombarded *C. reinhardtii* has no effect (Kindle *et al.*, 1989).

Mayfield and Kindle (1990) reported exponentially growing *C. reinhardtii* at 0.4×10^6 microalgae ml^{-1} to produce the highest yield of transformants. Exponentially growing *H. pluvialis* also provides bombardment transformants (Teng *et al.*, 2002), while *E. gracilis* is used in the late exponential phase (~ 6×10^6 microalgae ml^{-1}) (Doetsch *et al.*, 2001).

Synchronous cultures of *V. carteri* (Schiedlmeier *et al.*, 1994) and *H. pluvialis* (Teng *et al.*, 2002) have been

Table 5. Main variables of the particle bombardment methods used to transform eukaryotic microalgae[1]

Microalgae	Preparation					Bombardment			Results[7]			References[8]
	Promoter[2]	Gene[3]	P[4] (bars)	D[5] (cm)	Beads[6] (µm)	Cells ×10^{-6}	DNA/beads (µm mg^{-1})	Cell substrate	Col 10^{-6} cells	Sb	St	
Volvox carteri (gonidia)	TUB^{n}	*ble*	—	—	1.0	—	—	—	1	Yes	Yes	Hallmann and Rappel, 1999
Volvox carteri (gonidia)	upstream^{n}	*nitA*^{n}	6	9	2.0 ++	0.2	—	Filter paper	25	Yes	Yes	Schiedlmeier *et al.,* 1994+
Dunaliella salina	CaMV35S	*bar*	31	6	— ++	100	1.0/0.6	Liquid	89	Yes	—	Tan et al., 2005
Dunaliella salina	actin^{n}	*bar*	—	—	1.0	20	—	Agar	1.5	Yes	Yes	Guo-Zhong *et al.,* 2005
Haematococcus pluvialis	SV40	*ßgal*	41	6	0.6	—	—	Filter paper	50	No	—	Teng *et al.,* 2002
C. reinhardtii	—	*OEE1*^{n}	—	—	1.2	200	—	Liquid/agar	<0.1	Yes	Yes	Mayfield and Kindle, 1990+
C. reinhardtii	—	*nit1*^{n}	—	3	1.2	80	0.8/0.5	Liquid/agar	2.5	Yes	Yes	Kindle *et al.,* 1989 +
C. reinhardtii	—	*atpB*^{c}	—	3	1.2	6	0.4/—	Agar	8.0	Yes	No	Boynton *et al.,* 1988+
C. reinhardtii	atpA^{c}	*GUS*	76	—	1.0 ++	10	—	Agar	100.0	Yes	—	Ishikura *et al.,* 1999+
C. reinhardtii	—	*nit1*^{n}	—	—	—	30	—	Agar	3.0	Yes	—	Sodeinde and Kindle, 1993
C. reinhardtii	—	*YAC*	—	—	—	50	7.0/—	Agar	35.0	Yes	—	Vashishtha *et al.,* 1996
C. reinhardtii	—	*ASL*	—	15	—	10	0.4/0.5	Agar	5.0	Yes	—	Debuchy *et al.,* 1989
Navicula saprophila	acc1^{n}	*nptII*	76	8	0.5	300	1.0/3	Agar	0.1	Yes	—	Dunahay *et al.,* 1995
Cyclotella cryptica	acc1^{n}	*nptII*	76	8	0.5	30	1.0/3	Agar	0.3	Yes	Yes	Dunahay *et al.,* 1995
Thalassiosira weissflogii	fcp^{n}	*GUS*	—	6	1.1	5	1.0/—	Agar	<0.5	No	—	Falciatore *et al.,* 1999
Euglena gracilis	psbA^{c}	*aadA*^{n}	76	12	0.4	—	2.5/—	Durapore filter	—	Yes	—	Doetsch *et al.,* 2001
Chlorella kessleri	CaMV35S	*kan*	—	—	0.7	10	—	Millipore filter	—	Yes	Yes	El-Sheekh, 1999
Chlorella vulgaris	—	—	76	15	0.4	30	0.05/0.15	Agar	—	Yes	Yes	Dawson *et al.,* 1997
Porphyridium UTEX637	—	*AHAS*^{n}	89	9	0.7	100	0.8/0.5	Agar	0.8	Yes	—	Lapidot *et al.,* 2002
Phaeodactylum tricornutum	fcp^{n}	*ble/cat*	103	7	1.1	50	—	Agar	1.0	Yes	Yes	Apt *et al.,* 1996
Phaeodactylum tricornutum	fcp^{n}	*ble*	106	6	1.1	10	1/—	Agar	4.9	Yes	Yes	Falciatore *et al.,* 1999
Phaeodactylum tricornutum	fcp^{n}	*ble*	103	7	1.1	50	—	Agar	2.0	No	—	Zaslavskaia *et al.,* 2000

[1] —: not used, not found or not done. In the case of promoters, the selection was made by complementation of deficient mutants. [2] ^{n}: nucleus. ^{c}: chloroplast. acc1: Acetyl-CoA carboxylase gene promoter. actin: 5' upstream sequences of the actin gene. atpA: promoter from the chloroplast adenosine triphosphate gene. CaMV35S: *Cauliflower mosaic virus* 35S promoter. fcp: fucoxanthin chlorophyll binding protein promoter. psbA: promoter of the D1 protein of the PSII gene encoding the large subunit of ribulose bisphosphate-carboxylase/oxygenase. SV40: Simian virus promoter. TUB: *Volvox carteri* β-tubulin gene promoter. upstream: sequences located 5' upstream of the nitA gene. [3] ^{n}: nucleus. ^{c}: chloroplast. *aadA:* resistance to spectinomycin and streptomycin. *AHAS:* acetohydroxyacid synthase target of the sulphometuron methyl herbicide. *ASL:* argininosuccinate lyase. *atpB:* ß subunit of the chloroplast adenosine triphosphate gene. *bar:* phosphinothrim acetyltransferase conferring resistance to the active component of the herbicide Basta (bialaphos resistance gene). *ble:* resistance gene to Zeocin. *cat:* Chloramphenicol acetyltransferase gene. *GUS:* β-glucuronidase gene. *Kan:* kanamycin resistance gene. *nit1:* Nitrate reductase gene. *nptII:* Neomycin phosphotransferase *E. coli* gene conferring resistance to G418. *OEE1:* Oxygen-evolving enhancer protein. YAC: Yeast artificial chromosomes containing the FLA10 and PF14 locus of chromosome VI from *C. reinhardtii*. *ßgal:* beta galactosidase. [4] P, pressure-approximated by following the following conversions: 1 bar = 14.5 psi = 100 kPa = 100 KN m^{-2}. [5] D: distance in cm between the source of the beads and the target cell monolayers. [6] ++: gold beads. The rest were tungsten microparticles. [7] Col 10^{-6} cells: colonies of transformants obtained per million cotransfected microalgae. The number of transformants per million of transfected microalgae was the maximum reported and/or calculated from the reference data and approximated to the nearest round value. Sb: Southern blotting confirmation of the presence of inserted exogenous DNA (yes, exogenous sequences were present and no, exogenous sequences were not present). St: stable transformation (yes, growth in the presence of selection was maintained and no, growth in the presence of selection was lost). [8] +: Most of the reports employed the PDS-1000/He system from BioRad.

used to obtain bombardment transformants with greater reproducibility. Lapidot *et al.* (2002) report synchronised *Porphyridium* spp. can be obtained immediately after the dark period. When these divide in the presence of light, the naturally reduced thickness of their cell walls facilitates transfection and transformation.

The use of linearized or supercoiled circular plasmids does not significantly change the efficiency of transformation by bombardment in *C. reinhardtii* (Debuchy *et al.*, 1989; Mayfield and Kindle, 1990), *Cyclotella cryptica, N. saprophila* (Dunahay *et al.*, 1995) or *Ch. ellipsoidea* (Jarvis and Brown, 1991). However, conflicting results have been obtained for *C. reinhardtii* (Kindle *et al.*, 1989) and *P. tricornutum* (Apt *et al.*, 1996), in which the numbers of bombardment transformants decreased after linearization of the supercoiled DNA plasmids by 2 and 30-fold, respectively. Concerns have been raised regarding the correct interpretation of the above results since the possible linearization of supercoiled plasmids by friction might occur during bombardment (Dunahay *et al.*, 1995).

In contrast to the glass bead and electroporation methods, most authors report that, after bombardment, the presence of exogenous DNA in the genome of the transformants correlates positively with long term survival in the absence of selection (Table 5). This has been shown in *C. reinhardtii* (Kindle, 1990; Mayfield and Kindle, 1990), *V. carterii* (Schiedlmeier *et al.*, 1994; Hallmann and Rappel, 1999), *D. salina* (Guo-Zhong *et al.*, 2005), *C. cryptica* (Dunahay *et al.*, 1995), *Ch. kessleri* and *vulgaris* (Dawson *et al.*, 1997; El-Sheekh, 1999) and *P. tricornutum* (Apt *et al.*, 1996; Falciatore *et al.*, 1999). Mayfield and Kindle (1990) reported that although only ~20% of the *C. reinhardtii* transformants contained the exogenous gene they introduced, this was maintained for over 100 generations in the absence of selection. *V. carteri* transformants can be maintained for months (or > 100 cell divisions) in the absence of selection (Schiedlmeier *et al.*, 1994; Hallmann and Rappel, 1999). Two *P. tricornutum* clones are reported to have maintained the *ble* resistance gene after 50 cell divisions in the absence of selection (Apt *et al.*, 1996) and for at least one year (Falciatore *et al.*, 1999). In *C. cryptica,* transformants possessing the desired exogenous gene were maintained for more than one year in the absence of selection, although a number of individual microalgae were lost (Dunahay *et al.*, 1995).

Comparison of the glass bead, electroporation and bombardment methods

The advantages of glass bead agitation over the electroporation or the bombardment methods include its reproducibility in many laboratories, its simplicity, and the fact that it requires no specialised equipment. However, since cell wall-lacking microalgae are required for significant transformation to be detected, not all species can be transformed in this way.

Between ~ 100 and ~ 1,000 transformants per million transfected microalgae can be obtained by the glass bead (Table 3) and electroporation (Table 4) methods respectively. Both have been used for nuclear transformation but only for cell wall-lacking strains (Table 6). In contrast, the bombardment method can also be used for chloroplast transformation, resulting in ~ 100 transformants per million transfected cells. Bombardment can be used with almost any microalgal species, including the hard-walled diatoms (Table 5).

Cotransformation with two different plasmids, one containing a selectable gene and another containing the non-selectable gene of interest, has been widely used with microalgae because a high percentage of transformants manage to express both. Cotransformation has been most commonly used with the glass bead and bombardment methods, probably because of the introduction of multiple copies per microalga (Day *et al.*, 1990; Kindle, 1990). For instance, in *C. reinhardtii,* cotransformation rates of 10-50% (Kindle, 1990), ~ 6% (Tam and Lefevre, 1993) and ~ 10% (Berthold *et al.*, 2002) have been reported when using the glass bead method. Similarly, in *V. carteri,* bombardment cotransformation rates of 30% (Hallmann and Rappel, 1999), 40-80% (Schiedlmeier *et al.*, 1994) and 60% (Hallmann and Rappel, 1999) have been reported. In *P. tricornutum* the best figure achieved was 60-70% (Falciatore *et al.*, 1999), and in *Cyclotella cryptica* 20% (Dunahay *et al.*, 1995). In *Ch. ellipsoidea* (Chen *et al.*, 2001) and *Ch. vulgaris* (Chow and Tung, 1999), electroporation cotransformants have been obtained but no efficiencies have been reported.

The stability of transformants remains a major requirement if they are to be useful in any applied sense. Although confirmation of the presence of the exogenous DNA in the microalgae genome has been demonstrated in most reports by Southern (DNA),

Table 6. Comparison of the glass bead, electroporation and bombardment methods for transforming eukaryotic microalgae

Criteria	Glass beads	Electroporation	Bombardment
Required equipment	Simple	Complex	Complex
Predominant type of transformation[1]	Nucleus	Nucleus	Chloroplast
Numbers of transformants × 10^{-6} reported[2]	~100	~1,000	~100
Removal of cell wall required?[3]	Yes	Yes	No
Cotransformation rates	6-50%	?	20-70%
Demonstrated presence of exogenous DNA[4]	Yes	Yes	Yes
Demonstrated gene silencing[4]	Yes	Yes	Yes
Demonstrated episomal DNA[4]	Yes	Yes	Yes
Reports describing stability of transformants in the absence of selection[5]	66.6% (n = 3)	33.3% (n = 3)	90.9% (n = 11)

[1] The glass bead and electroporation methods penetrate the plasma membrane whereas bombardment penetrates both the plasma and organelle membranes. [2] This refers to the maximum number of transformants reported (data from Tables 3, 4, 5). [3] The removal of the cell wall to obtain protoplasts was required for efficient introduction of exogenous genes by the glass bead and electroporation methods (Berliner, 1981; Hawkins and Nakamura, 1999). [4] These aspects were studied in only a few reports and only in some of these were they actually demonstrated. [5] The number of reports describing the long-term stability of transformants in the absence of selection was calculated from the St column of Tables 3, 4 and 5 and expressed by the following formula: number of reports showing stability in the absence of selection/total number of reports studying stability in the absence of selection × 100.

Northern (RNA) or Western (protein) blotting, the maintenance of the acquired transformation phenotype during long-term culture is not so commonly reported. Thus, exogenous gene expression might be rapidly lost during culture even when selection is performed. This has been reported in glass bead transformants of *Ch. ellipsoidea* (Jarvis and Brown, 1991) and *Ch. vulgaris* (Hawkins and Nakamura, 1999), in electroporation transformants of *Ch. vulgaris* (Chow and Tung, 1999), and in bombardment transformants of *Thalassiosira weissflogii* (Falciatore *et al.*, 1999). Schiedlmeier *et al.* (1994) reported that some *V. carteri* bombardment transformants stopped growing and became bleached and died after a few days or weeks while others survived under selection conditions for more than a year (~ 1,500 divisions). Expression might also be lost after years of culture (Cerutti *et al.*, 1997; Wu-Scharf *et al.*, 2000), as reported for ~ 50% of *C. reinhardtii* glass bead transformants (Kindle, 1998; Schroda *et al.*, 2002).

Some of the above losses might be due to gene silencing. Changes in gene expression without modification of the genomic sequence may be attributable to gene silencing - for example by methylation (Wu-Scharf *et al.*, 2000). Babinger *et al.* (2001) reported bombardment transformants of *V. carteri* cultured for ~ 6 years (500-1,000 cell divisions) to show 6.4-fold more methylation events at the exogenous gene than in stable transformants maintained in parallel.

Loss of expression might also be due to loss of episomal DNA. Episomal DNA might be more abundant when using (as is commonly the case) circular supercoiled plasmids for transformation due to its greater resistance to intracellular DNAses than its linear derivatives. In *C. reinhardtii*, the presence of episomal DNA has been demonstrated on several occasions. Rochaix and VanDillewijn (1982) reported the episomal genes present in some of the first poly-L-ornithine transformants to be lost in the absence of selection. About 25% of bombardment transformants (inactivated in the chloroplast *atpB* gene) have also been reported to contain episomal plasmids after 65 generations under selection conditions, but that these were lost in the absence of selection (Boynton *et al.*, 1988). Tang *et al.* (1995) reported Southern blot analysis results suggesting the presence of plasmids in some electroporation transformants after 11 months of growth on selective media, a phenomenon they confirmed by plasmid rescue in *E. coli*. As mentioned above (Kindle, 1998), the thousands of transformants per million transfected microalgae that are obtained by electroporation (Tang *et al.*, 1995; Shimogawara *et al.*, 1998; Degui *et al.*, 2002) may be the result of many having episomal DNA. However, this has never been demonstrated. In other species, exogenous episomal genes have been detected under selection conditions, e.g., in 90% of *D. salina* electroporation transformants (Sun *et al.*, 2005) and in some *E. gracilis* bombardment transformants (Doetsch *et al.*, 2001). In one report in which

the glass bead method was used to obtain true *D. salina* transformants, the linearization of the *ble* codifying plasmid prevented the tendency of episomal plasmids being carried over for generations (Jin *et al.*, 2001).

In a few reports involving glass bead transformation (Table 3) and electroporation (Table 4), the presence of inserted exogenous DNA in the genome of transformants has correlated with their long term survival in the absence of selection. The long term stability of phenotypes in the absence of selection has been reported in 90.6% of papers whose authors used the bombardment method (Table 5), and in 66.6% and 33.3% of those that involved the use of glass beads and electroporation respectively (Table 6).

The maximum yields of eukaryotic microalgae transformants obtained have ranged from 100-1,000 per million transfected microalgae (Tables 3, 4 and 5) while in animal cells these figures are very often in ranges 10-100-fold higher (Colosimo *et al.*, 2000). The question therefore arises as to whether the efficiency of transfection/transformation obtained with microalgae can be increased to levels seen with these animal cells. With current techniques the expected number of transformants is sufficient for most experiments, but to be better able to select desired traits and to improve the stability of transformed cells (Table 1), larger numbers are desirable. Only improvements in the traditional methods or the development of new techniques can make this possible. It has recently been reported that the transformation of *C. reinhardtii* with *Agrobacterium tumefaciens* (T-DNA) (~350 transformants per million transfected microalgae) might be further improved and indeed used with other microalgal species (Kumar *et al.*, 2004). The transformants contained a variable number of copies of the T-DNA inserted into their genomes and grew for 18 months in the absence of selection. The simplicity of this method, the possibility of using cell wall-intact microalgae, the relatively high rate of stable transformants obtained (Table 6), and the precision of T-DNA integration, may allow its future use with other microalgal species. However, these first results need to be confirmed by independent groups.

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