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The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*

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Abstract A chimeric gene composed of the coding sequence of the *ble* gene from *Streptoalloteichus hindustanus* fused to the 5′ and 3′ untranslated regions of the *Chlamydomonas reinhardtii* nuclear gene *RBCS2* has been constructed. Introduction of this chimeric gene into the nuclear genome of *C. reinhardtii* by co-transformation with the *ARG7* marker yields Arg^+ transformants of which approximately 80% possess the *ble* gene. Of these co-transformants, approximately 3% display a phleomycin-resistant (Pm^R) phenotype. Western blot analysis using antibodies against the *ble* gene product confirms the presence of the protein in the Pm^R transformants and genetic analysis demonstrates the co-segregation of the *ble* gene with the phenotype in progeny arising from the mating of a Pm^R transformant to wild-type strains. Direct selection of Pm^R transformants was achieved by allowing an 18-h period for recovery and growth of transformed cells prior to selection. This work represents the first demonstration of stable expression and inheritance of a foreign gene in the nuclear genome of *C. reinhardtii* and provides a useful dominant marker for nuclear transformation.

Key words *Chlamydomonas* · Transformation · Dominant marker · *ble*

Introduction

Early attempts to develop a nuclear transformation system for the green unicellular alga *Chlamydomonas reinhardtii* relied on the introduction and expression of foreign genes in this organism. Rochaix and van Dillewijn (1982) reported complementation of the arginine-requiring mutant *arg7* with the yeast gene *ARG4* encoding argininosuccinate lyase. Hasnain et al. (1985) and Bingham et al. (1989) have also described foreign gene expression based on the expression of the bacterial neomycin phosphotransferase II gene. However, transformation rates in all cases were very low, often comparable with rates of reversion or mutation to spontaneous resistance. Moreover, putative transformants were usually unstable. Whilst efficient nuclear transformation has now been achieved based on the use of cloned *C. reinhardtii* genes to complement corresponding mutations (e.g. Debuchy et al. 1989; Kindle et al. 1989; Mayfield and Kindle 1990), the problem of foreign gene expression has remained (Day et al. 1990; Blankenship and Kindle 1992; Kindle and Sodeinde 1994). In spite of numerous attempts, a clear demonstration of stable integration, expression and inheritance of a foreign gene in *Chlamydomonas* has not been forthcoming. Possible explanations for this failure include the silencing of introduced genes by methylation (Blankenship and Kindle 1992), inappropriate codon usage in the foreign gene compared to the highly selective codon usage seen in *Chlamydomonas* genes (Rochaix 1987), ectopic effects (Kindle and Sodeinde 1994) and lack of introns or other non-coding elements (Stevens and Purton 1994). Recently, Hall et al. (1993) have reported the introduction of the *NPTII* gene into the nucleus of *Chlamydomonas* and demonstrated NPTII activity in these transformants. However, the co-segregation of the gene with NPTII activity in progeny resulting from sexual crosses was not tested.

In this work, we report the introduction and expression of the phleomycin (Pm) resistance gene (*ble*) from *Streptoalloteichus hindustanus* in *Chlamydomonas*. This gene was chosen for its small size and because its codon usage parallels that of *C. reinhardtii* genes. Analysis of phleomycin-resistant transformants demonstrates that

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they (i) harbour the *ble* gene; (ii) show stable resistance to phleomycin; (iii) synthesize the *ble* gene product, and (iv) show co-segregation of the introduced gene with the Pm^R phenotype. We also show that direct selection of Pm^R transformants is possible, making the *ble* gene a useful dominant marker for transformation.

Materials and methods

Strains and media

The *recA*$^-$ *Escherichia coli* strain XL1-Blue (Stratagene) was used in all recombinant DNA work. The *C. reinhardtii* strains used were the cell-wall deficient, arginine-requiring mutant 363 (*arg7-8* cw_d.mt−), the cell-wall deficient strains CC-278 (cw15.mt −) and CC-2656 (cw_d.mt−) and the wild-type strains CC-1021 (mt+) and CC-125 (mt+). These strains were obtained from the *Chlamydomonas* Culture Collection at Duke University (Harris 1989), with the exception of 363 which was a gift from R. Loppes (University of Liège). *C. reinhardtii* was grown in TRIS-acetate-phosphate (TAP) medium (Gorman and Levine 1965) supplemented with 50 mg/l arginine where required. Cultures were incubated in the light (45 μE/m^2/s) at 22° C. Inheritance of the Pm^R phenotype was investigated in crosses between transformant Pm65(mt−) and the wild-type strains as described by Harris (1989). Phleomycin resistance was scored by spotting 5 μl of cells grown in TAP to stationary phase onto TAP-agar plates containing 2 μg/ml Pm. Resistance levels in liquid culture were determined by adding 0.2 ml of cell culture to 25 ml of TAP containing a range of Pm concentrations and scoring for cell growth after 5 days.

Construction of *pSP105* and *pSP108*

The expression vector pSP105 was made by amplifying a 794-bp element from the 5′ untranslated region (UTR) of the *C. reinhardtii gene RBCS2* (Goldschmidt-Clermont and Rahire 1986) using the sense and antisense primers TGGACCGAAΔTTCGGAGTCCCCTGCACG and gggtcgacgcacgtGGCCATTTTAAGATGTT, respectively. (Upper case represents *RBCS2* sequence, Δ is a single base deletion that creates an *Eco*RI site, and lower case represents a novel sequence that introduces *Msc*I, *Pml*I and *Sal*I sites immediately downstream of the underlined initiation codon). The product was cloned into the *Hin*cII site of pBluescribe M13$^+$ (Stratagene) to make plasmid pSP104. A 231-bp *Eco*47III fragment from the 3′ UTR of *RBCS2* that includes the stop codon and the polyadenylation signal was cloned into the *Sma*I site of pSP104 to create pSP105 (Fig. 1). To make pSP108, the coding sequence of the *ble* gene was amplified from plasmid pUT430 (Drocourt et al. 1990) using the sense and antisense primers GTATCGATGGCCAAGcTGACCAGcGCCG and TTGGTCGaCGTCGGTtAGTCC, respectively [intiation codon is shown in bold, *Msc*I and *Sal*I sites used in cloning are underlined, lower case represents changes introduced to create the *Sal*I site or improve codon usage with respect to *C. reinhardtii* nuclear genes (Rochaix 1987)]. The 395-bp PCR product was cut with *Msc*I and *Sal*I and cloned into pSP105 to create pSP108 (Fig. 1). The *RBCS2-ble* junction was confirmed by DNA sequencing.

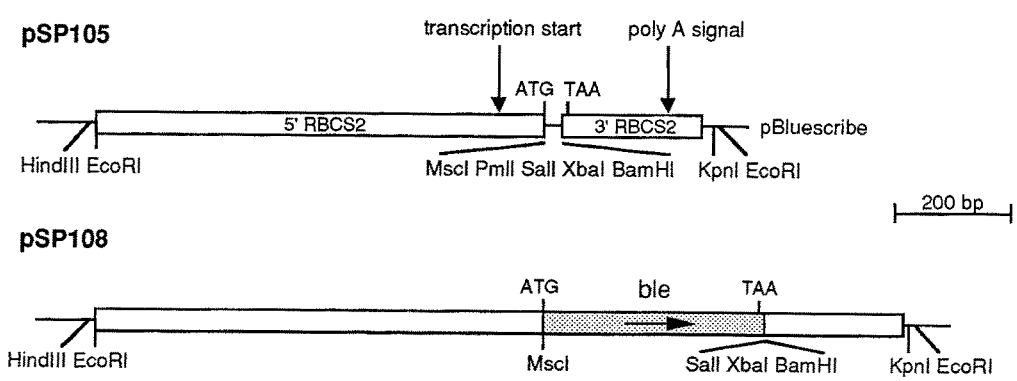
Nuclear transformation of *C. reinhardtii*

Co-transformation of strain 363 with the plasmids pARG7.8 (Debuchy et al. 1989) and pSP108 was carried out as described in Gumpel et al. (1994). For direct selection of phleomycin-resistant transformants, cells were grown to a density of 2×10^6/ml, harvested by centrifugation and resuspended in TAP to a final concentration of 2×10^8/ml. Cells (0.3 ml) were vortexed for 15 s with 0.3 g of 0.5 mm glass beads and 2 μg of pSP108, together with 100 μl of sterile 20% (v/v) polyethylene glycol (PEG) where required. The vortexed cells were diluted in 20 ml TAP liquid medium and left to express the *ble* gene by incubating at 25°C, 80 μE/m^2/s for 18 h with gentle shaking. Cells were then pelleted by centrifugation, resuspended in 4 ml of TAP containing 0.6% molten agar and poured onto the surface of a TAP agar plate containing 2 μg/ml phleomycin. Plates were incubated at 22° C in the light (45 μE/m^2/s). Transformants appeared after 7–8 days.

DNA analysis

Total genomic DNA was prepared from *C. reinhardtii* using the 'miniprep' method described by Rochaix et al. (1988). Restriction digestion, gel electrophoresis and transfer to nylon membranes (Hybond-N; Amersham) were according to standard protocols (Sambrook et al. 1989). Radiolabelled DNA probes were prepared using the method of Feinberg and Vogelstein (1984). Probes used were (i) the 395-bp PCR fragment containing the *ble* coding sequence; (ii) the 1.4-kb *Eco*RI fragment of pSP108, and (iii) the 650-bp *Bam*HI-*Nco*I region of *RBCS2* (Goldschmidt-Clermont and Rahire 1986). Hybridisation was at 42° C overnight in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 0.1% SDS, 0.1 mg/ml salmon sperm DNA. Washing of membranes was at 65° C in 0.1 × SSC, 0.1% SDS.

Fig. 1 Schematic diagram of plasmids pSP105 and pSP108. Sequences from the 5′ and 3′ untranslated regions of *RBCS2* are shown as *open boxes*. The transcription start site, putative polyadenylation signal, start codon (ATG) and stop codon (TAA) of *RBCS2* are indicated on pSP105. The start and stop codons of the phleomycin resistance gene, *ble* (*stippled box*), are indicated on pSP108. Unique restriction sites together with the two *Eco*RI sites are shown below each plasmid



Western analysis

Total soluble protein was isolated from 25-ml cultures of *Chlamydomonas* grown to 5×10^6 cells/ml. Pelleted cells were washed in protein sample buffer (5 mM HEPES pH7.5, 10 mM EDTA, 2 mM benzamidine, 2 mM DTT), repelleted and resuspended in 250 µl of the same buffer. Cells were lysed by freeze-thawing and insoluble cellular material removed by centrifugation. Protein samples were boiled for 1 min and separated by SDS-PAGE using a 17.5% TRIS-tricine gel as described by Schägger and von Jagow (1987). Proteins were transferred to nitrocellulose membrane (Towbin et al. 1979) and probed using anti-Sh.ble antibodies (Cayla, France). Antibody binding was visualised using an alkaline phosphatase assay kit (Bio-Rad) according to the manufacturer's instructions.

Results

Effect of phleomycin on *C. reinhardtii*

The glycopeptide phleomycin belongs to a family of related antibiotics that act by degrading DNA (Gatignol et al. 1988). These antibiotics exhibit activity against a wide range of prokaryotic and eukaryotic organisms and the *ble* gene from the bacterium *S. hindustanus*, encoding a 13.5 kDa phleomycin-binding protein, has been developed as a dominant marker in a number of species (e.g. Jain et al. 1992; Jefferies et al. 1993). The *ble* gene is particularly attractive as a potential marker in *Chlamydomonas*, firstly because of its small size (0.4 kb), and secondly because the codon bias in this GC-rich gene is very similar to that found in *C. reinhardtii* genes (only three of the codons in *ble* are rarely used in *C. reinhardtii* genes). In order to determine whether *ble* could be used as a dominant marker in *Chlamydomonas*, we investigated the effect of phleomycin on the wild-type strain CC-125 and the cell-wall-deficient strain CC-278. We found that *C. reinhardtii* is extremely sensitive to the drug, both in liquid medium and in solid medium, with minimum inhibitory concentrations (MIC) of approximately 0.02 µg/ml and 0.5 µg/ml, respectively. Furthermore, the absence of a cell wall has no effect on the MIC (data not shown).

Transformation rates using homologous markers are currently of the order of 10^{-5}–10^{-6}. We therefore investigated the incidence of spontaneous resistance to phleomycin in order to determine whether this antibiotic would be suitable for selection of transformants. 1×10^9 wild-type cells were plated on Pm at a concentration of 2 µg/ml. No Pm-resistant colonies were recovered, indicating that the spontaneous resistance rate is several orders of magnitude below the transformation rates.

Construction of the *Pm* resistance marker

A number of groups have reported the expression of foreign genes in *C. reinhardtii*. In each case, the gene has been linked to its own promoter (Rochaix and van Dillewijn 1982) or to another heterologous promoter such as the *nos* promoter of *Agrobacterium* (Hall et al. 1993) or the early promoter of simian virus 40 (Hasnain et al. 1985) and the evidence for expression of these genes has been open to question. Repeated attempts by ourselves to demonstrate expression of foreign genes linked to the cauliflower mosaic virus 35S promoter or to strong promoters from higher plants have been singularly unsuccessful (Day et al. 1990; our unpublished results). Conversely, several groups have successfully used *Chlamydomonas* promoters to create chimeric *Chlamydomonas* genes which are expressed in the alga (Davies et al. 1992; Kozminski et al. 1993; Nelson et al. 1994). We therefore constructed an expression vector (pSP105) based on the *C. reinhardtii* gene *RBCS2*, a highly, and constitutively, expressed gene (Goldschmidt-Clermont and Rahire 1986). Vector pSP105 contains the 5′ and 3′ untranslated regions of *RBCS2* separated by a multiple cloning site, and allows translational fusions of foreign genes at the initiation codon (Fig. 1). The phleomycin resistance gene *ble* was cloned into pSP105 to create pSP108, in which *ble* is fused in-frame with *RBCS2* (Fig. 1). During the construction, two of the non-optimal codons in *ble* were changed (TTG to CTG and AGT to AGC) to reflect the codon usage in *C. reinhardtii* genes (Rochaix 1987). In pSP108 the *ble* construct is downstream of the *lac* promoter and we have found that in the presence of IPTG, pSP108 confers phleomycin resistance to *E. coli* at a concentration of 5 µg Pm/ml (data not shown).

Generation of transformants

The *ble* gene was introduced into the nuclear genome of *C. reinhardtii* by co-transformation. Strain 363 was transformed with plasmids pSP108 and pARG7.8 (Debuchy et al. 1989) using the glass bead protocol of Kindle (1990). In two separate experiments, 100 Arg^+ transformants, arising from complementation of the *arg7-8* mutation in 363 by the cloned *ARG7* gene, were replica-plated to solid medium with or without 2 µg/ml phleomycin to score for transformants expressing the *ble* gene. In the first experiment, four Pm^R clones were identified. In the second experiment, two Pm^R clones were recovered. These six transformants were named Pm16, Pm19, Pm21, Pm65, Pm71 and Pm73. The Pm^R phenotype of these clones is stable in the absence of selective pressure. They have been maintained on TAP agar plates in the absence of phleomycin for more than 18 months and retain resistance to the drug at 2 µg/ml. DNA was isolated from the six Pm^R transformants and from nine Pm^S transformants, as well as from the untransformed strain. The DNA was digested with *Eco*RI, fractionated by gel electrophoresis, transferred to a nylon membrane and hybridised to a radiolabelled probe

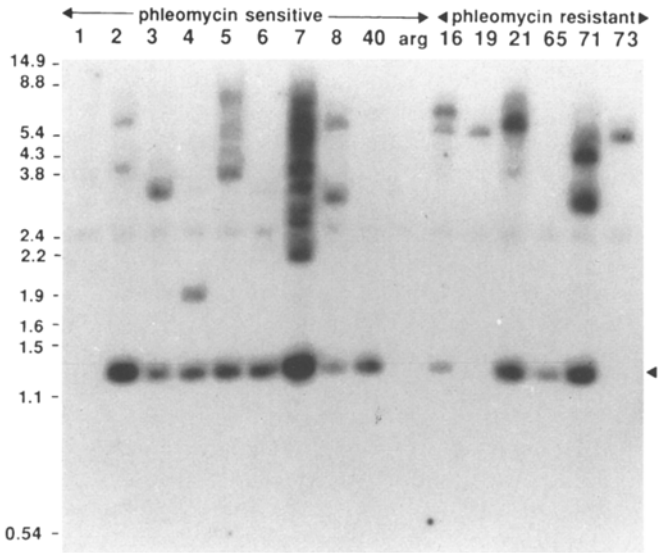


Fig. 2 Southern analysis of DNA extracted from Pm^S and Pm^R co-transformants and the untransformed strain 363 (arg). DNA was digested with *Eco*RI and hybridised with a probe for the coding region of *ble*. The 1.4 kb *Eco*RI fragment corresponding to the cassette in Fig. 1 is indicated by an *arrow*. Size markers are in kb

specific for the *ble* gene. As shown in Fig. 2, the gene is present in all of the Pm^R clones and eight of the nine Pm^S clones, but related DNA is not present in the genome of the untransformed strain. Analysis of additional Pm^S clones demonstrated that approximately 80% of Arg^+ transformants are co-transformants harbouring *ble* DNA (data not shown). The *RBCS2-ble* construct of pSP108 is flanked by *Eco*RI sites (Fig. 1). In all of the Pm^S co-transformants and four of the six Pm^R co-transformants this 1.4 kb *Eco*RI fragment has remained intact, with one or more copies present in the genome of each clone. In the case of two of the Pm^R clones (Pm19 and 73), one or both of the *Eco*RI sites appear to have been lost during the integration of the marker. Further Southern analysis has confirmed that Pm65 contains only a single copy of *ble* (data not shown) whereas Pm16, 21 and 71 contain multiple copies of the gene. Since the marker comprises *ble* flanked by the 5′ and 3′ regions of *RBCS2*, we investigated whether a gene targeting event had occurred in any of the Pm^R or Pm^S transformants, such that the endogenous *RBCS2* gene had been disrupted. Southern blot analysis revealed that *RBCS2* has remained intact in all the transformants (data not shown). This supports previous findings that homologous recombination occurs at a very low level in *Chlamydomonas* and that gene disruption events do not occur readily in this organism (Sodeinde and Kindle 1993; Gumpel et al. 1994).

Expression of *ble* in Pm^R transformants

Co-transformation occurs readily in *Chlamydomonas*, with the frequency of integration of a second unselected gene into the nuclear genome often being as high as 80% (Day et al. 1990; Diener et al. 1990; this work). It is therefore important to demonstrate that the observed phenotype of a transformant arises from the expression of the introduced gene and not from mutation within the transformant population. In order to demonstrate that the *ble* gene was being expressed in the Pm^R transformants we initially looked for *ble* transcripts by northern blot analysis and RNase protection analysis. In neither case could transcripts be detected (data not shown). Very low transcript levels for intact and chimeric transgenes in *Chlamydomonas* have been reported by various researchers (Kindle et al. 1989; Blankenship and Kindle 1992; Quinn et al. 1993) although the reasons for this are unclear. We therefore investigated whether the *ble* gene product was detectable in our transformants by western analysis using antibodies raised against the bacterial protein. Total soluble protein was extracted from four transformants, the untransformed strain and *E. coli* cells expressing the *ble* gene carried on plasmid pSP108. The protein extract was fractionated by SDS-PAGE, electroblotted to nitrocellulose and probed using the antibodies. The results are shown in Fig. 3. The native Sh.Ble protein is a small soluble protein of 13.5 kDa (Dumas et al. 1994). A single protein band of equivalent size is seen in both the bacterial and transformant extracts, but not in the untransformed *Chlamydomonas*, despite the similarity in amounts of extract loaded, as evidenced by the free chlorophyll at the bottom of the western blot. Further analysis has shown that the Sh.Ble protein is also detectable in the other two Pm^R transformants, but not

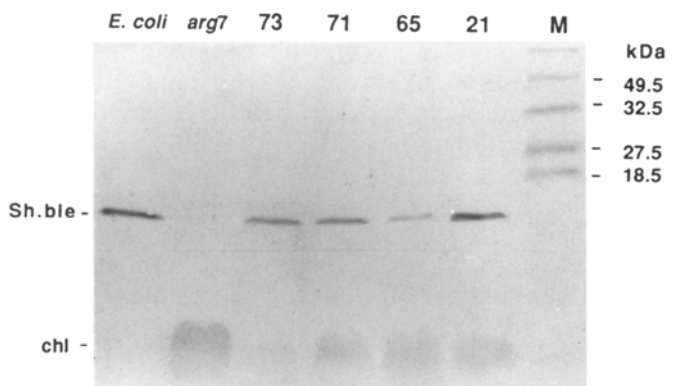


Fig. 3 Western analysis of four Pm^R *C. reinhardtii* transformants, the untransformed host 363 (arg7) and *E. coli*, transformed with pSP108 and grown in the presence of IPTG. Total soluble protein was fractionated on a 17.5% denaturing polyacrylamide gel, transferred to nitrocellulose and probed using anti-Sh.Ble antibodies. Free chlorophyll (chl) and pre-stained markers (M) are indicated

in the Pm^S co-transformants containing the *ble* gene (data not shown).

Inheritance of the Pm^R phenotype

Proof of stable integration and expression of a foreign gene in the nuclear genome of *Chlamydomonas* requires a clear demonstration of co-segregation of the gene with the phenotype in progeny arising from sexual crosses. We therefore examined the inheritance of *ble* in the Pm^R transformant Pm65(mt–), which contains a single copy of the gene, by mating to a Pm^S wild-type strain [either CC-125(mt+) or CC-1021(mt+)]. Recently we have found that the 363 strain used as the recipient for transformation carries one or more uncharacterised mutations that significantly affect mating efficiency and also prevent the generation of complete tetrads (Gumpel et al. 1995). As a consequence, we were able to obtain only a few progeny representing seven incomplete tetrads. However, these thirteen clones were scored for phleomycin resistance, the presence of a cell-wall, mating type, and arginine requirement. As shown in Table 1, seven of the thirteen are resistant to Pm. This phenotype is not tightly linked to the other phenotypic markers tested. In order to demonstrate the presence of the *ble* gene specifically in these seven progeny, DNA was isolated from all thirteen progeny, digested with *Eco*RI and hybridised to a probe representing the whole of the *ble* construct, including the *RBCS2* sequences. As shown in Fig. 4, the 1.4 kb *Eco*RI fragment harbouring the construct is present only in those seven progeny capable of growth on Pm. Although the number of progeny is low, the data provide firm evidence for the co-segregation of the *ble* DNA and the Pm^R phenotype.

Table 1 Phenotypes of progeny from the Pm65 (mt–) × WT (mt+) cross

Strain	Phenotypes[a]			
	Pm	cw	arg	mt
WT	S	+	+	+
Pm65	R	–	+	–
1A	S	+	+	+
1B	R	–	+	+
2A	S	+	–	–
2B	S	+	+	+
3A	R	–	+	+
3B	R	+	+	–
4A	R	–	+	+
4B	S	+	+	–
5A	S	+	+	+
5B	R	–	+	+
5C	S	+	–	–
6A	R	+	+	–
7A	R	–	+	ND

[a] The following traits were scored: sensitivity (S) or resistance (R) to Pm; presence (+) or absence (–) of a cell wall (cw); requirement for arginine (arg) in the medium (this phenotypic marker derives from the *arg7-8* mutation present in Pm65 but which is masked by complementation with the introduced *ARG7* gene); mating type (mt)

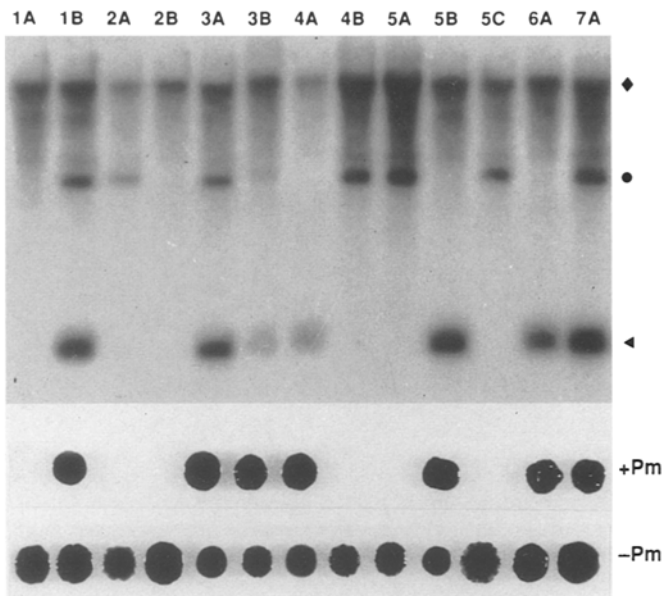


Fig. 4 The co-segregation of *ble* and the Pm^R phenotype in progeny from a Pm65(mt–) × WT(mt+) cross. DNA from the 13 progeny was cut with *Eco*RI and hybridised to the 1.4 kb *Eco*RI *RBCS2-ble* fragment (upper panel). The band corresponding to the endogenous *RBCS2* gene present in all the progeny is marked with a *diamond*. The *RBCS2-ble* transgene band derived from Pm65 is marked with an *arrow*. The presence of this transgene correlates with the Pm^R phenotype as confirmed by spot tests on solid medium +/– Pm (lower panels). An additional fragment of *RBCS2* (marked with a *circle*), which is derived from Pm65, does not co-segregate with the Pm R phenotype

Since the probe used for the Southern analysis also contains sequence from *RBCS2*, a high molecular weight band (marked with a diamond in Fig. 4) representing the native *RBCS2* gene is also seen in the Southern blot. An additional band is seen in seven of the progeny (marked with a filled circle) which does not co-segregate with the *ble* gene. This DNA is not present in the wild-type strains but is present in Pm65 (data not shown) and appears to represent additional *RBCS2* DNA derived from the pSP108 plasmid that has integrated at a second site unlinked to that of the introduced *ble* construct.

Direct selection for *PmR* transformants

Having demonstrated that the *RBCS2-ble* construct could be expressed in *Chlamydomonas* to confer Pm resistance, we tested whether this gene could be used as a dominant selectable marker for transformation. Strain 363 was transformed with pSP108 using the glass bead method of Kindle (1990) but maintaining

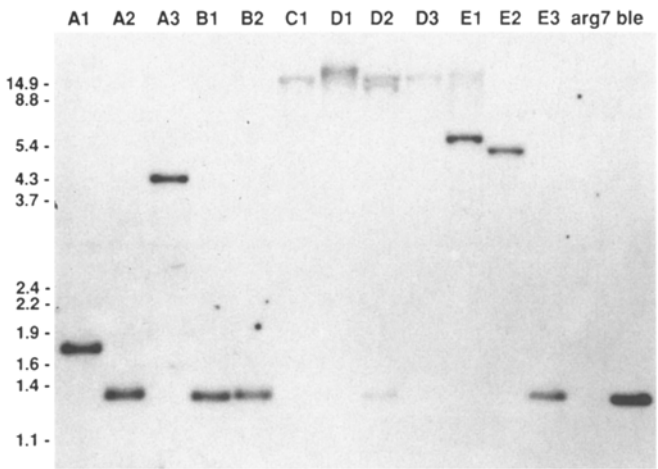


Fig. 5 Southern analysis of DNA extracted from directly selected transformants (A1–E3) and the untransformed strain 363 (arg7). DNA was digested with *Eco*RI and hybridised with a probe for the coding region of *ble*. Size markers are in kb

arginine in the medium throughout. We found that if the cells are transferred onto solid medium containing 2 μg/ml Pm immediately after vortexing with the beads, no Pm^R colonies are obtained. However, if the cells are allowed to recover and express the *ble* gene, by diluting cells in liquid medium and shaking in dim light for 18 hours prior to harvesting and plating on the solid medium, then Pm^R colonies appear after 7–9 days. Typically, 5–10 colonies are obtained per plate. Similar rates were obtained using the Arg^+ cell wall-deficient mutant CC-2656. To confirm that these colonies were true transformants, Southern blot analysis was carried out on twelve of the 363 colonies picked from five separate plates (A–E). The DNA was digested with *Eco*RI and probed using the *ble* DNA as before. As shown in Fig. 5, each transformant contains the *ble* DNA.

Kindle (1990) has previously demonstrated that glass-bead transformation using the *NIT1* marker is dramatically improved by the addition of 5% polyethylene glycol (PEG). However, transformation rates using *ARG7* were found to be unaffected by PEG (Purton and Rochaix 1995). We therefore examined what effect PEG had on transformation using the *ble* marker. Interestingly, we found that PEG had no effect on the transformation rate with CC-2656 (cw_d), but had a marked effect on CC-278 (cw15), improving the rate from only a few transformants to 40–50 transformants per plate. This finding suggests that it is the nature of the cell wall defect that determines whether the addition of PEG is beneficial, rather than the marker used.

Discussion

The development of a reliable and simple nuclear transformation system for *Chlamydomonas* has resulted in the exploitation of this alga as an excellent model system for molecular genetic studies. A variety of photosynthetic, auxotrophic and motility mutants have been characterised by complementation with cloned wild-type genes (Mayfield and Kindle 1990; Smart and Selman 1993; Debuchy et al. 1989; Kindle et al. 1989; Diener et al. 1990) and the high rate of transformation now makes possible the cloning of genes by complementation using gene libraries (Purton and Rochaix 1994; Zhang et al. 1994). Furthermore, the high incidence of co-transformation allows the efficient introduction of unselected genes into *Chlamydomonas* (e.g. Mitchell and Kang 1991). Finally, the apparently random integration of transforming DNA into the nuclear genome provides a simple way of generating 'tagged' mutants in which the affected gene is disrupted by the inserted DNA and therefore amenable to cloning (Tam and Lefebvre 1993; Davies et al. 1994). However, the development of nuclear transformation methodology has been hindered by the difficulties of expressing foreign genes in *Chlamydomonas*. This has limited the repertoire of dominant markers and reporter genes available and prevented the development of suitable markers for gene knockout experiments.

Our demonstration that a bacterial gene can be expressed in *Chlamydomonas* to provide a stable phenotype, and that this gene can be used as a dominant selectable marker, confirms that there is no intrinsic bar on the expression of foreign genes. We have shown that if the *ble* gene is introduced into the genome without selection (i.e. by co-transformation with *ARG7*) then approximately 3% of the co-transformants express the gene. It is not yet clear whether the silencing of *ble* in the majority of co-transformants is due to (i) mutations such as small deletions/insertions or rearrangements within the gene which are not detected by Southern analysis; (ii) insertion of the gene into transcriptionally inactive regions of the chromosomes; (iii) hypermethylation leading to gene silencing or (iv) a combination of the above, or some other undefined processes. Nonetheless, once a functional copy of *ble* is established in the nuclear genome it remains functional (even in the absence of selective pressure) through many generations of vegetative growth and after transmission to progeny resulting from sexual crosses.

The expression of *ble* under the control of the *RBCS2* promoter produces a sufficient cellular concentration of the Sh.Ble protein to confer resistance to phleomycin and to be easily detectable using antibodies raised against the bacterial protein. This raises the possibility that *Chlamydomonas* could be used as a host for the production of recombinant proteins. This is particularly appealing since this unicellular eukaryote is capable of photoautotrophic growth (or heterotrophic growth using the cheap carbon source acetate) and can be readily cultured in large quantity. As noted by Kindle and Sodeinde (1994), it may be possible to further improve

levels of expression by linking the foreign gene to scaffold-associated regions, as has been demonstrated in other organisms (e.g. Poljak et al. 1994).

The *RBCS2-ble* marker has a number of advantages over previously described selectable markers. Firstly, it is considerably smaller than all other available markers – 1.4 kb, compared to 9 kb for *NIT1* (Nelson et al. 1994) 7.6 kb for *ARG7* (Purton and Rochaix 1995) and 4.5 kb for *RBCS2-CRY1-1* (Nelson et al. 1994). It is portable as an *Eco*RI cassette and, unlike *ARG7*, it contains no repetitive DNA (Debuchy et al. 1989). These features simplify the design, construction and stability in *E. coli* of plasmids intended for the introduction of non-selectable genes into *Chlamydomonas*. Secondly, the *ble* gene confers phleomycin resistance in both *Chlamydomonas* and *E. coli*. Not only is this useful in the construction of *ble*-containing plasmids, but makes the *ble* marker a powerful tool for cloning genes by insertional mutagenesis. Previous work in several laboratories has shown that tagged mutants can readily be generated using *NIT1* or *ARG7* (Adam et al. 1993; Tam and Lefebvre 1993; McHugh and Spanier 1994; Gumpel et al. 1995). However, the vector DNA flanking the marker is often lost or rearranged, thereby preventing the simple isolation of genomic DNA at the integration site by plasmid (or marker) rescue to *E. coli* (Tam and Lefebvre 1993; Gumpel and Purton 1994). With the *ble* marker this problem does not arise since selection for Pm^R *Chlamydomonas* transformants ensures that the marker is intact and able to act as a selectable marker for DNA recovered to *E. coli*. Thirdly, the protocol for direct selection of Pm^R transformants using *RBCS2-ble* is somewhat simpler than that for direct selection of emetine-resistant transformants using *RBCS2-CRY1-1*. The latter involves inducing gametogenesis in transformed cells, culturing the gametes for 4 days and then allowing them to dedifferentiate back to vegetative cells prior to applying drug selection (Nelson et al. 1994). In our protocol, transformed cells are allowed to recover and express the *ble* gene by growing them in liquid medium for 18 h prior to selection on Pm-containing solid medium.

The development of the *RBCS2-ble* marker illustrates that foreign gene expression is feasible in *Chlamydomonas* and extends the repertoire of markers available for transformation. We are currently exploring various parameters which may increase the numbers of transformants that can be recovered, and the level of *ble* expression.

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