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ORIGINAL PAPER

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The flanking regions of *PsaD* drive efficient gene expression in the nucleus of the green alga *Chlamydomonas reinhardtii*

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Abstract The nuclear gene *PsaD* encodes an abundant chloroplast protein located on the stromal side of the Photosystem I complex. We have cloned and sequenced a genomic fragment containing the *PsaD* gene from the green alga *Chlamydomonas reinhardtii*. Sequence comparison with its cDNA revealed that the *PsaD* ORF contains no introns. Thus, the regulatory sequences required for high-level expression of *PsaD* must lie in the flanking promoter and untranslated regions. We used this genomic fragment to construct a vector that allows for high-level expression of endogenous and exogenous genes, as well as cDNAs that could not be expressed from existing vectors. It is also possible to use the PsaD transit sequence to target the expressed protein to the chloroplast compartment.

Keywords *Chlamydomonas reinhardtii* · Intron · Nuclear expression

Introduction

The unicellular green alga *Chlamydomonas reinhardtii* is a powerful eukaryotic model system for studying a number of biological processes such as photosynthesis, flagellar assembly and function, organelle biosynthesis, gametogenesis and mating, phototaxis and circadian rhythms (Harris 1989). This organism has recently become increasingly attractive, as reliable transformation methods have been developed for the nuclear, chloroplast and mitochondrial genomes (Rochaix 1995). The development of selectable markers, as well as the introduction of several technical improvements have made nuclear transformation simple and efficient (for review see Kindle 1998). In addition, it is possible to use gene tagging strategies and the rescue of nuclear mutants with genomic libraries to identify new genes and characterize their function (Tam and Lefevre 1993; Purton and Rochaix 1994; Zhang et al. 1994).

Despite these important advances, the expression of heterologous genes remains difficult and attempts to express reporter genes that are routinely used in other eukaryotic systems have met with limited success (Day et al. 1990; Blankenship and Kindle 1992; Kindle and Sodeinde 1994; Sizova et al. 1996; Stevens et al. 1996). The lack of efficient ectopic nuclear gene expression represents an important limitation on the genetic manipulation of *C. reinhardtii*. Several factors could account for the poor expression of genes introduced into the nuclear genome, such as position effects, inefficient transcription from heterologous promoters, inefficient RNA processing, mRNA instability and gene silencing (Cerutti et al. 1997). Furthermore, among the hallmarks of the *C. reinhardtii* nuclear genome are its high GC content (62%) and a codon bias favouring G or C at the third position (Harris 1989; Silflow 1998). It is probable that the translation of sequences that do not match this codon usage is adversely affected. This view is supported by the recent use of a synthetic gene encoding the green fluorescent protein (GFP) that can be efficiently expressed in *C. reinhardtii* (Fuhrmann et al. 1999). For this purpose, the GFP gene was completely re-synthesised to conform with the nuclear codon usage of *C. reinhardtii*.

Another feature of most *Chlamydomonas* nuclear genes is the presence of several small introns in their coding sequences. Although some intronless genes have been described, intronic sequences appear to play an important role in the regulation of gene expression (Fabry et al. 1995; Silflow 1998). Introns are not absolutely required for gene expression, as the rescue of mutants by transformation with cDNAs is possible (Diener et al. 1993; Auchincloss et al. 1999; Perron et al.

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1999; Boudreau et al. 2000). However, complementation of mutant strains with their respective cDNAs appears to be less efficient than with their genomic counterparts. It has also been clearly demonstrated that insertion of the first intron of the *RbcS2* gene into the *Ble* coding sequence increases expression levels from the *RbcS2* promoter by up to 30-fold. The improvement was found to be mediated in part by an enhancer element within the intronic sequence (Lumbreras et al. 1998). The *RbcS2* promoter has been widely used to drive gene expression in the nucleus of *C. reinhardtii* and it is considered as the strongest constitutive promoter available to date (Goldschmidt-Clermont and Rahire 1986; Kindle 1998). Recently it was shown that the strength of the *RbcS2* promoter can be further enhanced by inserting the HSP70A heat shock promoter upstream (Schroda et al. 2000). However, replacement of the *RbcS2* coding sequence by a gene of interest leads to the loss of important intronic sequences and to reduced expression levels. Introduction of *RbcS2* introns into the coding sequence, as has been performed for the selectable marker *Ble*, is possible, but time consuming. Alternatively, the intron can be inserted in non-coding flanking regions, but its efficiency appears to be reduced (Lumbreras et al. 1998).

In order to overcome the existing limitations, we have developed a new nuclear expression vector for *C. reinhardtii*, based on a genomic fragment containing the *PsaD* gene. This gene encodes an abundant protein of the Photosystem I complex and does not contain any introns in its coding sequence. The vector allows for high expression levels of both endogenous and exogenous genes. We can also make this vector more versatile by taking advantage of the *PsaD* chloroplast transit sequence to target expressed proteins to this compartment.

Materials and methods

Strains and media

C. reinhardtii wild-type and *cw15* cells were grown as described by Harris (1989). If necessary the media [TRIS-acetate phosphate medium (TAP) and high-salt minimal medium (HSM)] were solidified with 2% Bacto agar (Difco) and supplemented with zeocyn (Cayla) or arginine. The term "high light conditions" refers to illumination at 800 $\mu E/m^2/s$.

Nucleic acid techniques

Standard molecular procedures were performed as described by Sambrook et al. (1989). The bacterial host strain used was *E. coli* DH5α. The P cosmid library containing *C. reinhardtii* genomic fragments of 30–40 kb (Purton and Rochaix 1994) was screened with a radiolabelled 520-bp probe obtained by PCR amplification of the *PsaD* coding sequence, excluding the chloroplast transit sequence. A 5-kb *Pst*I fragment that hybridized to the probe was isolated from several cosmids and cloned into pKS (Stratagene). To obtain the pGenD expression vector, a 2.3-kb *Pst*I-*Apa*LI fragment containing the *psaD* gene was fully sequenced. A 2037-bp *Bam*HI-*Apa*LI fragment (blunted at the *Apa*LI site) was subcloned into pKS between the *Bam*HI and *Eco*RV recognition sites. Unique restriction sites for *Nde*I and *Eco*RI were introduced using single-tube PCR as described by Picard et al. (1994) and the following oligonucleotides: (*Nde*I) 5′-TCACAACAAGCC*CATATG*GCCG-TCATGATGCG-3′; and (EcoRI) 5′-CAGCTGCTGCCA*GAAT-TC*TTAGATCTCAGCAG-3′. The ATG of the *Nde*I site corresponds to the *PsaD* start codon and the *Eco*RI site is located just after the TAA stop codon of *PsaD*. The *Ble* gene, *Arg7* cDNA and the *PsaF* cDNA were amplified by PCR with specific oligonucleotides that introduced *Nde*I and *Eco*RI restriction sites at the 5′ and 3′ ends of the coding sequence, respectively. After endonuclease digestion, the amplified DNAs were cloned into pGenD to obtain the pGenD-Ble, pGenD-Arg7 and pGenD-PsaF constructs. The pGenD2 vector was obtained by cloning an *Xba*I-*Hin*dIII fragment of pGenD into pUC19 from which the *Nde*I-*Eco*RI polylinker fragment had been removed. This recloning step was necessary in order to introduce a unique *Nae*I site after the transit sequence of *PsaD*, as a *Nae*I site is already present in pKS. The *Nae*I restriction site was introduced as described above with the following oligonucleotide: (NaeI) 5′-AGGGGCAGCCT-CAGC*GCCGGC*GCGGACCAC CACG-3′. The different constructs produced are depicted in Fig. 1.

Transformation of *C. reinhardtii*

Nuclear transformation was performed as described by Kindle (1998). For direct selection on zeomycin, transformations were performed as described by Lumbreras et al. (1998), except that cells were plated directly on selective media without the use of molten agar. For comparison of the transformation efficiency between different constructs, transformations were carried out in parallel with the same cells, using 1 μg of DNA and 6×10^7 cells per transformation.

Preparation of chloroplast extracts and total proteins

Total protein extracts were prepared by resuspending cell pellets in protease inhibitor mix (10 mM EDTA, 5 mM ϵ-amino caproic acid, 1 mM benzamidine HCl, 25 μg/ml pepstatin A, 10 μg/ml leupeptin) and frozen at –20°C. After thawing, one volume of 2× lysis buffer was added (100 mM TRIS-HCl pH 6.8, 4% SDS) and the extracts were incubated at 37°C for 15 min. Chloroplasts were purified on discontinuous Percoll gradients as described by Zerges and Rochaix (1998).

Immunoblot analysis

Protein extracts were separated by SDS-PAGE on 15% gels. Proteins were quantitated with the bicinchoninic acid assay (Smith et al. 1995). In all cases 15 μg of total cell protein was loaded per gel slot. After electrophoresis the proteins were electroblotted onto nitrocellulose membranes before incubation with specific antibodies. Antiserum raised against the Ble protein was purchased from Cayla (France) and detection was performed with the enhanced chemiluminescence technique (Super Signal, Pierce).

Results

Cloning of the *PsaD* gene

PsaD is a 20-kDa subunit of the Photosystem I located on the stromal side of the thylakoids. PsaD is required for efficient electron transfer from Photosystem I to soluble ferredoxin (Zilber and Malkin 1988; Chitnis et al. 1989). A cDNA encoding the PsaD subunit from *C. reinhardtii* had already been cloned and sequenced in our laboratory (Farah et al. 1995). We used a fragment

of this cDNA to screen a cosmid library of total *C. reinhardtii* genomic DNA (Purton and Rochaix 1994). A 5-kb *Pst*I fragment that hybridized to the *PsaD* probe was isolated from several cosmids and cloned into pKS. A 2.3-kb *Bam*HI-*Apa*LI fragment was subcloned into pKS and fully sequenced (Fig. 1). It was found to contain a unique ORF corresponding to the *PsaD* gene. Surprisingly, the ORF sequence was identical to that of the cDNA, indicating that *PsaD* does not contain any introns in its coding sequence. Southern analysis showed that *PsaD* is present in a single copy in the genome of *C. reinhardtii* (data not shown).

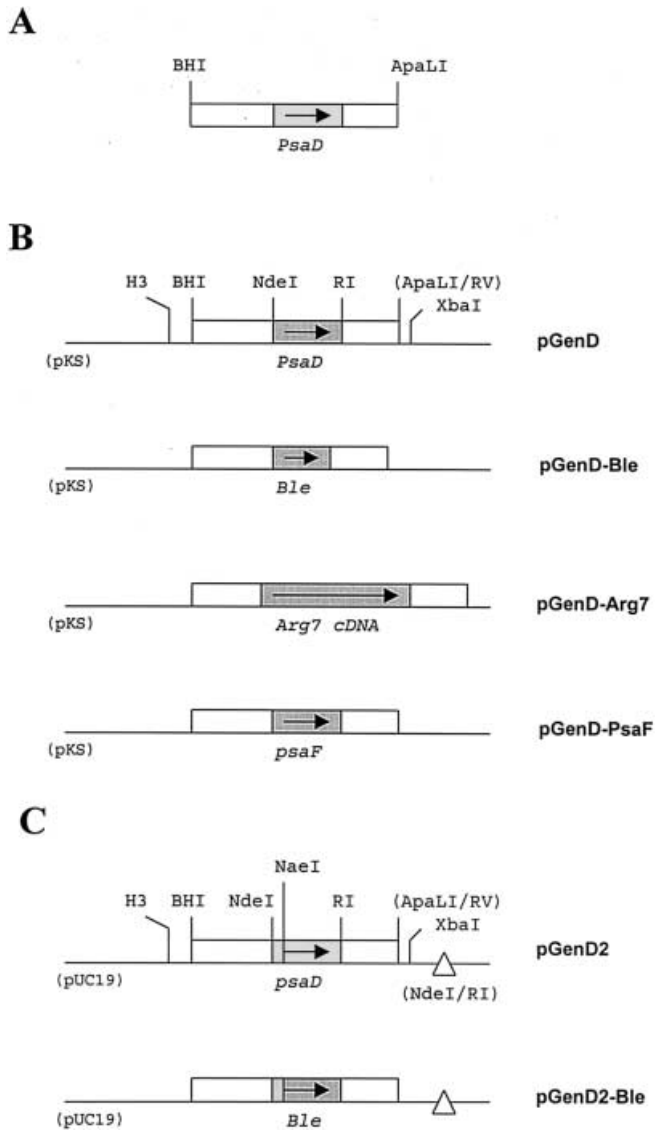

Fig. 1A–C Schematic representation of the different *PsaD*-derived constructs. **A** The 2.3-kb genomic fragment containing the *PsaD* gene. **B** The pGenD vector and pGenD-derived expression constructs *Ble*, *Arg7* and *PsaF*. **C** The pGenD2 and pGenD2-Ble constructs. The restriction sites used for subcloning are indicated on the genomic fragment, and on the pGenD and pGenD2 vectors, but were omitted for the other constructs. Abbreviations: BHI, *Bam*HI; RI, *Eco*RI; RV, *Eco*RV. The *Apa*LI/*Eco*RV junction is shown in *parentheses* to indicate that the restriction sites no longer exist. The *triangles* in **C** indicate the deletion of part of the pUC19 vector between the *Nde*I and *Eco*RI sites that removes these sites. The plasmid vector is indicated in *parentheses* on the *left* of each construct

Construction of the pGenD expression vector

Given the absence of introns in the coding sequence of *PsaD*, all the regulatory sequences leading to high expression are probably located in its flanking regions. We therefore reasoned that this gene could provide a favourable context for efficient nuclear expression of other genes. In order to construct such an expression vector, we subcloned into pKS a 2037-bp *Bam*HI-*Apa*LI fragment containing 822 bp of the *PsaD* 5′ upstream region, 591 bp of the *PsaD* coding sequence and 624 bp of the *PsaD* 3′downstream region. We subsequently introduced, by site-directed mutagenesis, a unique *Nde*I restriction site at the initiation codon of the *PsaD* ORF and a unique *Eco*RI restriction site after the stop codon to generate the pGenD vector (see Materials and methods; Fig. 1). These sites allow one to easily replace the *PsaD* ORF by other sequences.

Expression of selectable markers

We first tested the pGenD vector for the expression of the *Ble* gene and the *Arg*7 cDNA, two selectable markers used for nuclear transformation of *C. reinhardtii*. *Ble* is a bacterial gene that confers resistance to phleomycin and zeocyn, whereas *Arg* can complement mutant strains deficient in argininosuccinate lyase which are unable to grow in the absence or arginine (Debuchy et al. 1989; Stevens et al. 1996; Auchincloss et al. 1999). We constructed two plasmids, pGenD-Ble and pGenD-Arg7, in which the *PsaD* coding sequence was replaced by the *Ble* and *Arg* sequences, respectively (see Materials and methods).

We performed transformation experiments with pGenD-Ble and compared its efficiency with that of the plasmids pSP115 and pSP108 (Table 1). In pSP108 the expression of *Ble* is driven by the 740-bp promoter region of *RbcS2*, whereas in pSP115 the promoter has been shortened to 180 bp and the first intron of *RbcS2*

Table 1 Comparison of the transformation efficiencies of the different constructs under selection for expression of the *Ble* gene

Construct	Number of zeocyn-resistant transformants per μg DNA (n)[a]	Relative transformation efficiency
pSP 108	10 ± 4.2 (6)	1
PsaD genomic fragment	0 (1)	0
pSP115	93 ± 22.6 (6)	9.3
pGenD-Ble	274 ± 56.3(8)	17.4

[a]n, number of transformations

has been inserted into the *Ble* coding sequence. These modifications were shown to enhance the transformation efficiency 30-fold (Stevens et al. 1996; Lumbreras et al. 1998). Under our experimental conditions, the increase in transformation efficiency of pSP115 relative to pSP108 was approximately ten-fold. The pGenD-Ble plasmid leads to a 27-fold and 3-fold increase in transformation efficiency relative to pSP108 and pSP115, respectively (Table 1). Immunoblot analysis of total cell extracts from the transformants was performed to determine the steady-state levels of Ble protein (Fig. 2). Clearly, the expression levels observed with pGenD-Ble are at least as high as with pSP115 and the transformation efficiency of the constructs correlates with protein accumulation, indicating that the high transformation rates obtained with the pGenD-Ble vector are due to efficient expression of the selectable marker.

Similar experiments were performed to compare the transformation efficiencies of pGenD-Arg7, pKS18 (the *Arg7* cDNA cloned in pKS) and pARG7.8ϕ3 (bearing an *Arg7* genomic fragment) (Debuchy et al. 1989; Auchincloss et al. 1999). It can be seen that the *PsaD* promoter can drive the expression of the *Arg7* cDNA and leads to an almost ten-fold enhancement of transformation efficiency when compared to the cDNA alone (Table 2). However, the transformation efficiency obtained with pGenD-Arg7 was only 58% of that reached with the genomic *Arg7* DNA, indicating that the *PsaD* promoter does not provide all the necessary regulatory sequences for optimal expression of this gene.

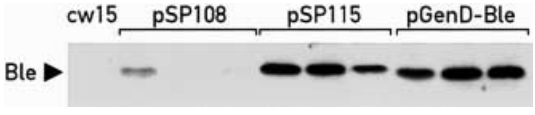

Fig. 2 Accumulation of the Ble protein. The protein concentration of the cell extracts from three transformants obtained independently with the plasmids pSP108, pSP115 and pGEND-Ble and from *cw15* was determined. Equal amounts (15 μg) of protein from each cell extract were loaded and fractionated by 15% PAGE and analyzed by immunoblotting using antisera raised against the Ble protein. The slight increase in the molecular weight of Ble in the pSP115 transformants is due to the presence of two extra codons in one loop of this protein (Lumbreras et al. 1998)

Table 2 Comparison of the transformation efficiencies of the different constructs under selection for expression of the *Arg7* gene

Construct	Number of arginine-prototrophic transformants per μg DNA (n)[a]	Relative transformation efficiency
pKS18	19 ± 5.5 (9)	1
PsaD genomic fragment	0 (2)	0
pGenD-Arg 7	177 ± 20 (9)	9.3
pARG7.8ϕ3	303 ± 20 (6)	15.9

[a]n, number of transformations

Expression of the *PsaF* cDNA

Some cDNAs appear to be particularly difficult to express in the nucleus of *C. reinhardtii,* especially when no direct selection can be applied and co-transformation experiments need to be performed. An example is the *PsaF* gene which encodes a lumenal subunit of the Photosystem I complex. The 3bF mutant strain carries a deletion in the *PsaF* gene, but is able to grow photoautotrophically (Farah et al. 1995). Although PsaF is required for photoautotrophic growth under high light conditions, it is not possible to select directly for *PsaF* expression (Hippler et al. 2000). Rescue of the mutant by co-transformation of a selectable marker together with the *PsaF* genomic DNA can be performed routinely (Hippler et al. 1998). However, expression of the PsaF protein from its cDNA could never be achieved in similar experiments (M. Hippler, D. Stevens and J-D Rochaix, unpublished results). In order to test the ability of the *PsaD* promoter to drive the expression of such a cDNA, we generated the pGenD-PsaF construct by introducing the *PsaF* cDNA into pGenD (Fig. 1). This plasmid was introduced into Chlamydomonas cells by co-transformation with pSP115, selecting for growth on medium containing zeocyn. Transformants were screened for *PsaF* accumulation by immunoblot analysis. Eight out of 80 transformants accumulated the PsaF polypeptide and two of these produced wild-type amounts of PsaF (Fig. 3). The ability of these transformants to grow photoautotrophically under high light intensity correlates with the level of PsaF accumulation, indicating that the expressed protein is functional. These results suggest that the pGenD expression vector is suitable for the expression of cDNAs that cannot otherwise be expressed without the inclusion of introns.

Chloroplast targeting of expressed proteins

The *PsaD* gene encodes a typical N-terminal chloroplast transit sequence of 35 amino acids, similar to that of

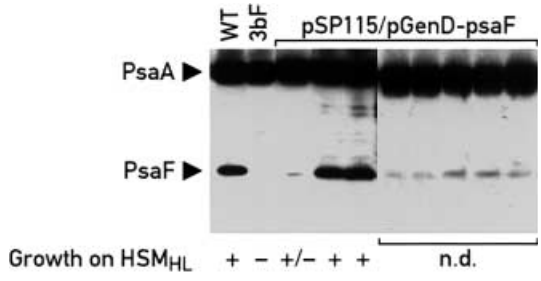

Fig. 3 Immunoblot analysis of total cell extracts from wild type, strain 3bF and nine transformants obtained by cotransformation with pSP115 and pGenD-psaF. Conditions for electrophoresis and immunoblotting were as in Fig. 2. The blot was incubated with antisera directed against the PsaA and PsaF subunits of the photosystem I complex. The ability of the transformants to grow photoautotrophically under high light is indicated at the bottom of each lane; n.d., not determined

other stromal proteins of *C. reinhardtii* (Franzen et al. 1990; Farah et al. 1995). We designed a new vector, pGenD2, which contains a unique *Nae*I restriction site just downstream of the transit sequence (see Materials and methods, Fig. 1). DNA fragments can be inserted into this site so as to generate fusion proteins that should be targeted to the chloroplast. We tested this possibility by introducing the *Ble* gene into pGenD2 and co-transformed a *cw15:arg7* strain with pARG7.8ϕ3 and pGenD2-Ble. Total cell extracts from transformants grown on medium lacking arginine were screened for *Ble* expression by immunoblotting analysis. Five out of 30 clones analysed accumulated Ble protein at high levels, but none was able to grow on medium containing 5 μg/ml zeocyn (data not shown). However, when *cw15* cells were transformed with pGenD2-Ble and directly selected for zeocyn resistance, colonies could be obtained, although the transformation efficiency was reduced about ten-fold compared to pGenD-Ble. Interestingly, in all the zeocyn-resistant transformants, the size of the Ble protein was variable and always larger than expected, suggesting that integration of the expression construct had generated Ble fusion proteins (Fig. 4, lane 5). In order to localize the Ble protein, we isolated chloroplasts from *cw15* cells transformed with pGenD-Ble or with pGenD2-Ble, selected either directly for zeocyn resistance or by cotransformation with pARG7.8ϕ3. Total cell proteins and chloroplast protein extracts of these strains were analysed by immunoblotting and probed with antisera directed against Ble, PsaD and the cytosolic marker eIF4A (Fig. 4). Clearly, in the strain that had been co-transformed with the pARG7.8ϕ3 and pGenD2-Ble vectors, the Ble protein is efficiently imported into the chloroplast (Fig. 4, lane 4). In contrast, the Ble protein expressed from the pGenD-Ble plasmid is found in total cell extracts (Fig. 4, lane 7), but not in the chloroplast (Fig. 4, lane 8). The same holds for the Ble fusion protein (Fig. 4, lanes 5 and 6). The Ble proteins in total and chloroplast extracts are identical in size, indicating that the transit sequence is cleaved after import across the chloroplast envelope. Interestingly, zeocyn sensitivity correlates with Ble targeting to the chloroplast, indicating that the Ble protein is unable to fulfill its protective function in the chloroplast of *C. reinhardtii* and that Ble is quantitatively imported into this compartment.

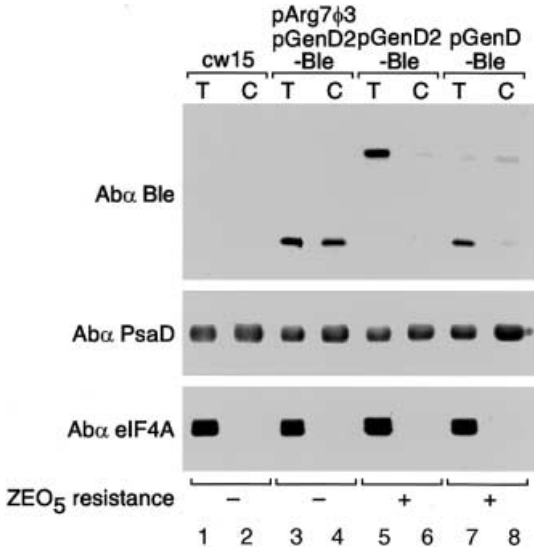

Fig. 4 Immunoblot analysis of extracts of total cells (T) or isolated chloroplasts (C) from *cw15* cells cotransformed with pARG7.8ϕ3 and pGenD2-Ble and selected for arginine prototrophy, and from cw15 cells transformed with pGenD2-Ble and pGenD-Ble and directly selected for zeocyn resistance. Conditions for electrophoresis and immunoblotting were as in Fig. 2. The blot was incubated with antisera raised against Ble, PsaD and eIF4A (a cytosolic marker). Resistance to 5 μg/ml zeocyn is indicated for each strain

Discussion

A characteristic feature of *C. reinhardtii* nuclear genes is the presence of numerous small introns (Silflow 1998). The presence of intronic sequences has been shown to increase gene expression (Lumbreras et al. 1998). Some intronless genes have been described (Fabry et al. 1995) and it is possible to rescue mutants with the corresponding wild-type cDNAs, suggesting that introns are not absolutely required for gene expression, at least for those genes which do not give rise to abundant transcripts (Auchincloss et al. 1999; Perron et al. 1999). Nevertheless, it has proven difficult to express cDNAs of highly expressed genes. In this respect, the structure of the highly expressed *PsaD* gene is unusual, as its coding sequence does not contain any introns. Introns have been described in untranslated regions of two genes and further work will be required to test whether this is also holds in the case of *PsaD* (Mitchell and Kang 1993; Sugase et al. 1996).

We have used a genomic fragment containing the *PsaD* gene to create a new nuclear expression vector for *C. reinhardtii*. This vector allows for the efficient expression of endogenous and exogenous selectable markers, as well as of the *PsaF* cDNA. The major advantage of the *PsaD* context is that all regulatory sequences appear to lie within its promoter and 5′ and 3′ untranslated regions. The *Psa*D-based vector is therefore particularly useful for gene expression, as it does not require the time-consuming insertion of introns to enhance expression.

We also took advantage of the *PsaD* chloroplast transit sequence to make the pGenD2 vector more versatile, allowing targeting of the expressed protein to the chloroplast. This feature is particularly advantageous for the study and engineering of processes that take place in the chloroplast. Interestingly, when the Ble protein is targeted to the chloroplast, cells are no longer resistant to zeocyn. In agreement with this, zeocyn resistance is only found in cells in which integration events have generated Ble fusion proteins that are not imported into the chloroplast. Recently, a Ble-GFP fusion has

been shown to be localized in the nucleus of *C. reinhardtii*, suggesting that Ble is a nuclear protein (Fuhrmann et al. 1999). Phleomycin and zeocyn are glycopeptides that damage DNA, and Ble prevents these deleterious effects by binding to the antibiotics with high affinity. Our results suggest that nuclear DNA is the target of these antibiotics, whereas the chloroplast genome appears to be less susceptible. This might be explained by a low permeability of the chloroplast envelope for these glycopeptides and/or by the higher copy number of chloroplast DNA molecules.

A number of recent technical advances have greatly facilitated the study of biological processes in *C. reinhardtii*. The availability of a new, efficient and versatile nuclear expression vector will certainly contribute to make this organism even more attractive for basic and applied research.

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