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Stable nuclear transformation of *Chlamydomonas reinhardtii* by using a *C. reinhardtii* gene as the selectable marker

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ABSTRACT **We have developed a stable nuclear transformation system for the unicellular green alga *Chlamydomonas reinhardtii*. Transformation was accomplished by introducing the cloned *C. reinhardtii* oxygen-evolving enhancer protein 1 (OEE1) gene into *C. reinhardtii* cells by bombardment with DNA-coated tungsten particles. The recipient strain was an OEE1-deficient, nonphotosynthetic, acetate-requiring mutant, which recovered photosynthetic competence after transformation, and was therefore able to grow in the absence of acetate. Analysis of several transformants indicates that transformation has proceeded via second-site integration of the cloned gene, leaving the endogenous mutant gene intact. In genetic crosses of transformants with wild type, both mutant and wild-type phenotypes were recovered, showing that the photosynthetic competence of transformants was due not to reversion of the original locus but rather to expression of the introduced gene. We suggest that the success of the present system is largely due to using a homologous *C. reinhardtii* gene, leading to stable maintenance and expression of the gene. Transformation with heterologous genes may be problematic because of poor expression due to an unusual codon bias in *C. reinhardtii*.**

Chlamydomonas reinhardtii is a unicellular green alga that is an ideal model organism for the study of a number of cellular processes, including cell–cell interactions during mating and the assembly and function of the flagellar and photosynthetic apparatus (1–4). Recently DNA-mediated transformation of the chloroplast has been demonstrated (5, 6), but, despite significant effort, an effective and reproducible method for transforming the *C. reinhardtii* nuclear genome has not yet been developed.

Two previous reports of transformation of the *C. reinhardtii* nuclear gene involved complementation of the *arg-7* mutant of *C. reinhardtii* by the yeast *ARG4* gene (7, 8). The reported rate of transformation was similar to the reversion rate of the marked strain, and although some arginine-independent colonies contained yeast DNA sequences, it was never demonstrated that this foreign DNA was responsible for the arginine-independent phenotype. A report of transformation of *C. reinhardtii* with a cloned kanamycin resistance gene from bacteria did not establish whether the resistance was due to the introduced gene or to spontaneous kanamycin resistance, which appears in *C. reinhardtii* at a high frequency (9). Other laboratories have been unable to achieve transformation by using either of these selection systems.

Although the previous reports of transformation have shown evidence for the integration of foreign DNA in some cases, none of these reports have demonstrated that the introduced DNA was stably expressed in the transformed cells. One reason that heterologous genes do not work well as selectable markers for *C. reinhardtii* transformation may lie in the highly biased codon usage of *C. reinhardtii* nuclear genes. Examination of codon usage in several nuclear genes (10–13) shows that adenine residues in the third codon position are generally excluded, except for stop codons (see Table 1). If tRNAs with rarely used codons were deficient in the cytoplasm of *C. reinhardtii*, the inefficient expression of bacterial and other heterologous genes would not be surprising. Alternatively, the high G+C content of *C. reinhardtii* nuclear DNA (approximately 65%) might result in promoter, transcription termination, RNA processing, or ribosome-binding sequences which are different from those of other organisms. Clearly many of these potential problems can be circumvented by the use of a *C. reinhardtii* gene for transformation.

Previous work identified a photosynthetic mutant of *C. reinhardtii* which provided a selectable phenotype (14). This mutant, Fud44, lacks a nuclear-encoded protein called oxygen-evolving enhancer protein 1 (OEE1) which is required for photosynthetic oxygen evolution. Absence of OEE1 renders the cell photosynthetically incompetent and therefore unable to grow on medium lacking an external carbon source [e.g., acetate (15)]. Fud44 contains a 5-kilobase (kb) insertion of a retrotransposon-like element in the second intron of the unique OEE1 gene (16), which results in the absence of detectable OEE1 mRNA or protein (14). The mutation has a spontaneous reversion rate of approximately 10^{-6} per generation. In these revertants the transposon excises imprecisely from the OEE1 locus, leaving behind a 577-base-pair (bp) fragment. A single revertant in which transposon excision left behind a 191-bp fragment has also been observed (16). We have never observed a revertant containing a wild-type OEE1 gene (>50 independent revertants examined). Thus, revertants of the Fud44 mutation should be readily distinguishable from transformants.

MATERIALS AND METHODS

Construction of an *Escherichia coli* Plasmid Containing the Coding Region of the *C. reinhardtii* OEE1 Gene. A λ phage genomic clone containing the entire coding region of the single OEE1 gene of *C. reinhardtii* was isolated and characterized (13). A genomic fragment from this clone that included 3 kb of DNA 5′ of the initiation codon and 2 kb 3′ of the stop codon of the OEE1 gene was cloned as an 8-kb *Eco*RI–*Kpn* I restriction fragment in pUC19 to form plasmid pSB101 (insert, diagrammed in Fig. 1). The plasmid was propagated in a *recA*$^-$ strain of *E. coli* and plasmid DNA was isolated by centrifugation on CsCl gradients by standard methods.

Introduction of Plasmid pSB101 into Fud44 Cells. Nuclear photosystem II mutant Fud44 cells were grown in complete medium (HSA) (15) under continuous light to early-logarithmic phase (4×10^5 cells per ml, experiment 1) or

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Abbreviation: OEE1, oxygen-evolving enhancer protein 1.

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Table 1. Codon usage in nuclear genes of *C. reinhardtii*

Amino acid	Codon	No.	%	Amino acid	Codon	No.	%
Ala	GCT	42	22	Leu	**TTA**	**0**	**0**
	GCC	137	73		TTG	0	0
	GCA	**0**	**0**		CTT	1	1
	GCG	10	5		CTC	2	2
Arg	CGT	7	8		**CTA**	**0**	**0**
	CGC	78	91		CTG	127	97
	CGA	**0**	**0**	Lys	**AAA**	**0**	**0**
CGG	1	1			AAG	96	100
	AGA	**0**	**0**	Met	ATG	64	100
	AGG	0	0	Phe	TTT	1	1
Asn	AAT	0	0		TTC	85	99
	AAC	77	100	Pro	CCT	1	2
Asp	GAT	14	15		CCC	53	90
	GAC	83	85		**CCA**	**0**	**0**
Cys	TGT	0	0		CCG	5	8
	TGC	32	100	Ser	TCT	12	10
Gln	**CAA**	**0**	**0**		TCC	55	44
	CAG	78	100		**TCA**	**1**	**1**
Glu	**GAA**	**0**	**0**		TCG	39	31
	GAG	111	100		AGT	0	0
Gly	GGT	22	16		AGC	19	15
	GGC	112	83	Thr	ACT	10	10
	GGA	**0**	**0**		ACC	85	88
	GGG	1	1		**ACA**	**0**	**0**
His	CAT	2	10		ACG	2	2
	CAC	19	90	Trp	TGG	22	100
Ile	ATT	14	21	Tyr	TAT	0	0
	ATC	54	79		TAC	58	100
	ATA	**0**	**0**	Val	GTT	5	4
					GTC	60	43
					GTA	**0**	**0**
					GTG	75	53
				Stop	**TAA**	**6**	**100**
					TAG	0	0
					TGA	0	0

Nuclear genes whose codon usage was considered include β1 and β2 tubulins (11), the small subunit of ribulose-bisphosphate carboxylase (12), and oxygen-evolving enhancer proteins 1 (13) and 2 (10). Note (boldface type) that A residues are generally excluded from the third position of all codons except the stop codon.

late-logarithmic phase (2×10^6 cells per ml, experiment 2). Cells were concentrated by centrifugation at 4000 × *g* for 5 min and resuspended in HS minimal medium (15). In experiment 1, approximately 4×10^7 cells were used for each bombardment, in experiment 2, approximately 10^8 cells were used.

In experiment 1, Fud44 cells were either spread on plates in soft agar (5) or uniformly spread as a 0.4-ml liquid suspension over the bottom of a 60-mm culture dish. The Petri dishes containing the Fud44 cells were bombarded with 1.2-μm tungsten spheres (21) (mean size 1.2 μm) coated with supercoiled or linearized (by digestion with *Eco*RI or *Kpn* I) plasmid DNA. In experiment 2, cells were either spread directly on top of selective agar plates and bombarded as before or bombarded in liquid HSA medium, diluted in liquid HSA for 18 hr of growth, and then plated on selective agar plates. All of the selective plates were placed in a 25°C incubator under cool white fluorescent light (4 W/m^2) under a 12-hr light/dark cycle. Phototrophic colonies, which appeared 2–3 weeks after bombardment, were replated twice on solid selective medium and then grown in complete liquid medium prior to isolation of DNA, RNA, or protein.

DNA, RNA, and Protein Isolation, Electrophoresis, and Blotting. Cells were grown in complete liquid medium to late-logarithmic phase and pelleted by centrifugation at 8000 × *g* for 10 min.

DNA isolation, restriction digestion, gel electrophoresis, and blotting to nitrocellulose were as previously described (17).

RNA was isolated with guanidinium hydrochloride as described (18). Total RNA, 5 μg per lane, was separated on denaturing agarose gels and electroblotted to nylon membranes as described (19). Both DNA and RNA blots were hybridized with ^{32}P-labeled DNA probes (as indicated in the figure legends) at 42°C in 50% (vol/vol) formamide/5× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.0/1 mM EDTA)/0.1% SDS/0.5% nonfat dry milk containing salmon sperm DNA at 25 μg/ml. The blots were washed three times for ½ hr each at 60°C in 1× SSPE/0.1% SDS. All blots were exposed to x-ray film with intensifying screens.

Protein isolation, gel electrophoresis, and Western hybridization were all performed as previously described (20).

RESULTS

Introduction of Cloned OEE1 Gene into Fud44 Cells and Identification of Transformed Strains. Introduction of the cloned OEE1 gene into mutant Fud44 cells was accomplished by bombardment with DNA-coated microprojectiles (21). After 2–3 weeks small green colonies appeared, and they were repicked onto minimal plates. All colonies able to grow photosynthetically were screened for the presence of pSB101 DNA. Table 2 summarizes the results of two different experiments.

Phototrophic colonies were classified as putative transformants if they contained DNA fragments hybridizing to pSB101 DNA which were in addition to the endogenous OEE1 gene (containing the transposon insertion). In the two experiments, 4 transformants were recovered among 17 phototrophic colonies. Each of the remaining 13 colonies was a revertant arising from transposon excision, and all showed Southern patterns identical to the spontaneous revertant Fud44-R2 (data not shown).

Analysis of OEE1 Genes from Genomic DNA of Transformed Strains. DNA was isolated from several independent transformants and analyzed by Southern analysis. DNAs were digested with *Pst* I, separated on agarose gels, blotted

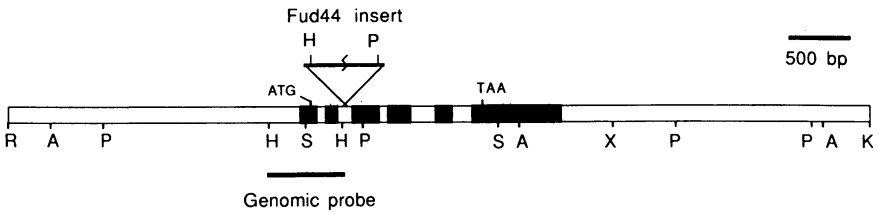

FIG. 1. Map of the cloned OEE1 gene used for transformation. The wild-type OEE1 gene was cloned from a λ-EMBL3 genomic library and subcloned into the *Eco*RI and *Kpn* I sites in the polylinker of pUC19 to form plasmid pSB101. Restriction sites for *Eco*RI (R), *Ava* I (A), *Pst* I (P), *Hin*dIII (H), *Sal* I (S), *Xho* I (X), and *Kpn* I (K) are shown. The Fud44 insert is not shown to scale; the actual size of the insert is approximately 5 kb. Coding regions of the single OEE1 gene, which contains five intervening sequences, are shown in black. The relative positions of the ATG start and TAA stop codons are also shown. The genomic probe used in the Southern analysis is indicated with a bold line.

Table 2. Recovery of photosynthetic transformants after bombardment with pSB101 DNA-coated particles and selection on minimal plates

Exp.	Protocol					Phototrophic colonies	Transformant colonies
	Cells	DNA digestion	Bombardment	After bombardment	Plating		
1	Early log	*Eco*RI	In liquid HSA	18 hr in liquid HSA	HS	2	1 (TX-18)
		Kpn I	On HSA agar	18 hr in liquid HSA	HS	2	0
2	Late log	None	In liquid HSA	18 hr in liquid HSA	HS	2	1 (3B)
		*Eco*RI	In liquid HSA	18 hr in liquid HSA	HS	3	1 (12F)
		Kpn I	In liquid HSA	18 hr in liquid HSA	HS	3	0
		*Eco*RI + *Kpn* I	In liquid HSA	18 hr in liquid HSA	HS	1	0
		None	On HS plates	—	—	0	0
		*Eco*RI	On HS plates	—	—	2	0
		Kpn I	On HS plates	—	—	1	1 (56Q)
		*Eco*RI + *Kpn* I	On HS plates	—	—	1	0

In experiment 1 (early-logarithmic-phase cells) approximately 4×10^7 cells were used in each bombardment, while in experiment 2 (late-logarithmic-phase cells) approximately 2×10^8 cells were used for each bombardment. pSB101 plasmid was digested to completion with the enzyme listed or undigested supercoiled DNA was used. Treatment of cells during and after bombardment is stated under protocol. The number of transformant colonies in the phototrophic colonies was determined by Southern analysis as shown in Fig. 2. Every phototrophic colony analyzed which was not a transformant contained OEE1 DNA fragments characteristic of revertant strains.

to nylon membranes, and probed with a cloned genomic fragment which is specific for the 5′ end of the OEE1 gene. This probe hybridizes to the *Pst* I fragment that contains the insertion site of the transposon in the Fud44 mutant and thus readily distinguishes wild-type, mutant, and revertant OEE1 genes. The results of this experiment are shown in Fig. 2*A*. The probe hybridizes to a 2.5-kb fragment in wild-type DNA, while in Fud44, which contains the 5-kb insertion, it hybridizes to a 7.5-kb fragment (Fud44; Fig. 2*A*). Spontaneous revertants of Fud44 contain a 577-bp remnant of transposon excision (16), so that the *Pst* I fragment labeled by this probe migrates as if it is 577 bp longer than wild type (Fud44-R2; Fig. 2*A*). All three transformants show hybridization to two or three fragments; in each case one fragment comigrates with the Fud44 fragment carrying the 5-kb insertion. Since the original mutation appears to be unchanged, there is no evidence for reversion at this locus or replacement of the Fud44 mutation by homologous recombination with the wild-type OEE1 gene. The additional fragments presumably represent newly introduced copies of the OEE1 gene from plasmid pSB101. In the case of transformant TX-18, the extra hybridizing fragment comigrates with the wild-type *Pst* I fragment, suggesting that the entire *Pst* I fragment has remained intact. However, in 56Q and 12F the hybridizing fragments are larger than wild type, suggesting that the transforming DNA has integrated into chromosomal DNA via a recombination event between the *Hin*dIII site marking the 5′ end of the probe and the *Pst* I site upstream. In 12F there are two fragments in addition to the Fud44 band, suggesting that two insertion events may have occurred.

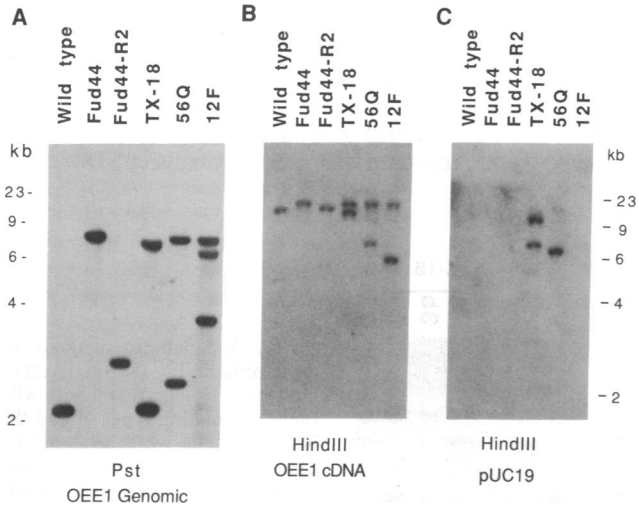

FIG. 2. Characterization of pSB101 sequences in Fud44 transformants. (*A*) DNA was isolated from wild type, Fud44, a spontaneous revertant of Fud44 (Fud44-R2), and three transformants isolated from plates bombarded with *Eco*RI-cut plasmid (TX-18, 12F), and *Kpn* I-cut plasmid (56Q). The DNA was digested with *Pst* I, separated on agarose gels, and blotted to nitrocellulose. The filter was probed with a 700-bp *Hin*dIII genomic fragment specific for the 5′ end of the OEE1 gene. (*B*) DNAs were digested with *Hin*dIII and probed with a OEE1 cDNA. (*C*) Probe was removed from the filter in *B* and the filter was reprobed with pUC19.

Southern analysis of the same DNAs digested with *Hin*dIII and probed with an OEE1 cDNA (Fig. 2*B*) shows that each of the transformants contains a fragment approximately 20 kb in length, which comigrates with the OEE1 gene of Fud44. In addition, each of the transformants has one additional fragment which hybridizes with the OEE1 cDNA. This filter was stripped of the OEE1 cDNA probe and rehybridized with nick-translated pUC19. As shown in Fig. 2*C*, pUC19-homologous sequences are found in the TX-18 and 56Q transformants, but not in the wild-type, Fud44, Fud44-R2, or 12F lanes. Sequences homologous to pUC19 were identified in the 12F transformant from DNA isolated immediately after this transformant was identified. The DNA used for the Southern blot presented in Fig. 2 *B* and *C* was isolated from cells which had been maintained for several months on nonselective media, suggesting that the pUC19 sequences were lost during this growth period. The presence of two fragments hybridizing to pUC19 sequences in the TX-18 lane suggests that there have been two integration events, that the integration site lies within the pUC19 vector sequences, or that there has been recombination or duplication of the pUC19 sequences after integration. With the limited number of transformants analyzed it is difficult to distinguish among these possibilities. However, these Southern analyses yield results which are consistent with second-site integration of an intact OEE1 gene with concomitant integration of pUC19 vector sequences.

Northern Analysis of OEE1 mRNA from Transformed Strains. To determine if the introduced OEE1 genes were expressed in the transformed strains, RNA was isolated from wild type, Fud44, and each of the transformants which had been characterized by Southern analysis. Samples (5 μg) of total RNA were separated on denaturing agarose gels, blotted to nylon filters, and hybridized with a ^{32}P-labeled OEE1 cDNA probe. All of the transformants accumulated OEE1 mRNA to levels similar to those of wild type (Fig. 3*A*), while the Fud44 cells were completely deficient in OEE1 mRNA, as previously demonstrated (14). When the same RNA blot

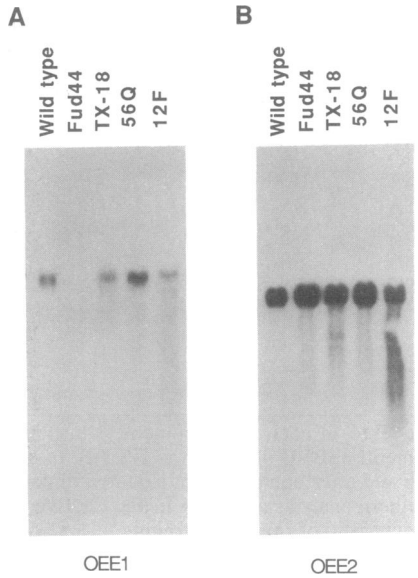

FIG. 3. OEE1 RNA of Fud44 transformants. RNA was isolated from wild type, Fud44, and three transformants grown in complete medium. Total RNA (5 μg per lane) was separated on a denaturing formaldehyde gel and electroblotted to nylon membrane. (*A*) The blot was hybridized with an OEE1-specific cDNA. (*B*) An identical filter was hybridized with a probe specific for another nuclear-encoded photosynthetic protein, the oxygen-evolving enhancer protein 2 (OEE2) polypeptide.

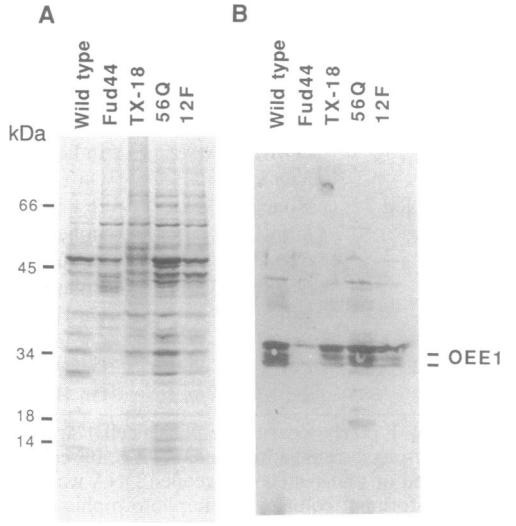

FIG. 4. OEE1 in Fud44 transformants. Protein was isolated from wild type, Fud44, and transformants. Proteins were separated on SDS/polyacrylamide gels and either stained with Coomassie blue R (*A*) or blotted to nitrocellulose and hybridized with antiserum to OEE1 (*B*). The OEE1–antibody complex was then allowed to react with goat anti-rabbit antiserum conjugated with alkaline phosphatase and visualized by alkaline phosphatase activity staining. Contaminating antibodies recognize a protein in each of the sample lanes which is unrelated to OEE1.

was probed with a cDNA for another nuclear-encoded mRNA (encoding the OEE2 protein), this mRNA was shown to accumulate to similar levels in all strains (Fig. 3*B*).

Analysis of OEE1 Proteins from Transformed Strains. To further demonstrate that the introduced OEE1 genes were being expressed, transformants were assayed for the accumulation of OEE1. Proteins were isolated from wild type, Fud44, and each of the transformants, separated on SDS/polyacrylamide gels, and either stained with Coomassie blue (Fig. 4*A*) or blotted to nitrocellulose for Western analysis. The protein blot was hybridized with crude OEE1 antisera, then with anti-rabbit antiserum conjugated with alkaline phosphatase, and developed with standard alkaline phosphatase staining reagents (Vector Laboratories). Approximately equal amounts of OEE1 accumulate in wild-type and transformant cells, while the mutant Fud44 completely lacks the polypeptide (Fig. 4*B*). Thus, the expression of the OEE1 gene appears to be similar in wild-type and transformed *C. reinhardtii* cells, in terms of both mRNA and protein accumulation.

Analysis of OEE1 Genes after Sexual Crosses of Transformants with Wild-Type Strains. Southern analysis showed that each of the transformants contained OEE1 sequences which were not present in the original Fud44 recipient. Furthermore, the mutant Fud44 gene, with its 5-kb insertion, is still present. However, there is a remote possibility that a reversion event that was undetectable at the DNA level was responsible for the photosynthetic competence of these cells and that the newly integrated OEE1 DNA is not functional. To test this, we crossed two of the transformants (TX-18 and 56Q) to wild-type cells to see whether we could recover the original Fud44 nonphotosynthetic phenotype. Sixteen tetrads were dissected from the cross of TX-18 × wild type and, although none of them was complete, both photosynthetic and nonphotosynthetic progeny were recovered. Four photosynthetic and three nonphotosynthetic colonies were picked at random and DNA was isolated. DNA was digested with *Hin*dIII and *Kpn* I and subjected to Southern analysis using an OEE1 cDNA as the probe. Of the three nonphotosynthetic colonies tested one carried only the original Fud44 gene (compare Fig. 5, lanes Fud44 and 1a) and did not carry either wild-type or TX-18 OEE1 fragments. Recovery of nonphotosynthetic progeny containing only the Fud44 OEE1 gene proves that the original OEE1 mutation has not reverted and shows that the photosynthetic phenotype in the transformants must be due to the expression of the introduced wild-type OEE1 gene. Interestingly, the other two nonphotosynthetic progeny recovered carry apparently altered wild-type fragments in addition to TX-18 fragments (Fig. 5, lane 3b). These altered fragments may be due to illegitimate recombination between the TX-18 and wild-type loci. Of the four photosynthetic progeny examined one contained only the wild-type fragment, two contained both wild-type and TX-18 fragments (lane 6a), and one contained Fud44 and TX-18 fragments (lane 7a). As expected no progeny were recovered which carried both wild-type and Fud44 fragments.

From the cross of 56Q × wild type only photosynthetic progeny were recovered (20 tetrads analyzed). Southern

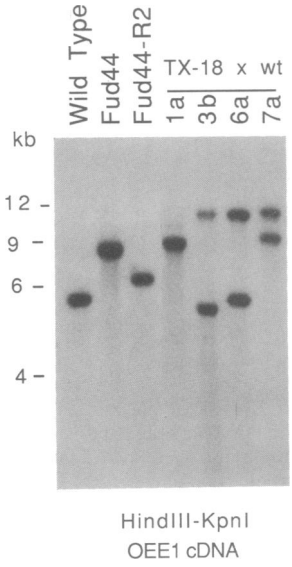

FIG. 5. Genetic analysis of transformant TX-18. TX-18 (mating type −) was crossed with wild type (wt; mating type +) and the daughter cells were analyzed by phototrophic growth. Both phototrophic and nonphototrophic colonies were recovered. DNA was isolated from wild type, Fud44, transformant TX-18, two nonphotosynthetic progeny (1a, 3b), and two photosynthetic progeny (6a, 7a). DNAs were digested with *Hin*dIII and *Kpn* I, separated on agarose gels, blotted to nylon membrane, and hybridized with an OEE1 cDNA probe as described in the legend of Fig. 2.

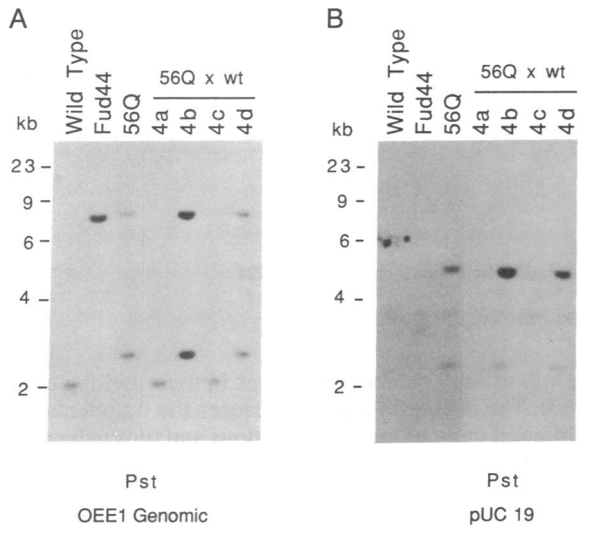

FIG. 6. Genetic analysis of transformant 56Q. 56Q was crossed with wild type and the daughter cells were analyzed for photosynthetic growth. All progeny were able to grow on minimal medium (i.e., were photosynthetic). DNA was isolated from wild type, Fud44, 56Q, and four complete tetrads (DNA from a single tetrad is shown). DNAs were digested with *Pst* I, separated on agarose gels, and blotted to nylon membrane. (*A*) The filter was probed with a 700-bp *Hin*dIII fragment (see Fig. 1). (*B*) The OEE1 probe was removed and the filter was reprobed with pUC19.

analysis of DNAs isolated from four of these tetrads shows that the 56Q OEE1 gene segregates with the Fud44 OEE1 gene in each of the crosses (Fig. 6*A* shows one Southern blot) and suggests that 56Q is linked to the Fud44 OEE1 gene. Hybridization of the same Southern blot with nick-translated pUC19 shows that the pUC19 sequences segregate with the 56Q OEE1 gene and therefore may be linked as well (Fig. 6*B*).

DISCUSSION

We have demonstrated stable nuclear transformation of *C. reinhardtii* by using the *C. reinhardtii* OEE1 gene as the selectable marker. The transformation rates reported here are low, about 1/5th the reversion rate. Despite this low transformation rate, transformants can readily be distinguished from revertants, since revertants arise from imprecise transposon excision and always contain a remnant of the transposon (16).

The transforming DNA appears to integrate in single or low copy number. The introduced DNA and transformed phenotype are stably maintained for at least several hundred generations under nonselective growth conditions, although in some cases (12F) the pUC19 sequences which cotransformed with the OEE1 gene may not be stably maintained. In addition, the introduced DNA is stably transmitted in sexual crosses. In the two crosses reported here, the phototrophic phenotype and introduced DNA segregate together as a single Mendelian trait. In one case (56Q), the introduced DNA is linked to the endogenous OEE1 gene, although it has not replaced the mutant allele.

We speculate that the use of a homologous gene as the selectable marker is crucial to the success of the present method. The *C. reinhardtii* gene for nitrate reductase (*nit-1*) can also be used for nuclear transformation of *nit-1* mutants of *C. reinhardtii*, using the particle gun to introduce the DNA into the nucleus (22). The requirement for homologous DNA for transformation may be due to unusual codon usage in *Chlamydomonas*.

Transformants have arisen from both linear and supercoiled DNA. A period of growth under nonselective conditions is not required to recover phototropic transformants. This differs from chloroplast transformation in *Chlamydomonas*, where a period of nonselective growth greatly enhances the recovery of transformants (5, 6).

The introduced DNA is expressed at near wild-type levels for both mRNA and protein under heterotrophic growth conditions. Under these growth conditions the transformants grow at wild-type rates. However, under phototrophic growth conditions transformants grow slower than wild type or Fud44 revertant strains. It will be interesting to determine if the expression of the introduced OEE1 gene is altered under phototrophic growth conditions. Relatively high expression of functional OEE1 is probably required for phototrophic growth. If the efficiency of expression of the introduced OEE1 gene is dependent upon its site of integration, only a fraction of integration events may result in expression at a level sufficient to allow phototrophic growth.

A more detailed examination of these and other transformants will be necessary to understand the processes which take place during transformation in *C. reinhardtii* and to develop methods which will give the higher rates of transformation necessary for a full exploitation of *C. reinhardtii* nuclear transformation.

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