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One step modification of *Chlamydomonas reinhardtii* BACs using the RED/ET system

Chlamydomonas BAC vectörlerinin RED/ET yöntemiyle tek basamakta modifikasyonu

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ABSTRACT

With the availability of the complete genome, *Chlamydomonas* BACs are being used extensively in functional genomics analysis. The following aspects of their construction, however, make them less than optimal for some types of analysis. (1) These BACs do not contain a gene to allow direct selection of transformants in complementation analysis. (2) Co-transformation using the BAC and an independent vector with a selectable marker has a low efficiency. (3) Most BACs have more than one gene, necessitating sub-cloning of each gene into a different vector (relying on the use of restriction enzymes). To simplify this process, we modified *Chlamydomonas* BACs by inserting 2 selectable marker cassettes, using the RED-ET system. We ligated a eukaryotic and a prokaryotic selectable marker cassette and used it in a one-step modification instead of a two-step counter selection protocol and showed the expression of both cassettes. This method will decrease the time needed for use of BACs in functional genomics analysis in *Chlamydomonas*.

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ÖZ

Tüm genom sekansının tamamlanması üzerine, *Chlamydomonas* BAC'ları fonksiyonel genomik analizlerinde yaygın bir şekilde kullanılmaktadır. Fakat bu vektörlerin hazırlanmalarında kullanılan aşağıdaki sebeplerden dolayı bu tip analizlerde kullanımları optimalden daha düşük olmaktadır: (1) Bu BAC'larda komplementasyon analizlerinde elde edilen transformantların direk seçimini sağlayan bir gen yoktur. (2) BAC ve bağımsız bir vektörün ko-transformasyon (birlikte transformasyon) olarak kullanımında birlikte transformasyon oranı düşüktür. (3) BAC'ların çoğu birden fazla gen içermektedir, bu da her genin farklı bir vektöre sub-klon (alt klonlama) yapılmasını gerektirmektedir (bu da restriksiyon enzimlerine bağımlılık demektir). Bu çalışmada, BAC transformasyonu işlemini kolaylaştırmak için, RED/ET tekniğini kullanarak *Chlamydomonas* BAC'larına 2 selectable marker cassette (seçilebilir ekspresyon kaseti) eklenmiştir. Bir ökaryotik ve bir prokaryotic ekpresyon kaseti ligasyon edilmiş ve BAC'ların iki basamak modifikasyonu yerine, tek basamakta modifiye edilmesi sağlanmıştır ve bu kasetlerin ekspresyonu kanıtlanmıştır. Böylelikle BAC'ların *Chlamydomonas*'da fonksiyonel genomik analizlerinde kullanımları için harcanacak zaman süresi azalacaktır.

1. Introduction

Chlamydomonas reinhardtii is a eukaryotic green alga that has been used as a model system for the analysis of many biological functions including photosynthesis, motility, sexual development, mating etc. Harris (1989). Besides being an excellent model system it is also used in commercial applications, such as algal hydrogen production Ghirardi et al. (2000). The *Chlamydomonas* genome has been sequenced and efforts continue on functional annotation of the genes (Merchant et al. 2007; Blaby et al. 2014). With the completion of genome sequencing, large numbers of genes with unknown function have become available. The analysis of gene function will require use of BACs for complementation analysis.

Insertional mutagenesis has been used extensively in *Chlamydomonas* (Kindle 1990; Li et al. 2016) and results in insertion of the mutagen (plasmid DNA) randomly in the genome and in some cases causing large deletions (Aksoy et al. 2014). If the insertion occurs in the coding or regulatory regions of a gene, it disrupts the function of the gene, leading to an observable phenotypic change. Different techniques have been used for determination of the site of insertion including TAIL-PCR Dent et al. (2005) and RESDA-PCR Gonzalez-Ballester et al. (2005).

After finding the insertion site in the genome and showing the insertion is genetically linked to the mutant phenotype, the wild type genomic sequence is reintroduced to complement the mutation. This step still remains to be improved. Many researchers use BACs as their resource for the wild type gene sequence and some researchers amplify the sequence to be introduced by PCR. If the sequence to be amplified is large, PCR may not be the best choice, because correct amplification of a large sequence even with most high fidelity polymerases is difficult. Therefore, BAC complementation is more practical and less costly. Application of CRISPR-Cas9 (Jiang et al. 2014; Baek et al. 2016; Ferenczi et al. 2017) will allow researchers to make mutations in any gene they want to study and this will result in increased use of BACs in these functional genomics studies.

Chlamydomonas BACs have no selectable marker except for a chloramphenicol cassette which can be expressed only in *E. coli*. Because of this difficulty, different methods have been used for BAC complementation: (1) Transformation of cells with the BAC and observation of complementation of the mutant phenotype by appropriate selection techniques (can only be used if the complemented cells have a selectable phenotype of their own). (2) Co-transformation of the BAC along with an independent plasmid which has a selectable marker that can be expressed in *Chlamydomonas* (co-transformation of BAC and the additional plasmid is expected to be low), (3) Sub-cloning of the region of interest from the BAC into a different vector which has selectable marker for *Chlamydomonas*. This method relies on restriction enzymes or PCR, which is not always practical especially for larger genes.

To overcome the disadvantage of the above complementation methods, we applied RED/ET recombination (recombineering) (Zhang et al. 1998; Muyrers et al. 2001) to modify *Chlamydomonas* BACs by inserting a selectable marker gene that can be expressed in *Chlamydomonas*. This way cells can be transformed with only the modified BAC and transformants can be selected on antibiotic without the need to do co-transformations. To do this, we created an insert which has two selectable markers; a kanamycin cassette for expression in *E. coli* and a hygromycin cassette Berthold et al. (2002) for expression in *Chlamydomonas*. There are no reports of a dual expression construct for *Chlamydomonas*. By creating this fusion construct, we have reduced the two-step counter selection protocol (BAC modification with the counter selection cassette followed by replacement of counter selection cassette with a sequence of interest) into one step. The insert we created is not large, therefore the recombination frequency is not affected drastically and there is no need to modify the RED/ET protocol.

The other advantage of this technique is that most of the *Chlamydomonas* BACs contain several genes, making it more difficult to determine which gene is responsible for the complementation. Our insert can be used to replace any of the genes on a BAC and then the modified BAC can be used for complementation.

2. Materials and Methods

2.1. E. coli and Chlamydomonas Strains

E. coli strain DH10B was used as host for the entire recombination protocol and JM109 was used for routine bacterial techniques. *sr-u-2-23* mating type minus (CC 275) was the *Chlamydomonas* strain used.

2.2. BAC Clones and BAC Isolation

The *Chlamydomonas* BACs 33A3 and 37P6 were obtained from the Clemson University Genomics Institute (www.genome.clemson.edu). For BAC isolation, Qiagen Plasmid Mini or Midi kits (Qiagen, Valencia, CA, USA) were used.

2.3. Media and Antibiotics

E. coli strain DH10B hosting the BACs was maintained in LB medium containing 15 μg ml-1 chloramphenicol. The following antibiotic concentrations were used in recombination as suggested by the RED/ET protocol: Tetracyclin (3 μg ml^{-1}), kanamycin (15 μg ml^{-1}) and chloramphenicol (15 μg ml^{-1}).

Chlamydomonas cells were grown in liquid or solid TAP medium Harris (1989). TAP plates supplemented with 10 μg ml^{-1} hygromycin were used for selection after transformation with modified BACs. All antibiotics were from Sigma.

2.4. Agarose Gel Electrophoresis

All the gels were 0.9%. All purpose Hi-Lo DNA Marker (Bionexus, Oakland, CA, USA) was used in all the gels.

2.5. Construction of the Insert

2.5.1. Fusion of a Prokaryotic and a Eukaryotic Expression Cassette

First the *hyg3* (*hygromycin3*) cassette Berthold et al. (2002) was moved to pBluescript SK- from pHyg3 to create a NotI site needed to create our fusion cassette as explained below. The *hyg3* cassette was cut out of the original vector (pUCBM20) by digesting pHyg3 with EcoRV (Promega, Madison, WI, USA) and KpnI (Promega, Madison, WI, USA). The cassette (1733 bp) was gel purified using the QIAquick Gel Extraction Kit (Qiagen) and ligated into pBluescript SK-. To do this ligation, pSP124S, a plasmid available in many *Chlamydomonas* research labs Lumbreras et al. (1998), was also digested with EcoRV and KpnI to produce pBluescript SK- that had the same restriction ends as the *hyg3* cassette. This digestion released the *ble* cassette from pSP124S and removed 44 bp from the MCS of pBluescript. The digestion products were run on a gel and pBluescript was gel purified. The *hyg3* cassette was ligated into the purified vector using T4 DNA ligase (Promega, Madison, WI, USA). The insert to vector ratio was 3:1 in the ligation reaction. JM109 competent cells (Promega, Madison, WI, USA) were transformed with 2 μl of the ligation reaction by heat shock method. Briefly, JM109 cells and plasmid to be transformed were incubated on ice for 30 sec, then incubated at 42 °C for 45 sec in a water bath. After this step cells were incubated on ice for 2 min and then 900 μl of SOC media was added. Thereafter cells were shaken for 60 min at 37 °C and

transformants were selected on 100 μg ml^{-1} ampicillin containing LB plates. This new plasmid was named pHyg3MA (4647 bp) to differentiate it from the original pHyg3. The plasmid was isolated with the Qiagen Plasmid Mini Kit. To make sure the *hyg3* cassette was still functioning, pHyg3MA (in both circular and linear form) was used to transform *sr-u-2-23 mt-* (CC 275) cells and many hygromycin resistant colonies were obtained.

Inserting only the *hyg3* cassette into the BACs wouldn't allow us to select modified BACs in *E. coli*, because the cassette has a *Chlamydomonas* promoter and would therefore be expressed only in *Chlamydomonas.* In order to select for the modified BACs, we needed a dual expression cassette (a cassette that can be expressed both in *Chlamydomonas* and in *E. coli*). We have not seen a report of such a cassette. We therefore ligated a prokaryotic expression cassette next to the *hyg3* cassette in pHyg3MA. The Tn5-*neomycin* cassette, which was included in the BAC modification kit, was used for this purpose. This cassette was amplified by PCR using Epicentre Taq polymerase (Epicentre Biotechnologies, Madison, WI, USA) using primers Tn5-neo-f (5'-TGGACAGCAAGCGAACCGGAATTGC-3') and Tn5-neo-r (5'- TCAGAAGAACTCGTCAAGAAGGCG-3'). The product was gel purified and ligated into a pGEMT Easy vector (Promega, Madison, WI, USA). 2 μl of the ligation product was used to transform JM109 cells and transformants were selected on LB plates containing 50 μg ml^{-1} ampicillin and 50 μg/ml kanamycin. The plasmid was isolated using the Qiagen Plasmid Mini Kit and the Tn5-neomycin cassette was excised with NotI (Promega, Madison, WI, USA). This excision from the pGEMT Easy vector introduced 40 bp to the 948 bp cassette, making it 988 bp. The cassette was gel purified and ligated into NotI digested, gel purified pHyg3MA. This ligation was again used to transform *E. coli* and the transformed *E. coli* were again selected on LB plates containing 50 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin. This plasmid was named pHyg3MA-Tn5neo (5635 bp). pHyg3MA and pHyg3MA-Tn5neo was digested with SspI (Fermantas, Glen Burnie, MD, USA) to confirm the ligation of Tn5-*neo* cassette.

2.5.2. Generation of the Insert with Homology Arms by PCR

pHyg3MA-Tn5neo was used as a PCR template for creating the selectable marker insert (*hygromycin* and Tn5-*neomycin* fusion cassettes) to be used for modification of the BACs. To create the insert with homology arms, 50 bp homology arms were added to the 5'end of the primers. The sequences of the oligonucleotides with the homology arms are: NARHA-T7: 5'-GAATTCCCCGGATCATGCCGCCAGTAGCACGAGTGAAGAGGTTCATGAGG**GTAATACGACTCACTATAGGGC**-3' and NARHA-BGal: 5'-CGCAGCGATTTACCCGTGTTGCGGAAATAGAGCGTGTCGGCAGTGGCGTA**ATGACCATGATTACGCCAAGC**-3'. The underlined regions are the regions homologous to the *nar2/nit8* gene that is found in all *Chlamydomonas* BACs and the bold regions are the priming regions for the pHyg3MA-Tn5neo. All the oligonucleotides were from Operon Biotechnologies.

PCR with homology arm primers produced low amounts of product. We therefore first amplified the fused cassettes with short primers (T7 and BGalf primers, bold regions). Cycling conditions for this reaction were 98 °C 2 minutes (hot start) then 30 cycles of 98 °C for 10 seconds, 58 °C for 30 seconds and 72 °C for 1 minute followed by a 5 minutes final extension at 72 °C. For the second reaction, this 3 kb (2969 bp) product was diluted 1:10 and used as a template with the homology arm primers (NARHA-T7 and NARHA-BGal) to make the insert to be used for recombination. Cycling conditions were 98 °C for 2 minutes (hot start) then 30 cycles of 98 °C for 10 seconds, 58 °C for 30 seconds and 72 °C for 1½ minutes followed by 5 minutes final extension at 72 °C.

Phusion (Phu) Hot Start DNA Polymerase (New England Biolabs, Ipswich, MA, USA), which shows very high accuracy, was used in both reactions. In both reactions HF buffer was used with Phusion Hot Start DNA Polymerase. DMSO was added to the second PCR with homology arm primers to get more product.

The PCR product was digested with DpnI (Promega, Madison, WI, USA), which digests the circular plasmid template, but not the PCR product. After digestion, the DpnI was heat inactivated and the PCR product was gel purified. This gel purified sample was used for recombination.

2.6. pRED/ET System and Recombination

RED/ET system (Quick & Easy BAC Modification Kit) was purchased from Gene Bridges (Gene Bridges, Heildelberg, Germany). The protocol for growth of bacteria and electroporation was followed as suggested in the manual, except that the electrocompetent bacteria were prepared by shaking at 200 rpm rather than the suggested 1000 rpm. This speed was used at each step of recombination. Electroporation was done using the BioRad (Hercules, CA, USA) Gene Pulser electroporator. Settings were 1.8 kV, 200 Ohms and 25 μF.

2.7. Verification of Correct Recombination

The BACs were analyzed by PCR to verify the recombination.

The primers for amplification of the *hyg3* cassette were: Hyg3-f2 (5'- AGAGCACCAACCCCGTACT-3') and Hyg3-r2 (5'- GAAGGCGTTGAGATGCAGTT-3').

PCR for the Tn5-*neo* cassette was performed with primers: Tn5-neo-f (5'- TGGACAGCAAGCGAACCGGAATTGC-3') and Tn5-neo-r (5'- TCAGAAGAACTCGTCAAGAAGGCG-3').

PCR to show correct replacement was done with primers: NarCh-f-2091 (5'-GAACGAAACAGTCGGGAAAA-3') and pBACmn6020 (5'-GGCCCAAGTATTCGCTATCA-3').

Restriction analysis was done with KpnI and BglII (Fermentas).

2.8. Transformation of Chlamydomonas with BACs

5-10 μg of circular modified or unmodified BACs were used to transform the cells. Transformants were selected on TAP hygromycin (10 μg ml^{-1}) plates. Unmodified BACs were used as a negative control to check the effectiveness of hygromycin killing. *Chlamydomonas* was also transformed with 1 μg of the circular pHyg3MA as a positive control to compare the expression of the *hygromycin* cassette in the modified BACs.

3. Results

3.1. Summary of the Recombination Process

The diagram shown in Figure 1 summarizes the steps of the protocol we used. The plasmid pHyg3 contains the *aphVII* cassette for hygromycin B resistance Berthold et al. (2002). First, pHyg3MA-Tn5neo was created by ligating the *hyg3* and Tn5-*neo* cassettes into pBluescript SK-. pHyg3MA-Tn5neo was then used as a template to make the insert; both cassettes were amplified with primers T7 and BGalf (short primers). This PCR product was then amplified with homology arm primers to create the insert to be used in recombination. We decided to insert this product into the *nar2/nit8* gene that is present in all *Chlamydomonas* BACs. The *nar2/nit8* gene which encodes a subunit of the nitrate transporter had been inserted into the BACs during creation of the BAC library, to be used as a selectable marker for *nit8* deficient strains (which cannot transport nitrate therefore cannot grow on nitrate as a sole nitrogen source). However, the gene was later found to be non-functional (P.A. Lefebvre, personal communication). Therefore, we decided to replace a region of this gene with our insert.

3.2. Creation of the Insert with Homology Arms

3.2.1. Ligation of hyg3 and Tn5-neo Cassettes

First the *hyg3* cassette was ligated into pBluescript SK- producing pHyg3MA. Then the Tn5-*neo* cassette was ligated into Not I digested pHyg3MA to create pHyg3MA-Tn5neo. To make sure the ligation occurred correctly, pHyg3MA and pHyg3MA-Tn5neo were digested with SspI which cuts once in the pBluescript vector and once in the multiple cloning site (removing a 553 bp from the vector). The difference in size in the upper band (~1 kb larger) is proof of ligation of the 988 bp Tn5-neo cassette into pHyg3MA (Fig. 2A).

3.2.2. PCR for Creation of the Insert with Homology Arms

First, the fused *hyg3* and Tn5-*neo* cassettes were amplified with primers T7 and BGal-f (short primers) using pHyg3MA-Tn5neo as a template. To create the insert with homology arms, homology arm primers were used to amplify the product of the short primers (Fig. 2B). The expected size of pHyg3MA-Tn5neo is 2969 bp (Fig. 2B. lane 1). For comparison, PCR was also done for pHyg3MA (Fig. 2B. lane 2); this product is 1.8 kb.

The PCR products of the short primers and homology arm primers of pHyg3MA-Tn5neo were run on a gel. As expected, the product of the homology arm primers was 100 bp larger then the short primer product (Figure 2C).

3.3. Recombination

Recombination was done using the RED/ET system as described above. Although we did not get large number of recombinants on each plate, we were able to get sufficient recombinant colonies in each experiment to use for our complementation analysis. The fact that we shook the *E. coli* at 200 rpm, rather than the suggested speed of 1000 rpm might

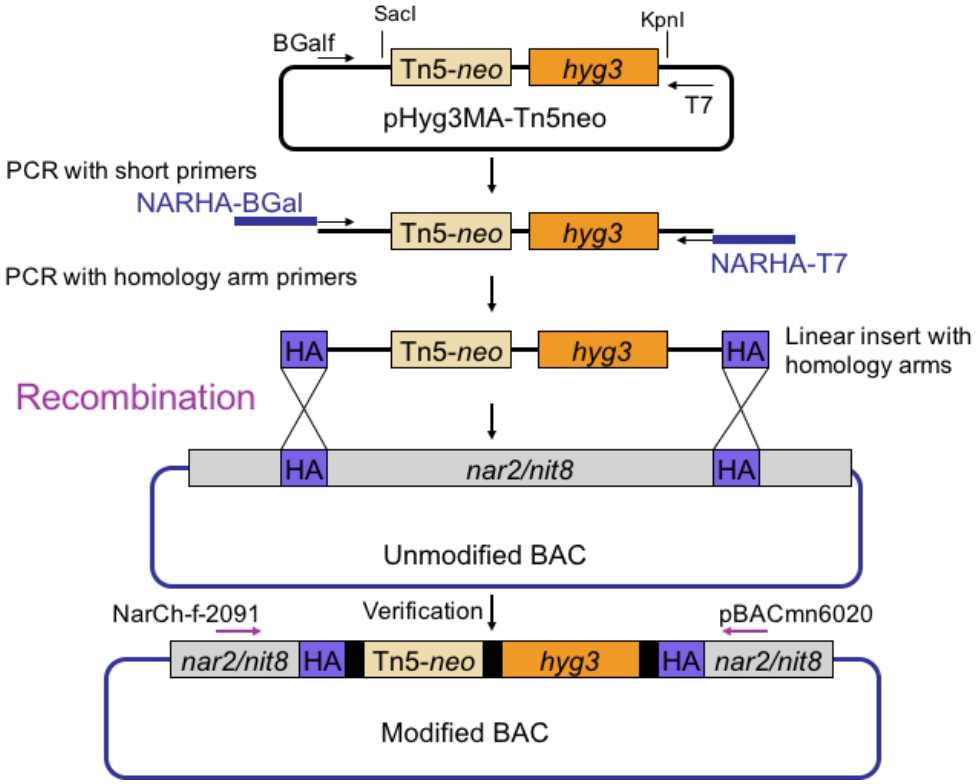

Figure 1. One step BAC modification. pHyg3MA-Tn5neo which contains *hyg3* and Tn5-*neo* cassettes was used as template for PCR to create the insert. In the first step, both cassettes were amplified with short (without homology arms) primers, T7 and BGalf. In the second step, the PCR product of T7 and BGalf was used to create the insert with homology arms using homology arm primers. This product was used in recombination to replace a region of the *nar2/nit8* gene in the BAC. Arrows on the modified BAC show the position of primers used for verification of recombination.

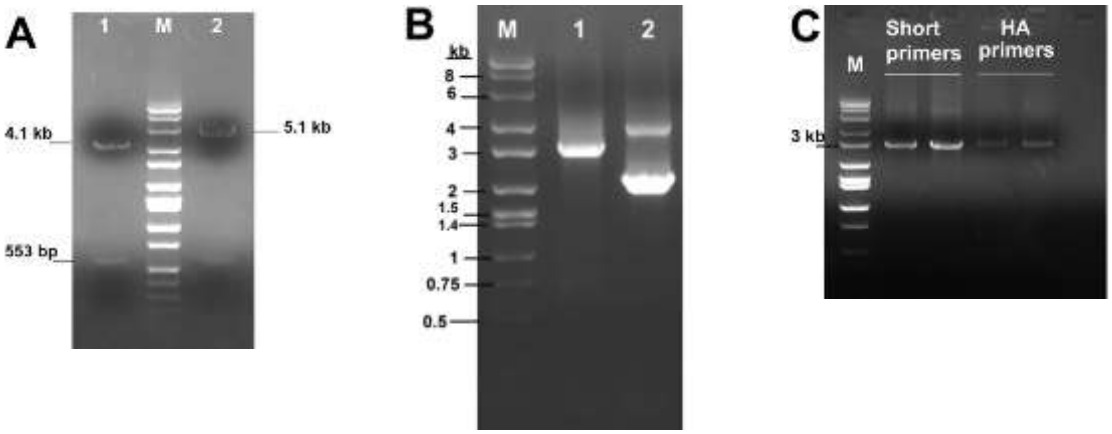

Figure 2. Creation of the Insert for Recombination. A. pHyg3MA (lane 1) and pHyg3MA-Tn5neo (lane 2) following SspI digestion. Hyg3MA-Tn5neo is 988 bp larger because of the Tn5-*neo* cassette. B. Creation of the insert with homology arms by PCR. Lane 1 is the pHyg3MA-Tn5neo product (2969 bp), lane 2 is the pHyg3MA product (1.8 kb). C. PCR products of short primers (BGalf and T7) and homology arm primers (NARHA-BGalf and NARHA-T7). HA products are 100 bp larger than the no HA (short primers) products. M: molecular weight marker.

explain why we did not get hundreds of recombinants as described in the protocol. Since we produced sufficient numbers of recombinants for our purposes, we didn't try to improve this. Following the recombination step, we picked the recombinant colonies and transferred them onto fresh chloramphenicol and kanamycin plates to make sure that they expressed the kanamycin resistance gene (Tn5-*neo* cassette). All of the recombinants grew well on kanamycin plates (data not shown).

3.4. Verification of Correct Recombination

3.4.1. PCR Analysis

We first did PCR to show the presence of the *hyg3* cassette in the modified BACs. All of the recombinant BACs gave the expected 638 bp product. PCR was also done to amplify the Tn5-*neo* cassette. All of the recombinant BACs again gave the expected product which is 948 bp. The results of these PCR reactions are not shown.

We then did PCR with primers that surround the insert. To show the replacement of part of *nar2/nit8* with our insert, we then designed primers that surround the insert in the BAC. Because we replaced a 3175 bp sequence of *nar2/nit8* with a 2969 bp insert, the PCR should give a 206 bp smaller product for the modified BACs. The primers used in this PCR were NarCh-f-2091 and pBACmn-6020; the product for the unmodified BACs should be 3949 bp (Fig 3). We also designed primers that would bind to the insert; in these PCR reactions, as expected, we found that only the modified BACs produced a PCR product (results not shown).

3.4.2. Verification of Successful Recombination by Restriction Digestion

After checking the replacement in the modified BACs by PCR, we performed a restriction analysis to confirm the results. We digested the modified and unmodified BACs with BglII and KpnI and compared their restriction patterns.

Figure 4A shows BAC 37P6 clones digested with BglII. As expected, all modified BACs had a restriction pattern different from the unmodified BAC. Clones 2 and 4 had the same banding pattern and it was different from the unmodified BAC. These results confirm the result we obtained by PCR (Fig. 3), that clones 2 and 4 were correctly modified. While clones 1 and 3 also had an insertion it appeared have occurred in a different region of the BAC (as discussed in Fig. 3). These results emphasize the importance of verifying the recombination, as suggested by the RED/ET protocol.

We then digested a correctly modified BAC (see Fig. 3) and an unmodified one for both BAC 33A3 and BAC 37P6. Modified BAC clone #7 of 33A3 and #4 of 37P6 were tested. Both modified clones had a different restriction pattern then the unmodified clones (Fig. 4B).

3.5. Transformation of Chlamydomonas with Modified BACs

We used the modified BAC clones to transform the CC 275 strain of *Chlamydomonas.* We were able to get hygromycin resistant transformants in each experiment. Three of the transformants are shown growing on a hygromycin plate in Figure 5.

4. Discussion

With the *Chlamydomonas* genome available, BACs are being used extensively in functional genomics analysis. BACs are an important source for complementation analysis, because they carry the full sequence of a gene of interest and possibly the cis acting regulatory elements. The disadvantage of using *Chlamydomonas* BACs is that, they don't carry a selectable marker gene. Therefore, cells transformed with BACs cannot be selected directly, e.g., on antibiotic plates. Most researchers do co-transformation to overcome this, but co-transformation of two different vectors occurs at a low efficiency.

We therefore decided to apply RED/ET recombination to *Chlamydomonas* BACs. We created an insert that can be used in a single step BAC modification. The insert is only 2969 bp and can be used in the RED-ET system without any modifications.

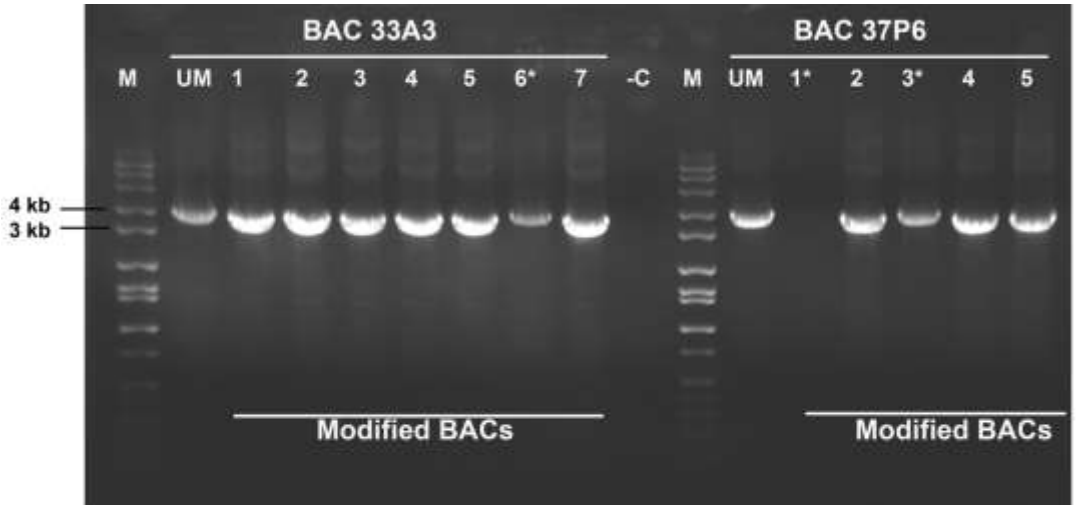

Figure 3. PCR with primers surrounding the insert. The PCR product of the correctly recombined modified BACs is 206 bp smaller than the product of unmodified BACs (UM). Modified BAC #6 of BAC 33A3 and #1 and #3 of BAC 37P6 were not modified correctly; indicated with * (the cassette inserted elsewhere in the BAC). These BACs therefore gave the same size product as the unmodified BACs (UM). Modified BAC #1 of 37P6 had a small amount of PCR product that is barely visible in the picture. In repeated PCRs this BAC always gave very little product which let us conclude that this BAC had a secondary modification. -C: PCR control with no DNA template. M: molecular weight marker. UM: Unmodified.

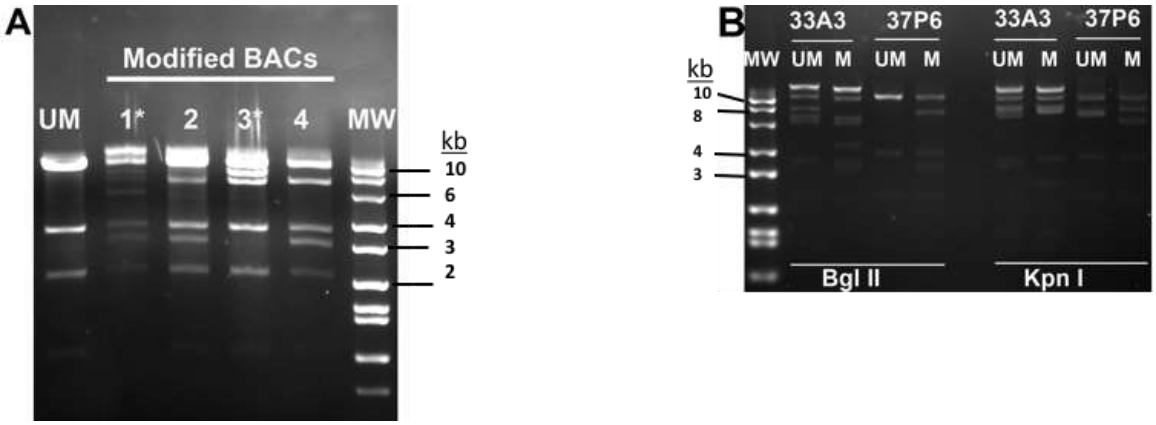

Figure 4. Verification of correct recombination by restriction analysis. A. BAC 37P6 clones digested with BglII. Clones 2 and 4 had he same pattern, which was different from the unmodified BAC, showing that they were correctly modified. The patterns of 1 and 3 were also different from the unmodified BAC (* indicates not correctly modified BACs), but we had previously shown that they were not correctly modified (see Fig. 3). B. Clone 7 of BAC 33A3 and clone 4 of 37P6 (see Fig. 3) are digested with BglII and KpnI. According to both PCR and restriction pattern, these 2 modified BACs are correctly modified. Therefore, they were used in complementation experiments. MW: molecular weight marker. M: modified. UM: unmodified.

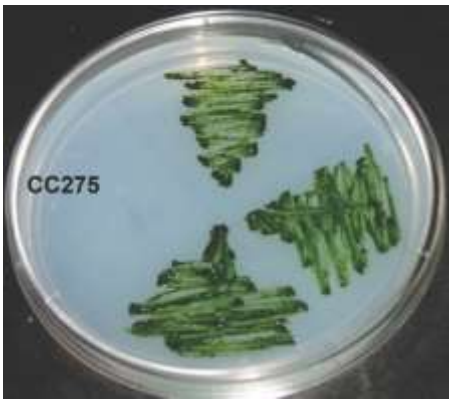

Figure 5: Expression of the Inserted *hyg3* Cassette in *Chlamydomonas. Chlamydomonas* strain CC 275 was transformed with modified BACs and transformants were selected on 10 μg ml^{-1} hygromycin TAP plates. 3 of the transformants are shown here growing on hygromycin plate. Section of the plate labeled as CC 275 is the untransformed cells which cannot grow on hygromycin.

It has the *hyg3* cassette for expression in *Chlamydomonas* and the Tn5-*neo* cassette for expression in *E. coli.* We showed the expression of the Tn5-*neo* cassette in *E. coli* and the *hyg3* cassette in *Chlamydomonas*. The modified BACs were used to transform *Chlamydomonas* and hygromycin resistant transformants were obtained.

We replaced a region of the *nar2/nit8* gene which has no functionality in *Chlamydomonas* BACs, but the insertion could also be done in other regions of the BAC that don't have any function for the vector. Because our insert is a selectable marker, it can be used for recombination for different purposes. For instance, most BACs contain more than one gene. Therefore, in order to determine which gene product is necessary for complementation, each gene can be deleted one at a time (or multiple genes can be deleted at once, leaving one gene in the BAC) and the modified BAC can then be used for complementation, to see which gene will rescue the mutation. In addition, this system maybe used to mutate *Chlamydomonas* genes in *E. coli* by homologous recombination.

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