Chapter 9

Conversion of BAC Clones into Binary BAC (BIBAC) Vectors and Their Delivery into Basidiomycete Fungal Cells Using *Agrobacterium tumefaciens*

Shawkat Ali and Guus Bakkeren

Abstract

The genetic transformation of certain organisms, required for gene function analysis or complementation, is often not very efficient, especially when dealing with large gene constructs or genomic fragments. We have adapted the natural DNA transfer mechanism from the soil pathogenic bacterium *Agrobacterium tumefaciens*, to deliver intact large DNA constructs to basidiomycete fungi of the genus *Ustilago* where they stably integrated into their genome. To this end, Bacterial Artificial Chromosome (BAC) clones containing large fungal genomic DNA fragments were converted via a Lambda phage-based recombineering step to *Agrobacterium* transfer-competent binary vectors (BIBACs) with a *Ustilago*-specific selection marker. The fungal genomic DNA fragment was subsequently successfully delivered as T-DNA through *Agrobacterium*-mediated transformation into *Ustilago* species where an intact copy stably integrated into the genome. By modifying the recombineering vector, this method can theoretically be adapted for many different fungi.

Key words Recombineering, Binary vector, Bacterial artificial chromosome, Basidiomycete, *Ustilago*, *Agrobacterium*-mediated transformation

1 Introduction

Genetic complementation is required to prove the function of genes identified either by homologous search or by map-based cloning. To functionally analyze entire genes with regulatory elements, in their native genomic setting, or complete clusters of genes, it is highly desirable to be able to transfer these large DNA fragments to recipient cells. Large genome fragments, represented by bacterial artificial chromosome (BAC) clones, are routinely used for positional cloning, physical map construction, and whole genome sequencing because of their capability to replicate stably in *E. coli* [1, 2]. Typically, genomic DNA fragments contained in BAC clone libraries range in size from 50 to 150 kb. However, cloning of such BAC-size fragments into plasmid constructs that

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can be used for genetic transformation, using conventional cloning methods such as restriction enzyme digestion and ligation, are often time-consuming and inefficient because of the big sizes and few convenient restriction sites available for cloning in such fragments. An alternative approach to manipulate large DNA fragments uses a yeast-based recombineering system [3] or recombineering technology based on Lambda (λ) phage biology [4–7]. In addition, genetic transformation using chemical procedures or electroporation, in particular for large DNA constructs, is not efficient for some fungi. An alternative transformation procedure using *Agrobacterium tumefaciens*, shown initially to be able to genetically transform fungi [8], was further developed in our laboratory for transformation of *Ustilago* species [9]. Previously it had been shown that large genomic DNA fragments could be delivered by *A. tumefaciens* into tobacco plants by developing a BAC vector suitable for maintenance in and transfer from this bacterium as a so-called binary vector [10] which was termed a BIBAC vector; this also required the proper placement of transfer (T)-DNA border sequences [11].

We combined different techniques and made use of the λ phage-based in vivo genetic engineering technology to convert BAC clones with *Ustilago*-specific selection markers into binary BAC (BIBAC) constructs by homologous recombination in *E. coli*. This recombineering technology is based on three λ RED-encoded genes (*Exo*, *Beta*, and *Gamma*) that have been transferred to the chromosome of the recombineering-proficient *E. coli* strain SW102 by integrating a λ prophage [12]. The Exo protein is a 5′ to 3′ exonuclease that binds to the end of introduced linear dsDNA and degrades one strand to make a 3′ ssDNA overhang [6]. Beta is a ssDNA binding protein that binds to the 3′ overhang and promotes its annealing and homologous recombination with the complementary DNA strand of the BAC clone [6, 13]. Gamma encodes an inhibitor protein of *E. coli* RecBCD and SbcCD nuclease activities to protect the linear DNA and make it available for recombination with the BAC clone [6, 14, 15].

To convert BAC clones into BIBAC constructs that can be delivered by *A. tumefaciens* to the host cells (fungi), some specific functions such as a bacterial selectable marker, T-DNA specific border sequences (a left border LB and a right border RB sequences) and a broad-host range origin of replication need to be introduced. These essential functions are supplied by a linear DNA fragment produced by the Polymerase Chain Reaction from the recombineering vector (Fig. 1). A crucial part of this linear REC fragment is the presence of two 40–50 bp sequences on each end that are complementary to the selection marker in the BAC clone such as the chloramphenicol acetyl transferase gene (CAT resistance gene) present in most BAC vectors/clones (Fig. 1, Cat-1 and Cat-2). This provides the necessary homologous sequences for the λ RED

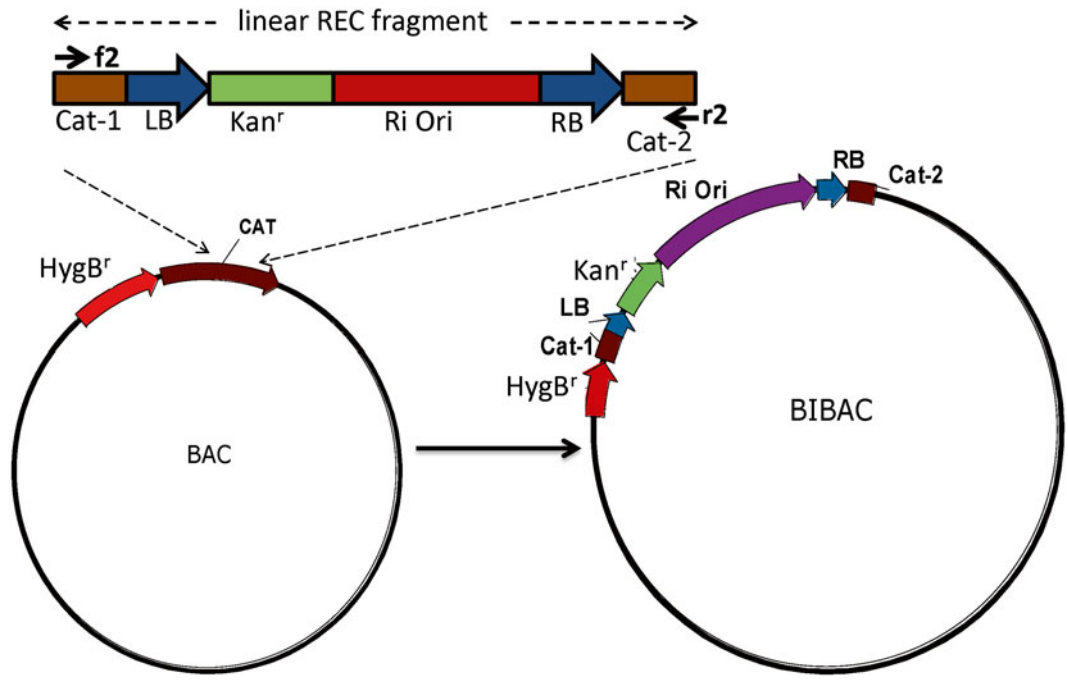

Fig. 1 Schematic representation of the BAC to BIBAC conversion method. Conversion of a Bacterial Artificial Chromosome vector, BAC, harboring genomic inserts (*black circle*), into a binary BAC (BIBAC) vector using a linear DNA fragment (linear REC fragment) amplified by PCR with primers f2 and r2 from recombineering vector pFT41 [16]. The REC fragment recombines into the chloramphenicol resistance gene (CAT) present on the BAC clone, using homologous regions present on both the left (Cat-1) and the right end (Cat-2) of the REC fragment (*dashed arrows*). Recombinants are selected for kanamycin resistance (Kan^r) present on the REC fragment and screened for loss of chloramphenicol resistance indicating proper integration. The resulting BIBAC constructs are then transformed into a suitable *A. tumefaciens* strain for subsequent transformation into *Ustilago*. Any DNA present between the right border (RB) and *left border* (LB) sequence elements in BIBAC clones functions as T-DNA and is transferred by the *A. tumefaciens* machinery to a host cell where it is integrated into the genome. *Ustilago* transformants were selected on hygromycin B; a *Ustilago*-specific hygromycin B phosphotransferase cassette present on the BAC vector (HygB^r, *red arrow*) placed this selection cassette after recombination with the REC fragment on the T-DNA of the BIBAC construct. The figure is not drawn to scale

proteins to integrate the linear REC fragment into the BAC clone, but also provides counter selection by conversion to chloramphenicol sensitivity indicating proper recombination.

Such a Rec vector was developed by Takken et al. [16] for use in ascomycete fungi by introducing a fungal-specific selectable marker cassette to allow *Agrobacterium*-mediated transformation (AMT) of *Fusarium* and *Aspergillus* species. We used two different types of REC vectors to convert BAC clones into BIBAC constructs. For one kind of BAC conversion, we used the pFT41 backbone [16] to convert the previously developed *Ustilago* specific BAC vector, pUsBAC5 [17]. This BAC clone already has a *Ustilago*-specific hygromycin B (HygB^r) selection cassette under control of the HSP70 promoter and terminator signals [18] and

therefore no fungal selection marker was required in the REC vector [9]. In the second BAC conversion approach, we replaced the Ascomycete-specific phleomycin resistance cassette from pFT41 with the *Ustilago*-specific HygB resistance cassette (S. Ali, J. Xu, and G. Bakkeren, unpublished). This REC vector can be used for conversion of any BAC clone that does not have a *Ustilago* (fungal) selectable marker.

We used *A. tumefaciens* to deliver these BIBAC clones into *U. hordei* and *U. maydis* and showed that *Agrobacterium* can efficiently deliver large DNA fragments into these basidiomycete fungi [9]. AMT has proved very efficient for delivery of foreign DNA into several fungal species that are refractory to genetic transformation with conventional transformation methods [19–23]. In addition to the higher efficiency of AMT in different fungi, it also usually results in single-copy T-DNA integration of mostly intact DNA fragments into the host genome at random sites [24–26]. This method has been used to transfer large DNA fragments into different fungi and plant cells [9, 16, 27]. In this chapter we will describe in detail the method for BAC to BIBAC conversion and their subsequent transfer into the basidiomycete fungus *Ustilago* by *Agrobacterium*.

2 Materials

Prepare all solution using ultrapure deionized water and analytical grade reagents.

2.1 Bacterial and Fungal Strains, Growth Media, and Culture Conditions

1. *E. coli* recombineering strain SW102 (a DY380 derivative with genotype *λcI*857, no *cro-BioA* but *tet*) can be requested from Dr. Neal Copeland (National Cancer Institute, Frederick, MD) [4, 12]. Supervirulent *Agrobacterium tumefaciens* strain COR309 is a recA-deficient C58 nopaline strain UIA143 harboring disarmed pTiB6 derivative plasmid pMOG101 [10] and a special vir helper plasmid pCH32, which provides extra copies of the *virA* and *virG* two-component signaling genes. *A. tumefaciens* strain COR308 is similar to COR309 except that it has disarmed pTi derivative plasmid pMP90 instead of pMOG101; they can be obtained from Cornell University (http://www.biotech.cornell.edu/BIBAC/BIBACHomePage.html). *Ustilago hordei* haploid strain Uh4857-4 (alias Uh364, *MAT-1*) has been described [17] and *U. maydis* haploid strain 324 (mating type *a2b2*) is Um521 [28].
2. Luria–Bertani (LB) medium: 1 % NaCl w/v, 1 % Bacto Tryptone w/v, 0.5 %, Bacto Yeast Extract w/v. LB broth and agar plates supplemented with appropriate antibiotics for plasmid selection in *E. coli* and *Agrobacterium*.

3. SOC medium: 2 % Bacto Tryptone w/v, 0.5 %, Bacto Yeast Extract w/v, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7.0.
4. LC medium: 0.8 % NaCl w/v, 1 % Bacto Tryptone w/v, 0.5 % Bacto Yeast Extract w/v.
5. Induction medium: 10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4 \cdot 7H_2O$, 0.7 mM $CaCl_2 \cdot 2H_2O$, 9 μM $FeSO_4$, 4 mM $(NH_4)_2SO_4$, 10 mM glucose supplemented with tetracycline (5 μg/mL), kanamycin (50 μg/mL), 40 mM MES, and 0.5 % glycerol.
6. Complete medium (CM): per liter, 5 g Casamino acids, 1.5 g Ammonium nitrate (18 mM end concentration), 10 g Yeast extract, 60 mL *Ustilago* macro salts (see below), set pH 7.0 with NaOH; (for plates, add 15 g agar). After autoclaving, add 20 mL sterile 50 % Glucose to 1 %.
7. *Ustilago* macro salts: per liter, 16 g KH_2PO_4, 4 g Na_2SO_4, 8 g KCl, 2 g $MgSO_4 \cdot 7H_2O$, 1 g $CaCl_2 \cdot 2H_2O$, 8 mL Trace Elements Solution.
8. Trace Elements Solution: per liter, 60 mg H_3BO_3 (boric acid), 140 mg $MnCl_2$, 400 mg $ZnCl_2$, 40 mg $Na_2Mo_4 \cdot 2H_2O$, 100 mg $FeCl_3$, 400 mg $CuSO_4$.
9. Selection medium: CM supplemented with Cefotaxime 200 μg/mL to kill off *A. tumefaciens* and 300 μg/mL hygromycin B to select for *Ustilago* transformants.
10. Acetosyringone (AS) 200 mM stock in DMSO (*see* **Note 1**).
11. Incubator and shaker at 20, 24, 28, and 30 °C.
12. Shaking water bath at 42 °C.
13. High speed refrigerated centrifuge with swing out rotor for 50 and 15 mL Falcon tube and refrigerated microcentrifuge for small tubes.
14. ME-25 filters (Schleicher and Schuell, 0.45 μm pore size, 47 mm diameter).

2.2 DNA Extraction

1. Sterilized acid-washed glass beads of 0.5 mm in size.
2. Lysis buffer: 0.5 M NaCl, 2 M Tris–HCl pH 7.5, 10 mM EDTA pH 8.0, 1 % SDS (*see* **Note 2**).
3. Phenol–Chloroform–Isoamylalcohol 24:24:1.
4. Ethanol 70 %.
5. Isopropanol.
6. TE pH 8.0 (10 mM Tris–HCl pH 7.5, 1 mM EDTA).
7. Prechilled mortars and pestles.

2.3 DNA Blotting, Probe Labeling, and Hybridization

1. 20× SSC buffer: 0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0, and 0.1 % w/v sodium dodecyl sulfate (SDS).
2. 1× Tris–Borate–EDTA (TBE) buffer: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0.
3. Saran Wrap, X-ray film (e.g., Kodak Biobax film, Kodak Canada, Toronto, ON, Canada), and developing reagents.
4. Agarose gel apparatus.
5. Transfer tray and alkaline transfer buffer. Alkaline transfer buffer is composed of 0.4 N Sodium hydroxide (NaOH) and 1 M sodium chloride (NaCl).
6. Hybond-N^+ nylon membrane (Amersham Biosciences/GE healthcare) or similar product from other suppliers.
7. αP^{32}-dCTP for radioactive labeling of probes.
8. Rediprime II Random Prime Labeling system (Amersham Biosciences/GE healthcare) or similar product from other suppliers.
9. Hybridization buffer, ULTRA-hyb buffer (Ambion) or similar product from other suppliers.
10. Hybridization oven and hybridization tube.

3 Methods

3.1 Preparation and Transfer of the Target BAC Clones in Recombineering Proficient E. coli SW102 Cells

1. To prepare the BAC plasmid, streak a BAC clone for single colony selection from a glycerol stock at −80 °C on LB plates with appropriate antibiotic (Cm).
2. Transfer a single colony to 5 mL LB medium in a 13 mL snap-cap tube with appropriate selective antibiotic, incubate overnight at 37 °C with vigorous shaking at 200–230 rpm.
3. Take 500 μL of the starter culture and inoculate into 150 mL LB media with selective antibiotic in a 500 mL conical flask and grow at 37 °C for 14–16 h with vigorous shaking.
4. Harvest cells at 3,700 × *g* for 15 min at 4 °C and follow the protocol for plasmid midi preparation (e.g., QIAGEN's QIAprep Spin Midi Kit and Protocol or a similar product from other suppliers (*see* **Note 3**).
5. Quantify the BAC DNA using a spectrophotometer; pure DNA should have an OD_{260}/OD_{280} ratio of >1.8. Use 1 μL of the BAC DNA solution for PCR analysis to confirm the plasmid, and use about 3–5 μg for restriction analysis.
6. Transfer 1–5 μL (0.2–2 μg) of freshly prepared target BAC constructs (*see* **Note 4**) into 50 μL electro-transformation-competent recombineering proficient *E. coli* SW102 cells

(*see* Subheading 3.3), on ice. As a negative control for plasmid transformation, use 5 μL of ddH_2O for 50 μL cells.

7. Pipette the *E. coli* cells with the BAC DNA into a prechilled 2 mm gap electroporation cuvette.
8. Set the electroporator to 2.5 kV/cm, 25 μ FD, 200 Ω. This setting is used for a Gene Pulser (Bio-Rad) using an electroporation cuvette with 2 mm gap. For 1 mm gap cuvettes, change the voltage to 1.2–1.6 kV/cm. Other electroporation apparatuses can be used following the manufacturer's instructions.
9. Flick the cuvette gently to mix and knock on the table to remove air bubbles. Dry the cuvette with a tissue and place into position in the electroporator. Proper transformation normally occurs when no arching is observed and a time constant of 4–5 is obtained.
10. Immediately remove the cuvette from the chamber and add 1 mL SOC medium without antibiotics to the cuvette. Remove all the contents of the cuvette using a sterile Pasteur pipette and transfer it to a 13 mL snap-cap tube.
11. Incubate the cultures at 28 °C to avoid premature induction of the phage recombineering (RED) genes in a shaking incubator for 60 min.
12. Plate out 50–100 μL of the cells on LB agar plates with appropriate antibiotic (Cm) and incubate overnight at 28 °C. No-DNA controls are treated the same and should be included.
13. Colonies should appear on selection plates where cells received BAC DNA whereas controls should not. Select a single colony and perform colony PCR with specific primers to confirm proper transformation with the target BAC clone. Make a glycerol stock of the confirmed colony and store at −80 °C for future use.

3.2 Generation of the REC Fragment by Polymerase Chain Reaction (PCR)

1. Design primers to generate a PCR product with 40–50 bp termini homologous to a selectable marker in the BAC construct. In REC vector pFT41, the CAT gene was targeted and primers cat-f2 (CCGTTGATATATCCCAATGGC) and cat-r2 (ACAAACGGCATGATGAACCT) were used.
2. Use a proof reading polymerase that is optimized for the generation of long PCR products such as *TaKaRa LA Taq* polymerase (TAKARA Bio INC) or a 18:1 blend of SuperTaqPlus (Ambion) and Pfu (Promega) or similar products from other suppliers.
3. For the amplification of long PCR products for recombineering, use the following program on a thermal cycler: an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of

30 s at 95 °C, 40 s at 58 °C (depending on the annealing temperature of the primers; this should be empirically determined) and 8 min at 68 °C, with a final extension at 68 °C for 15 min (*see* **Note 5**).

4. Separate the generated PCR molecules from the template REC plasmid DNA, primers and dNTPs by agarose gel electrophoresis. PCR purification kits can also be used for cleaning up the PCR product for the next enzymatic reaction (*see* **Note 6**). Approximately 150 ng of the final, clean PCR product is needed per transformation.
5. Careful removal (destruction) of the template DNA (the REC substrate plasmid) is required to eliminate background transformation. Digest the PCR product with restriction enzyme Dpn1, which recognizes a 4-base pair site (GATC), and only targets methylated DNA. Template DNA plasmid is usually methylated as most commonly used bacterial strains are dam^+ while the linear PCR product is usually not methylated. Dpn1 works well in PCR buffer and **step 4** can be eliminated by directly adding 2–4 μL of Dpn1 in 50 μL PCR reaction and incubating for 1 h at 37 °C. Scale up this digestion reaction when using larger volumes of PCR reaction.
6. Separate the PCR-amplified fragments from the digested template DNA by agarose gel electrophoresis and isolate the fragment as in the **step 4** above. Elute the DNA in sterile ddH_2O to avoid addition of any salts which will interfere in the electrotransformation step. If the concentration of eluted DNA is low, precipitate the DNA by adding 1/5 volume of 3 M sodium acetate and 2 volumes of 96–100 % ethanol. Mix and incubate the tube on ice for 30 min and centrifuge for 15 min at $20{,}000 \times g$ at 4 °C. Discard the supernatant and wash the pellet with 70 % ethanol and dissolve in a small volume of ddH_2O.

3.3 Preparation of Electro-competent Recombineering-Proficient E. coli SW102 Cells

Before starting the electro-competent cell preparation, cool down sterile ddH_2O, 10 % glycerol, and the electroporation cuvettes on ice for 1–2 h.

Remember to grow this strain at 28 °C because you do not want to induce the λ RED genes which are under the control of the temperature-sensitive λ-repressor, prematurely.

1. To prepare the electro-competent cells, streak *E. coli* strain SW102 or SW102 strains previously transformed with target BAC clones, from glycerol stocks for single colony selection on LB plates (with appropriate selection). To avoid any contamination, use tetracycline at 12.5 μg/mL for SW102 and use additional appropriate antibiotics for strains that harbor target BAC clones.

2. Transfer a single colony to 5 mL LB medium in a 13 mL snap-cap tube with appropriate selective antibiotic, incubate overnight at 28 °C with vigorous shaking at 200–230 rpm.
3. Take 500 μL of the starter culture and inoculate into 50 mL LB media with selective antibiotic in a sterile 250 mL conical flask. Incubate for 3–6 h at 28 °C in a shaking incubator until the density reaches an OD600 of 0.4–0.5. To monitor cell growth, take a sample for measuring the OD after 3 h and repeat each 30 min; use LB media as a reference when measuring the OD.
4. When the desired OD is reached, cool down the flasks containing the bacteria for 5 min on an ice slurry and transfer 40 mL of the culture into precooled 50 mL Falcon tubes.
5. Harvest the cells at 3,700 × *g* for 10 min at 0 °C and resuspend the cell pellet in 2 mL ice cold 10 % glycerol by gently shaking the tube in the ice slurry. When the cells are fully resuspended, add 13 mL 10 % glycerol to a final volume of 15 mL.
6. Invert the tube several times and centrifuge the cells at 3,700 × *g* for 5 min at 4 °C.
7. Remove the supernatant, resuspend the cells as in **step 5**, and spin once more as in **step 6**.
8. Remove all supernatant gently by inverting the tube. One needs to be very careful at this step to not lose the pellet, which should be kept on ice all the time.
9. Resuspend the cells gently on ice in 120–160 μL sterile ddH_2O or 10 % glycerol (use 10 % glycerol if the cells need to be stored at −80 °C for future use). This should give approximately 10^9 cells per mL. Aliquot 40 μL of the cells in a 1.5 mL precooled Eppendorf tube on ice.
10. Freeze remaining cells in 50 μL aliquots in liquid N_2 and store at −80 °C.

3.4 Heat Shock Induction of the λ RED Recombination Proteins and Recombineering Step

1. For induction of the λ RED proteins, grow strains that contain the target BAC clones at 28 °C to avoid premature induction. When the OD600 of the bacterial culture reaches 0.4–0.6, transfer 30 mL into a 250 mL conical flask and incubate in a shaking incubator at 42 °C for 15 min to induce the phage recombination RED genes, the expression of which is under control of a temperature-sensitive λ-repressor. The rest of the culture remains at 28 °C as non-induced control.
2. After 15 min, put both flasks containing induced and non-induced bacterial culture on ice for 20 min to cool them down and pour into 50 mL prechilled Falcon tubes; keep on ice.
3. Prepare electro-competent cells of both heat-induced and non-induced bacterial cultures as described above, starting

with **step 5** in Subheading 3.3, until 40 μL aliquots with a cell density of approximately 10^9 cells per mL are obtained in a 1.5 mL precooled Eppendorf tubes on ice (**step 9** in Subheading 3.3).

4. Add 150 ng of the clean, Dpn1-digested REC PCR product from **step 6** in Subheading 3.2 and pipette the *E. coli* cells with the DNA into a prechilled 2 mm gap electroporation cuvette.
5. Set the electroporator to 2.5 kV/cm, 25 μ FD, 200 Ω. This setting is used for a Gene Pulser (BIO-RAD) using an electroporation cuvette with 2 mm gap. For 1 mm gap cuvettes, change the voltage to 1.2–1.6 kV/cm. Other electroporation apparatuses can be used following the manufacturer's instructions.
6. Flick the cuvette gently to mix and knock on the table to remove air bubbles. Dry the cuvette with a tissue and place into position in the electroporator. Proper transformation normally occurs when no arching is observed and a time constant of 4–5 is obtained.
7. Immediately remove the cuvette from the chamber and add 1 mL SOC medium without antibiotics to the cuvette. Remove all the contents of the cuvette using a sterile Pasteur pipette and transfer it to a 13 mL snap-cap tube.
8. Incubate the cultures at 28 °C to avoid premature induction of the phage recombineering (RED) genes in a shaking incubator for 60 min.
9. Plate out 100–200 μL of the cells on LB plates with 50 μg/mL kanamycin and incubate at 28 °C. In the non-induced cells, no or very few colonies will appear while on the plate with induced cells, 100–200 colonies will grow.
10. Test at least 100 colonies for sensitivity to chloramphenicol and resistance to kanamycin. This can be done by taking a colony with a sterilized tooth pick and streaking it first on a LB + Cm plate and then, using the same toothpick, on a LB + Kan plate. The whole plate can also be replica-plated using sterilized pieces of velvet.
11. Colonies that are resistant to Kan and sensitive to Cm likely contain properly recombined BIBAC clones as correct recombination of the Rec vector into the BAC clone will have disrupted the chloramphenicol acetyltransferase (CAT) gene. This should be further verified with PCR and restriction enzyme digestion. However, there will be some colonies resistant to both Kan and Cm and could be the result of REC DNA integration into chromosomal DNA or non-homologous integration of the REC fragment into the BAC clone at other locations than the proper CAT gene.

3.5 Fungal Transformation

1. Transfer of desired BIBAC constructs into electro-competent *A. tumefaciens* strains is done by standard electroporation method as described for *E. coli* above, except with a longer incubation time (90–120 min) before plating on selection medium. It is important to use an *A. tumefaciens* strain that contains an extra copy of *virA* and *virG* genes since these have been shown to be important for the transfer of large T-DNA fragments to plant cells [27]. In our experiments we have used strains COR308 and COR309 that are recA deficient and contain extra copies of *virA* and *virG* on a helper plasmid pCH32. To maintain the helper plasmid, grow these strains under 5 μg/mL tetracycline selection at all times (*see* **Note 7**).
2. Streak the *A. tumefaciens* strains with the BIBAC construct from glycerol stocks on LB media with appropriate antibiotic and incubate at 28 °C.
3. Inoculate a single colony into 10 mL LC medium with selection for both the helper and BIBAC plasmid and incubate overnight.
4. Spin down 10 mL of BIBAC-containing *A. tumefaciens* culture for 10 min at 3,700 × *g*, at 4 °C, remove the supernatant and resuspend the pellet in 10 mL induction medium (IM) supplemented with 40 mM MES, 0.5 % glycerol, and 0.2 % glucose.
5. Resuspend the pellet to an OD_{600} of 0.4 in 5 mL IM and supplemented with 200 μM AS.
6. Incubate the *Agrobacterium* at 28 °C for 6–8 h to reach OD_{600} of 0.5–0.6. Incubate control cells in the same medium but without AS.
7. Grow fungi that you want to transform in liquid medium. In our laboratory, we grow *Ustilago* species in CM [29]; *U. hordei* at 22 °C and *U. maydis* at 28 °C. Re-inoculate 20 mL fresh CM to an OD_{600} of 0.15 and subsequently grow these cultures until an OD_{600} of 0.5 is reached.
8. Dilute the fungal cell cultures tenfold in IM and mix with an equal volume of AS-induced *A. tumefaciens*. This will result in an approximate ratio of 10:1 *Agrobacterium* to fungal cells (*see* **Note 8**).
9. 20 min after mixing the two cell cultures, inoculate 100–200 μL of the mixture onto ME-25 filters (0.45 μm pore size, 47 mm diameter) and place them on co-cultivation medium (IM, but with 0.1 % glucose and 200 μM AS). IM without AS can be used as a negative control for co-cultivation medium.
10. Air-dry the membranes for 30 min and incubate at 20–24 °C for 2–5 days or till individual colonies appear (*see* **Note 9**).

11. To select for *Ustilago* cells properly transformed with the T-DNA delivered from the BIBAC vectors by *A. tumefaciens*, transfer the membrane to selection plates containing CM medium supplemented with antibiotics such as HygB for which the resistance cassette is present on the T-DNA. Use cefotaxime at 200 μg/mL in the selection medium to kill off the *A. tumefaciens*. After 4 days, transfer individual colonies to CM with selective antibiotic and cefotaxime as above.

3.6 DNA Isolation from Fungal Cells

3.6.1 DNA Isolation for PCR Analysis

1. For PCR analysis, genomic DNA can be isolated using a mini prep protocol for *Ustilago* that is optimized in our laboratory and modified from a protocol used for yeast [30].
2. Inoculate transformed as well as untransformed control fungal cells from a fresh CM plate (*see* **Note 10**) in 5 mL CM with appropriate antibiotic in 13 mL snap-cap tubes until late-log growth, at an approximate OD_{600} of 1.5–2 (*see* **Note 11**).
3. Transfer 2 mL of the culture to a 2 mL Eppendorf tube and centrifuge for 2 min at 20,000 × *g* and aspirate off all the supernatant using regular laboratory vacuum.
4. Add 0.2–0.5 g of sterilized acid-washed glass beads of 0.5 mm in size to each tube. In our laboratory we are using a scoop made from cap of a microfuge tube and add one scoop to each tube (*see* **Note 12**).
5. Add 500 μL of lysis buffer and 250 μL of Phenol–Chloroform–Isopropanol (PCI) and vortex at high speed for 3 min. Do this step in a fume hood.
6. Centrifuge the tube for 3 min at 20,000 × *g* to separate the phases. Carefully transfer 450 μL of the upper aqueous phase to a new tube, avoiding touching and taking along the cell debris at the interface.
7. Add 250 μL PCI to the aqueous phase, vortex for 30 s, and centrifuge for 1 min at 20,000 × *g*. Carefully transfer 400 μL of the upper aqueous phase to a new tube.
8. To precipitate the genomic DNA, add 0.6 volumes of isopropanol, mix and keep on ice or at –20 °C for 30 min or more. Overnight storage at –20 °C works better.
9. Centrifuge for 15 min at 20,000 × *g* at room temperature; a white pellet should be visible which contains genomic DNA and a large amount of RNA. Remove supernatant by pipette or inverting.
10. Add 1 mL of 70 % room-temperature ethanol, centrifuge for 5 min at 20,000 × *g* at room temperature and remove supernatant. Be careful, since the pellet may be loose. Invert the tube on a paper towel for 10–20 min or until the pellet is dry. The pellet may also be dried in a speedVac but avoid over-drying since the pellet will be difficult to dissolve.

11. Dissolve in 100 μL TE buffer pH 8. This protocol yields both DNA and RNA. The quality and yield of the DNA can be checked by running 5 μL of the sample on 1 % agarose mini gel.

3.6.2 DNA Isolation for Gel Blot Analysis

To get a higher yield and a more pure quality of genomic DNA for gel blot analysis, isolate the DNA using the following protocol:

1. Inoculate a single colony of the transformed as well as untransformed fungi into 1 mL CM medium with appropriate antibiotic and incubate overnight. Transfer this 1 mL culture into 100 mL CM medium in 500 mL conical flasks and grow until late-log growth, at an approximate OD_{600} of 1.5–2.
2. Harvest the fungal cell culture in 50 mL Falcon tubes at 3,700 × *g* for 15 min at 4 °C. Remove the supernatant and freeze the pellet rapidly in liquid nitrogen.
3. Grind frozen cell pellets with prechilled mortars and pestles using liquid nitrogen and isolate the genomic DNA using the DNeasy Plant Maxi Kit (QIAGEN) following the manufacturer's instructions. Similar kits from other suppliers can also be used.

3.7 Verification of Fungal Transformation by Molecular Analysis

Standard molecular biological techniques such as PCR and genomic DNA blot analysis can be used to confirm proper transformation of fungal cells with T-DNA as expected from the BIBAC constructs.

1. To verify integration of intact T-DNA, perform PCR analysis for the presence of left border, right border, and selection marker.
2. For DNA blot analysis, use two probes one for the left border and one for the right border. For *Ustilago*, digest 8–10 μg of genomic DNA with appropriate restriction enzymes that cut the T-DNA not more than 2 kb from the border.
3. Run the digested DNA slowly on a 0.8 % (w/v) agarose gel in 1× Tris–borate–EDTA (TBE) buffer. Stain the gel with ethidium bromide, photograph with a ruler alongside, making sure size standards are matched up with the ruler. Treat the gel with a 0.25 M HCl solution by gently shaking for 15 min to reduce the sizes of the genomic DNA fragments and immediately transfer the gel to a 0.4 N NaOH solution and shake gently for 15 min. Transfer the DNA overnight from the gel to a Hybond-N^+ nylon membrane using a 0.4 N NaOH solution. Use a standard setup. After transfer, dismount the assembly and float the membrane carefully on a 6× SSC solution for 5 min, then air-dry on filter paper.
4. To detect specific fragments in the genome by hybridizations, label probes with a αP^{32}-dCTP, using the Rediprime II Random Prime Labeling system (Amersham Biosciences/GE healthcare) or a similar product from other suppliers. Nonradioactive methods can also be used for labeling the probes and several kits from different suppliers are available.

5. Hybridize labeled and denatured probes to the membrane using ULTRA-hyb buffer (Ambion) at 42 °C according to the manufacturer's instructions.
6. Wash the blot twice for 5 min each with 2× SSC plus 0.1 % w/v sodium dodecyl sulfate (SDS) solution, followed by two washes each of 15 min with 0.1× SSC plus 0.1 % SDS solution. Do all the washes at 42 °C.
7. Depending on signal strength, expose the blot to Kodak BioMax film (we used Kodak Canada, Toronto, ON, Canada) or other type film for 5–8 h. If the signals are weak, overnight exposure or the use of an intensifying screen and exposure at −80 °C might give better results. Detection using phosphor-imaging screens and laser detection can also be used.

4 Notes

1. Make a 200 mM acetosyringone solution in DMSO, filter-sterilize and dispense in aliquots of 500 μL in 1.5 mL micro-tubes and store in a −20 °C freezer until use.
2. Make the lysis buffer fresh each time. To make 10 mL of lysis buffer, mix together 6.8 mL of ddH_2O, 1 mL of a 5 M NaCl stock solution, 1 mL of a 2 M Tris–HCl (pH 7.5) solution, 0.2 mL of a 500 mM EDTA (pH 8.0) solution, and 1 mL of 10 % SDS solution (all autoclaved separately).
3. It is important to use midi or maxi kits designed for BAC plasmid preparation because of their big sizes and low copy numbers. The mini plasmid kits yield too little DNA and not of sufficient quality to be used for transformation in this protocol; larger-scale preparations normally work better.
4. It is important to use freshly prepared BAC DNA since large constructs deteriorate over time; older DNA preparations stored at 4 °C transform with less efficiency.
5. The extension time depends on type of polymerase used, however use 1 min per kb as general rule or follow the manufacturer's protocol provided with the polymerase.
6. Direct PCR purification should be used only when a clean single band of the expected size is amplified. Standard procedures can be used for the isolation of the PCR product from agarose gels. Different DNA-fragment isolation kits are commercially available that can be used according to the manufacturer's instructions. Depending on the method used, DNA loss can be substantial and several PCR reactions combined may be required.
7. *Agrobacterium tumefaciens* strains COR308 and COR309 were used for the transformation of BIBAC into *Ustilago* species.

Both COR308 and COR309 are recombination (rec) deficient strains and contain extra copies of two virulence genes: *virA* and *virG* on a helper plasmid pCH32. The rec deficient strains reduce instability of larger DNA fragments in *A. tumefaciens* and extra copies of these virulence genes, the two-component acetosyringone-sensing and virulence region inducers, increase its virulence and transformation efficiency into the host cells [10, 28].

8. Here the ratio of *Agrobacterium* to fungal cells is 10:1; however, to optimize the protocol for other fungi, different ratios of *Agrobacterium* to fungal cells, such as 1:1, 2:1, 5:1 and 20:1, can be tested.
9. Drying the membrane for different times, changes the transformation efficiency of different fungi [31]. To get the optimal transformation efficiency, different drying times, such as 10, 20, 30, 40, 50, and 60 min, can be tested.
10. Take the inoculum from a fresh agar plate not more than 7–10 days old. Inoculation from old agar plate stored for few weeks at 4 °C will fail to grow or will grow very slowly.
11. For *U. hordei*, it usually takes 2 days to reach the desired OD_{600} of 1.5–2 when growing at 22 °C, while *U. maydis* grows faster at 28 °C and may take only 24 h to reach the desired OD. Incubating *U. maydis* for more than 30 h will yield DNA that will be challenging to perform PCR with, or to cut with restriction enzymes.
12. Make sure glass beads do not stick near the rim of microfuge tube; failing to do so makes the tube not to close properly and leakage of phenol will happen during the vortexing step.

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