

Multigene Engineering in Rice Using High-Capacity *Agrobacterium tumefaciens* BIBAC Vectors

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Abstract

The high-capacity binary bacterial artificial chromosome (BIBAC) vector system permits the insertion of large fragments of DNA, up to 150 kb, into plants via *Agrobacterium*-mediated transformation. Here, we describe an optimized protocol for transformation of japonica rice (*Oryza sativa* L.) using this system. Calli derived from mature embryos are transformed using *Agrobacterium* strain LBA4404 that carries the BIBAC vector and the super-virulent helper plasmid pCH32. Transformed calli are then regenerated using optimized media and tested for transgene integration by PCR, GUS assay, and Southern blot analyses.

Key words Binary bacterial artificial chromosome (BIBAC), Transformation of large DNA fragment, *Agrobacterium tumefaciens*, Rice (*Oryza sativa* L.)

1 Introduction

Binary vector systems such as the pGreen series and the pCAMBIA series have been widely used in *Agrobacterium*-mediated transformation of plants [1]. Whereas plant transformation with DNA fragments below 20 kb is routine, success in stable plant transformation with DNA fragments larger than 50 kb has been limited [2].

A reliable system for transforming large fragments of DNA (>50 kb) into plants makes it feasible to introduce a natural gene cluster or multiple genes into a single locus. Thus, several disease resistance and/or pest resistance genes, or a gene cluster encoding the enzymes of a metabolic pathway, could be simultaneously introduced in one transformation step. Large insert transformation would make it feasible to study the expression of plant genes or gene clusters in their native genomic context and might eliminate integration site-dependent gene expression, which can be a serious problem in plant transformation experiments.

The high-capacity binary bacterial artificial chromosome (BIBAC) [3] can replicate in both *Escherichia coli* and *Agrobacterium tumefaciens* and permit the insertion of large fragments of DNA

(up to 150 kb) in tobacco [4] and tomato [5]. BIBAC libraries can not only be used for large-insert DNA library construction [6–8], but also facilitate gene discovery and functional studies by direct transformation of BIBACs carrying multiple genes or gene clusters into plants [9]. This transformation system opens the way for metabolic engineering in plants [10, 11].

Rice (*Oryza sativa* L.), a monocotyledonous plant, is a staple food for almost two-thirds of the world's population. In addition, its relatively small genome makes it an important model cereal for genome research and breeding. While *Agrobacterium*-mediated transformation of rice has been widely reported [12], the introduced DNA fragments have generally not been larger than 20 kb. In this chapter, we described a BIBAC-based protocol for transforming large fragments of DNA into rice.

2 Materials

2.1 Plants, Bacteria, and Vectors

1. Mature seeds of the *japonica* rice cultivar. There are various *japonica* rice cultivars available, but the seeds that we routinely use are from H1493, an early growing *japonica* variety initially bred in China [13].
2. *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen).
3. BIBAC2 vector [3] carrying the gene(s) of interest. BIBAC library construction was described previously [7]. The vector is available from Cornell University (<http://www.cctec.cornell.edu/express%20licensing/materials/BIBAC/>).
4. pCH32 virulence helper plasmid with *virG* and *virE* loci, containing *virE1* and *virE2* [3], also available from Cornell University.

2.2 Antibiotics and Selective Agents

1. Rifampicin.
2. Streptomycin.
3. Tetracycline.
4. Cefotaxime.
5. Kanamycin.
6. Hygromycin.

2.3 Media

1. Induction medium N6I: 4 g/L Chu (N6) medium with vitamins (Plantmedia, Dublin, OH, USA), 1.0 g/L proline, 0.4 g/L Casein Enzymatic Hydrolysate (CH) (Sigma), 2.0 g/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 45 g/L sucrose, 3 g/L phytigel (Sigma), pH 5.9.
2. Pre-cultivation medium N6P: 4 g/L N6 medium with vitamins, 0.6 g/L proline, 0.6 g/L CH, 2.0 g/L 2,4-D, 30 g/L maltose, 3 g/L phytigel, pH 5.6 (*see Note 1*).

3. *Agrobacterium* resuspend and infection medium N6A: 4 g/L N6 medium with vitamins, 0.6 g/L proline, 0.6 g/L CH, 2.0 g/L 2,4-D, 30 g/L maltose, pH 5.6. Add 100 μ M aceto-syringone before using.
4. Co-cultivation medium N6C: N6A supplemented with 3 g/L phytagel.
5. Resting medium N6R: 4 g/L N6 medium with vitamins, 1.2 g/L proline, 0.4 g/L CH, 2.5 g/L 2,4-D, 30 g/L sucrose, 3 g/L phytagel, pH 5.8. Add 400 mg/L cefotaxime before using.
6. Selection medium N6S: 4 g/L N6 medium with vitamins, 1.2 g/L proline, 0.4 g/L CH, 2.5 g/L 2,4-D, 30 g/L sucrose, 3 g/L phytagel, pH 5.9. Add 250 mg/L cefotaxime, 50 mg/L hygromycin before using.
7. Pre-regeneration medium MSP: 4.2 g/L Murashige and Skoog (MS) medium with vitamins, 2 g/L CH, 2 mg/L kinetin, 0.2 mg/L naphthalene acetic acid (NAA), 30 g/L maltose, 3 g/L phytagel, pH 5.8.
8. Regeneration medium MSR: 4.2 g/L MS medium with vitamins, 3 mg/L kinetin, 0.5 mg/L NAA, 30 g/L maltose, 3 g/L phytagel, pH 5.8.
9. Root-growing medium MSG: 2.1 g/L MS medium with vitamins, 0.2 mg/L NAA, 10 g/L sucrose, 2.5 g/L phytagel, pH 5.8. Add 50 mg/L hygromycin before using. Solidify in 30 $\times$ 200 mm tubes.
10. Solid LB medium supplemented with 25 mg/L rifampicin, 25 mg/L streptomycin, 50 mg/L kanamycin, and 5 mg/L tetracycline.

2.4 Other Materials for Transformation

1. Sterile filter paper.
2. 70 % ethanol.
3. 20 % bleach with 0.1 % Tween 20.
4. Parafilm.
5. Spectrophotometer.
6. Electroporator.
7. Laminar flow hood.
8. Unlit growth chamber.
9. Greenhouse.
10. Pots.

2.5 GUS Assay

1. X-gluc buffer: 100 mM sodium phosphate buffer (NaH_2PO_4 - Na_2HPO_4), pH 7.0, 1 mM X-gluc, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1 % Triton X-100.
2. Methanol.

2.6 PCR

1. DNA isolation kit.
2. Thermocycler.
3. *Taq* DNA polymerase and appropriate buffer.
4. Primers: *gus* sequence primers (5'-TCGCGAAAACGTGTGGAATTGATC-3' and 5'-AGCCGACAGCAGCAGTTTCAT-3'), *hpt* sequence primers (5'-GATGTAGGAGGGCGTGGATATGTC-3' and 5'-CTTCTACACAGCCATCGGTCCAGA-3'), and *nptII* sequence primers (5'-TCGGCTATGACTGGGCACAACAGA-3' and 5'-AAGAAGGCGATAGAAGGCGATGCG-3').
5. Agarose gel and equipment for DNA electrophoresis.

2.7 Southern Blot

1. ³²P-dCTP labeling kit.
2. Primers for *hpt*, *nptII*, or the gene of interest.
3. *EcoRV* and *HindIII* restriction enzymes, with appropriate buffer for double digest.
4. Agarose gel and equipment for DNA electrophoresis.
5. Hybond-N⁺ nylon membrane filters (Amersham, England).
6. 0.2 M HCl.
7. 0.4 M NaOH.
8. Pre-hybridization buffer: 5× saline-sodium citrate (SSC), 5× Denhardt's reagent, 0.5 % sodium dodecyl sulfate (SDS) and 100 µg/ml denatured, fragmented, salmon sperm DNA.
9. Wash buffer A: 1× SSC, 0.1 % SDS. A 20× stock solution of SSC contains 3 M sodium chloride and 0.3 M sodium citrate, adjusted to pH 7.0 with HCl.
10. Wash buffer B: 0.5× SSC, 0.1 % SDS.
11. Shaking incubator.
12. X-ray film and equipment for autoradiography.

3 Methods

3.1 *Agrobacterium* Transformations

1. Transfer the virulence helper plasmid pCH32 to *Agrobacterium* strain LBA4404 by electroporation using an electroporator at 1.8 kV/0.1 cm.
2. Introduce the BIBAC plasmid with a large insert fragment containing the gene(s) of interest to *Agrobacterium* strain LBA4404 harboring pCH32 by electroporation (*see Note 2*).

3.2 Rice Transformation and Regeneration

3.2.1 Callus Induction

1. Dehusk 100–120 mature, fresh rice seeds (sufficient for one transformation).
2. Soak the seeds in 70 % ethyl alcohol for 1 min, then surface-sterilize in 20 % bleach with 0.1 % Tween-20 for 30 min with shaking.

3. Rinse the seeds four to five times with sterile water in a laminar flow hood.
4. Dry the seeds on sterile filter paper.
5. Partially submerge 10–12 seeds on a single plate containing solid N6I medium with the embryo facing up.
6. Seal the plate with Parafilm and place it inside an unlit growth chamber at 26 ± 2 °C for 3–4 weeks. Calli are formed from the scutella after 3–4 weeks in culture.

3.2.2 *Callus Subculture and Pre-cultivation*

1. Dissect out the light yellow, compact, and relatively dry calli and subculture on the same N6I medium for another 2–3 weeks.
2. Select the actively growing, healthy looking, embryogenic calli and inoculate onto pre-cultivation medium N6P for 4 days.

3.2.3 *Agrobacterium Preparation, Infection, and Co-cultivation*

1. Grow *Agrobacterium* strain LBA4404 with pCH32 (harboring the BIBAC plasmid with a large insert fragment) for 2–3 days at 28 °C on solid LB medium supplemented with 25 mg/L rifampicin, 25 mg/L streptomycin, 50 mg/L kanamycin, and 5 mg/L tetracycline.
2. Resuspend agrobacterial cells in 25 ml liquid medium N6A with shaking (150 rpm) at 28 °C for 1.5–2 h to an OD₆₀₀ = 1.0 (*see Note 3*).
3. Immerse the embryonic calli from the 4-day culture in agrobacterial suspension for 15 min with gently shaking (80 rpm).
4. Remove the excess bacteria by decanting the liquid.
5. Transfer the calli onto sterile filter paper for blot-drying, and then place them on co-cultivation medium N6C in the dark at 24 °C for 3 days (*see Note 4*).

3.2.4 *Resting and Selection*

1. Wash the infected calli in sterile water several times, until the water becomes clear.
2. Wash twice (15 min each time) with sterile water containing 400 mg/L of cefotaxime with gently shaking (80 rpm).
3. Dry the calli on filter paper and transfer to resting medium N6R at 26 ± 2 °C for 1 week (*see Note 5*).
4. Transfer the calli to selection medium N6S and sub-culture them every 2 weeks. Keep the cultures in the dark at 26 ± 2 °C for 6–8 weeks until resistant calli proliferate (*see Note 6*).

3.2.5 *Regeneration and Rooting*

1. Move the hygromycin-resistant calli to pre-regeneration medium MSP for 1 week at a temperature of 26 ± 2 °C in a culture room at photoperiodic regime of 16/8 (light/dark) cycle.
2. Culture the growing calli on regeneration medium MSR for 2–4 weeks (*see Note 7*).

3. When the shoots develop into 2–4 cm plantlets, transfer them to magenta boxes containing 30 mL root-growing medium MSG (*see Note 8*).
4. Grow the plantlets under the same conditions stated above for 2–3 weeks.
5. Transfer well-rooted plants to soil in pots and grow them in a greenhouse.

3.3 GUS Assay

T-DNA delivery into calli and plantlets can be confirmed by histochemical assays for GUS.

1. Incubate tissue segments in X-gluc buffer at 37 °C overnight.
2. Wash leaf segments twice in 99 % methanol for 2 h before visual examination.

3.4 PCR Analysis

T-DNA delivery can also be confirmed by PCR amplification of the inserted sequences.

1. Isolate genomic DNA from young leaves of the control (untransformed plant), T0, T1 (from selfed seeds of T0 transformants), and T2 (from selfed seeds of T1 transformants) transgenic rice plants [14], using a DNA isolation kit or standard protocol.
2. Set up a 25 µl PCR reaction for each sample containing 20 ng of genomic DNA, 1 µM each of forward and reverse primer (for *gus*, *nptII*, or *hpt*), 1× PCR buffer, and 0.5 units of *Taq* DNA polymerase.
3. Run the following program for PCR: 94 °C for 5 min for 1 cycle; 94 °C for 50 s, 55 °C for 50 s, 72 °C for 80 s for 34 cycles; 72 °C for 10 min for 1 cycle.
4. Analyze PCR products by gel electrophoresis on 1 % agarose gels. The predicted specific sequences should be 998 bp for *gus*, 722 bp for *nptII*, and 852 bp for *hpt*.

3.5 Southern Analysis

1. To create labeled probe, the PCR-amplified specific fragments of *hpt/nptII* gene and insert genes are labeled with ³²P-dCTP by random priming according to the manufacturer's recommendation (Promega, USA).
2. Digest 5 µg of genomic DNA with 20 U *EcoRV* and 20 U *HindIII* at 37 °C overnight.
3. Run the digest on 1 % agarose gel at 4 V/cm for 16 h.
4. Soak the gel in 0.2 M HCl for 8 min, then in water for 1 min.
5. Transfer the DNA fragments onto a Hybond-N+ nylon membrane filter under alkaline conditions (0.4 M NaOH) for 24 h.
6. Add labeled probe to the pre-hybridization buffer (see above).

7. Hybridize overnight at 65 °C in an incubator with gentle shaking.
8. Wash the membrane at 65 °C, with wash buffer A for 20 min, then with wash buffer B for 15 min.
9. Blot dry, wrap in plastic wrap and autoradiograph for 3–5 days depending on the strength of the hybridization signals.

4 Notes

1. It has been reported that acidic pH during co-cultivation enhances the expression of *vir* genes [12]. Therefore, the same acidic condition (pH 5.6) during pre-cultivation would be beneficial to the transformation of large DNA fragments.
2. Additional *vir* genes as well as the *Agrobacterium* genetic background are very important for the transformation of large DNA fragments in rice. Among the various strains of *Agrobacterium* tested, only the strains with additional pCH32 could successfully produce transformants. A significant enhancement of transformation efficiency was observed when the strain LBA4404 with pCH32 contains additional *vir* genes [15].
3. The concentration of *Agrobacterium* cells is considered to be a critical factor for the efficiency of transformation. Low concentrations can reduce the frequency of T-DNA transfer. With the BIBAC system, a high concentration (OD600 = 1.0–1.2) of *Agrobacterium* cells improves the efficiency of transformation.
4. The addition of acetosyringone (100 µM) during co-cultivation is essential for transformation. Other factors, such as an acidic pH, relatively lower culture temperature (24 °C) and the duration of co-cultivation (2–4 days) were optimized to develop an efficient transformation procedure using the BIBAC system. It was observed that 2–4 days (depending on the bacterial density used for infection) of co-cultivation resulted in the highest GUS activity. Longer co-cultivation periods (>4 days) resulted in an abundant proliferation of bacteria and consequently decreased the regeneration frequency of the selected calli.
5. Excessive bacteria may put serious stress on plant cells and affect their regeneration potential. Calli are often completely colonized by the *Agrobacterium* making elimination of bacteria in subsequent stages more difficult. Controlling *Agrobacterium* overgrowth after co-cultivation is crucial, but prolonged exposure to the antibiotic cefotaxime or timentin, when combined with hygromycin selection, has a detrimental effect to infected calli. To overcome this problem, the co-cultivated calli are first washed with cefotaxime, then placed on

resting medium containing cefotaxime without hygromycin, before being transferred to selective plates. Using this method, *Agrobacterium* growth can be fully restrained without exposing the calli to the combined stresses of *Agrobacterium* and selection agent (hygromycin).

6. The duration of selection is very important. In our experiments, most regenerated shoots obtained after only 2–3 weeks selection were shown not to be transgenic. Therefore, a longer selection time of 6–8 weeks on 50 mg/L hygromycin was needed.
7. In our experience, shoot regeneration was delayed or absent when regeneration medium contained hygromycin. Hence, calli selected by hygromycin for several cycles were regenerated without hygromycin, thereby maintaining a balance between stringency of selection and regeneration efficiency.
8. Transformation efficiency is significantly improved using this procedure, involving selection of calli first on regeneration medium MSR and then on root-growing medium MSG. A low sucrose content of 10 g/L in MSG was found to be optimal for improving selection efficiency and reducing false positives. Sucrose is required for shoot growth, but is not critical once the shoots are fully developed.

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