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Technical Development for Large DNA Fragment Transformation in Plants

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Abstract

For large DNA fragment transformation in dicots and monocots, BIBAC2 vector system was applied to *Arabidopsis thaliana* and *Oryza sativa* L. cv. Jinmi as a model plant, respectively. For *Arabidopsis*, the *Th1* gene in T23L3 BAC clone whose size is about 90 kb was used as the target gene source for transformation. Because T23L3 BAC clone was originally constructed in pBelloBAC11, the target gene was reconstructed into BIBAC2. As the results of reconstruction, 476 colonies were survived in selection medium containing 40 mg/L kanamycin. In colony hybridization analysis, 24 out of 476 colonies exhibited positive signals. In the pulsed-field gel electrophoresis analysis, 11 out of 24 positive clones exhibited the band at the location of 90 kb. In Southern hybridization, positive signal band at the location of 90 kb was observed in all 11 transformants. Using these verified clones, *Agrobacterium*-mediated transformation was applied to *Arabidopsis thaliana th1*-201 mutant for genetic complementation test. Twelve thousands T_1 seeds were harvested, and antibiotic selection test is being analyzed to verify whether these seeds were transformed. For rice, COR356 that contains 150 kb human genomic DNA in a BIBAC2 vector was used as the target gene. As the results of transformation, 151 out of 210 co-cultivated calli were survived in selection medium containing 5 mg/L hygromycin, and 45 out of 151 survived calli were regenerated into plants. Transformation efficiency was 21.6%. Progeny test using T_1 seeds is being analyzed now. These results provide the potential that large DNA fragments can be transferred into both dicots and monocots by *Agrobacterium*-mediated transformation system.

Introduction

The vector system for transferring specific genes into plant genome can provide a strong potential to breed new cultivars, because the transferred genes may change certain genetic characteristics of target plants (Hernalsteens, 1980; Barton and Chilton, 1983; Klee et al., 1985). For this reason, a great deal of efforts has been made to develop a useful transformation vector. With the advent of these vectors, several systems for gene transformation, such as the *Agrobacterium*-mediated transformation (Fraley et al., 1983; Shah et al., 1986; Fishhoff et al., 1987), the particle bombardment-mediated transformation (Klein et al., 1987; Gordon-Kamm et al., 1990; Christou et al., 1991; Songstad et al., 1992) and the microinjection-mediated transformation (Capecchi, 1980; Neuhaus et al. 1987), have been developed. Using these gene transformation systems, single gene transformation system for creating agriculturally important plants has been well developed and documented in dicots (Shah et al., 1986; Fishhoff et al., 1987) and monocots (Chan et al., 1993; Hiei et al., 1994).

In recent, transformation of large DNA fragments into plants has been extremely required in con-

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junction with increasing concerns on plant genomic study. However, conventional systems for gene transformation include a critical limitation that the size of the transferred gene is restricted to less than 10-40 kb (Martom and Brouse, 1991). To overcome this limitation, a variety of vectors have been developed so far (Burke et al., 1987). For instance, based on the BAC (bacterial artificial chromosome) (Willetts, 1987; Shizuya, 1992), BIBAC2 (binary bacterial artificial chromosome) and pBACwich vectors had been developed by Hamilton et al. (1996) and Choi et al. (2000), respectively. Especially, Hamilton et al. (1996) indicated a couple of advantages of BIBAC2; 1) it had the characteristics of Ti plasmid DNA, and 2) it had the capability of replication in both *E. coli* and *A. tumefaciens*.

Using these characteristics, Hamilton et al. (1996) showed that large DNA fragments (150 kb human genomic DNA fragments) could be transferred into tobacco plants. However, in spite of these technical advances, it has been considered that large DNA fragment transformation is hard due to the difficulties in handling large DNA fragments. In monocots, although a few studies on single gene transformation using *Agrobacterium* have been reported in rice (Rhodora and Hodges, 1996; Zhang et al., 1997; Lee et al., 1998), there has been no report on large DNA fragment transformation using *Agrobacterium* so far.

Thus to establish large DNA transformation system, the present study had focused on the technical aspects for large DNA fragment transformation in dicots and monocots. Here we report that large DNA fragment transformation may be used as a effective tool for studying functional genomic, or transformation of genes related to the quantitative traits in plants.

Procedure of *Agrobacterium* -mediated large DNA transformation in dicots

Large DNA sources and preparation for transformation

For the dicots, *Arabidopsis thaliana* (Lendsberg) was used as a model plant. The *th1*-201 mutant lack of thiamin biosynthesis (Komida et al., 1988) was used as a target plant for large DNA transformation. Target gene, *Th1* (thiamin monophosphate pyrophosphorylase), was originated from *Arabidopsis* T 23L3 BAC clone which size was about 90 kb (Kim et al., 1998). Because T23L3 clone was originally constructed in pBelloBAC11 (Choi et al., 1995), target gene used for transformation in the present study was reconstructed into BIBAC2. To prepare large DNA sources, the T23L3 BAC clone was grown onto solid LB medium containing 12.5 mg/L chloramphenicol for overnight at 37℃. A single colony was inoculated into 2 liter culture flasks containing 1 liter of liquid TB medium containing 12.5 mg/L chloramphenicol. The inoculum was grown for 18 hrs at 37℃ with shaking (200 rpm) until cell density reached to 1.0-1.5 at OD_{600} Cells were harvested by centrifugation at 4℃ for 15 mins at 4,000 rpm (Supra 22K, Hanil Science Industrial Co., Ltd., Korea).

Plasmid DNA was isolated by alkaline lysis method (modified by Birnboim and Doly, 1979). After isopropanol precipitation and resuspension in $T_{10}E_1$ (10 mM Tris, 1 mM EDTA, and pH 8.0), plasmid DNA was further purified by cesium chloride density gradient centrifugation with ethidium bromide in the Beckman NVT90.1 rotor at 78,000 rpm for 5 hrs at 20℃. Plasmid band was isolated, and then ethidium bromide was removed by several extractions with equal volume of saturated butanol (Sigma, U.S.A.). DNA sample was diluted with two volume of the distilled water, precipitated with the absolute ethanol, washed with 70% ethanol, and resuspended in 100 μL of $T_{10}E_1$. Total 5 μg of DNA were initially digested with 50 units of *Not*I (New England Biolab. Inc., U.S.A), corresponding buffer, 4 mM spermidine and 10 × BSA for an hour at 37℃. After the initial enzymatic reaction, secondary enzymatic treatment was conducted with 25 units of *Not*I for 4 hrs. Digested plasmid DNA was separated by 1% low melting point agarose gel using the pulsed-field gel electrophoresis (CHEF-DR Ⅲ, Bio-rad, U. S.A.) under the conditions of 4.0 V/cm, 4s pulse, 1 x TAE buffer for 4 hrs at 14℃ (modified by Chu et al., 1986). Then, DNA fragment was separated from the gel. The slice was melted in a water bath for 5 mins at 65℃, and digested with GELase (Epicentre, Madison, WI, U.S.A) for an hour at 45℃ in a water bath. It was frozen in aliquots, and stored at -20℃.

Reconstruction of th1 gene into BIBAC2 vector

BIBAC2 provided from the Cornell University (Ithaca, NY, U.S.A.) was grown on solid LB medium containing 40 mg/L kanamycin and 10% sucrose for overnight at 37℃. Subsequent procedures for large DNA preparation were same as mentioned earlier. Total 6 μg of BIBAC2 vector DNA were digested with 30 units of *Not*I, corresponding buffer, 4 mM spermidine and 10 × BSA for an hour at 37℃. After initial enzymatic reaction, secondary enzymatic treatment was conducted with 20 units of *Not*I for 2 hrs, and then inactivated for 15 mins at 65℃. To prevent self-ligation of the vector, digested BIBAC2 plasmid DNA was dephosphorylated by five units

of SAP (Shrimp Alkaline Phosphotase, Behringer Mannheim, Germany) for an hour at 37℃, and then inactivated for 20 mins at 65℃. Because BIBAC2 has two *Not*I sites in cloning site, two DNA fragments, small and large size, are produced after restriction enzyme treatment. Thus, to remove small DNA fragments, BIBAC2 digested with *Not*I was separated by 1% low melting point agarose gel in TAE buffer at 60V for 3 hrs. The selected DNA fragments were cut out from the gel. The slice was melted in a water bath for 5 mins at 65℃, and treated with GELase for an hour at 45℃ in a water bath. Prepared *Th1* DNA fragments were ligated to the vector digested with *Not*I by T_4 DNA ligase (Promega, WI, U.S.A.). The flow chart for reconstruction of *Th1* gene from pBelloBAC to BIBAC2 in *E. coli* is illustrated in Figure 1.

Preparation of competent cells

E. coli DH10B was provided from the Caltech (CA, U.S.A). Its genotype is F⁻ *mcr*A △(*mrr-hsd*RMS-*mcr*BC) ϕ80d *lacZ*△M15△*lacX74* *deo*R *rec*A1 *end*A1 *ara*D139 △(*ara, leu*)7697 *gal*U *gal*Kλ- *rps*L *nup*G (Hanahan et al., 1991). This strain was cultured for overnight in SOB medium (Table 1) without magnesium, incubated with shaking (200 rpm) at 37℃.

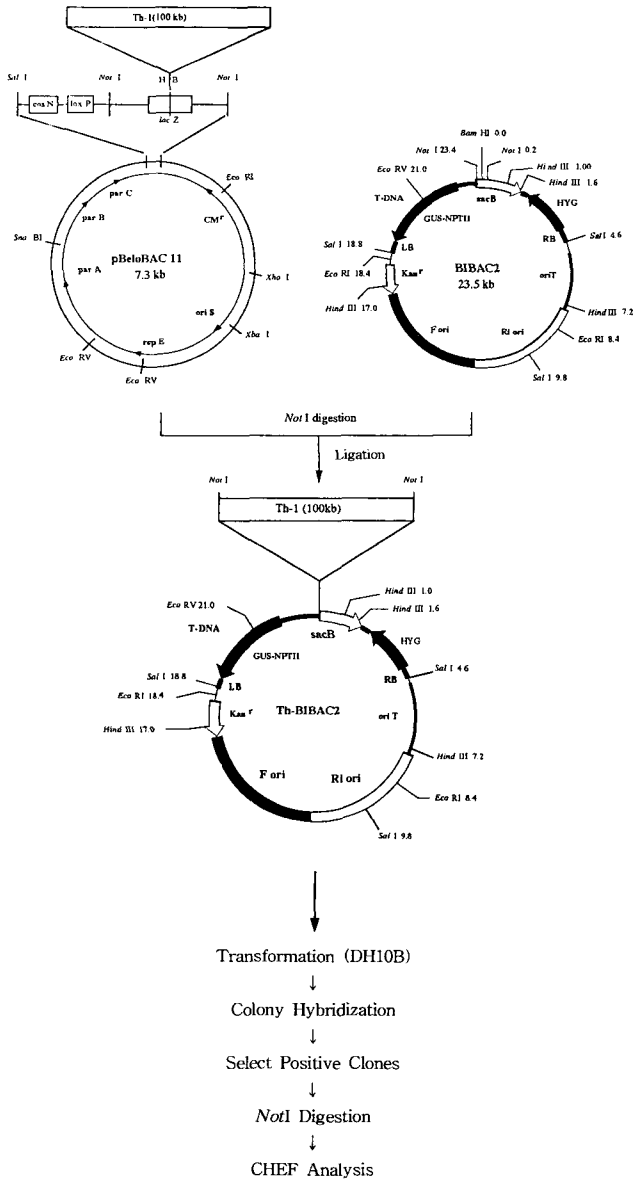

Figure 1. Flow diagram of the strategy for reconstruction of *Th1* gene from pBelloBAC to BIBAC2 vector.

Table 1. Media used for tissue culture and transformation.

Media	Composition
N6 callus induction	N6 salts and vitamins, 30 g/L sucrose, 2 mg/L 2,4-D, 8 g/L Agar, and pH 5.8
AB	3 g/L K_2HPO_4, 1 g/L NaH_2PO_4, 1 g/L, NH_4Cl, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.15 g/L, KCl, 0.01 g/L $CaCl_2$, 2.5 mg/L $FeSO_4 \cdot 7H_2O$, 5 g/L glucose, 15 g/L agar, and pH 7.2
N6 co-culture	N6 salts and vitamins, 30 g/L sucrose, 10 g/L glucose, 2 mg/L 2,4-D, 30 mg/L acetosyringone, and pH 5.2
N6 selection	N6 salts and vitamins, 30 g/L sucrose, 2 mg/L 2,4-D, 8 g/L agar, 500 mg/L carbenicillin, 5 mg/L hygromycin, and pH 5.8
AA suspension	AA salts and amino acids, B5 vitamin, 20 g/L sucrose, 2 mg/L 2,4-D, 0.2 mg/L kinetin, 30 g/L acetosyringone, and pH 5.8
Shoot regeneration	MS salts and vitamins, 30 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, 1 mg/L NAA, 3 g/L Bacto yeast extract, 2 mg/L BAP, 500 mg/L carbenicillin, 5 mg/L hygromycine, and pH 5.8
MS hormone free for root induction	MS salts and vitamins, 30 g/L sucrose, 5 mg/L hygromycin, and H 5.8
SOB	2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 nM glucose, and pH 7.0
SOC	2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 nM glucose, 20 mM glucose, and pH 7.0
YEP	10 g/L Bacto peptone, 10 g/L Bacto yeast extract, 5 g/L NaCl, 15 g/L agar, and pH 7.2

Small amounts of overnight cultured cells were transferred into fresh SOB medium, subcultured with vigorous shaking until OD_{550} reached to 0.7, and then harvested by centrifugation at 4℃ for 15 mins at 4,000 rpm. The pellet was resuspended by the equal volume of initial culture volume with 10% glycerol, and centrifuged under the same condition. This step was repeated twice. The supernatant was discarded. Then harvested cells were resuspended, dispensed in aliquots, and stored at -70℃.

Reconstruction of Th1 gene from pBelloBAC to BIBAC2 in E. coli

To dialyze out ligase buffer, ligation mixture was transferred onto 0.025 μm Millipore filter (Millipore Co., MA, U.S.A.) that was placed in a petri-dish containing 25 mL $T_{10}E_1$ at room temperature. Thirty μL DH10B competence cells and 1-2 μL ligates were used for transformation. Ligated DNA was transformed into *E. coli* DH10B competent cells by electroporator (Invitrogen, USA). Electroporation conditions were examined by various condition of voltages (11, 12, 13, 14, 15 and 16 kV/cm) and cuvette sizes (0.1 and 0.2 cm mouth) under the basal conditions of capacity 50 μF, impedance 150 Ω and 1800 V_{max}. From the preliminary test, 11 kV/cm voltage and 0.1 cm cuvette size exhibited the best transformation result (data not shown). After electroporation, cells were incubated in 0.4 mL SOC medium (Table 1) with shaking (200 rpm) for one hour at 37℃, and spread on LB medium containing 40 μg/mL kanamycin and 10% sucrose. As the results, 476 colonies were selected on LB medium containing 40 μg/mL kanamycin and 10% sucrose. To confirm whether the selected colonies had the correct size of the inserted gene, three analyses, such as the colony hybridization, the pulsed-field gel electrophoresis, and Southern hybridization, were conducted.

The analysis for transformant selection

After colony was inoculated onto Hybond-N^+ membranes (Amersham) and incubated for overnight at 37℃, colony hybridization was performed by ECL™ direct nucleic acid labeling and detection systems (Amersham). Hybridization temperature was 42℃. The filter was washed once for 20 mins at 42℃ by washing buffer (6 M Urea, 0.4% SDS, and 0.5 × SSC), and then washed twice for 5 mins at room temperature by 5 × SSC buffer. About 10 kb fragment of *Th1* gene digested by *Hin*dIII was used as probes for colony hybridization. Total 24 out of 476 colonies exhibited positive signals in this analysis (Figure 2). From these results, we assumed that most of the selected colonies contained partial sequences that did not match to the 10 kb probe sequence.

To verify whether 24 colonies contain the full length of the inserted DNA (90 kb), pulsed-field gel electrophoresis analysis was conducted. After plasmid DNA isolation from 24 candidate clones, plasmid DNA was digested with *Not*I. Then, pulsed-field gel electrophoresis analysis was performed for 16 hrs on 1% Pulsed Field Certified Agarose gel under the conditions of 6 V/cm, initial switch time 50s, final switch time 90s, temperature 14℃, 120 degree angle, and 1 × TBE buffer. From this analysis, 11 out of 24 positive clones exhibited the band at the location of 90 kb (Figure 3A). From these results, we confirmed that the clones included the full length DNA of *Th1* gene. Finally, Southern hybridization by ECL™ system was performed to examine whether the gene located at 90 kb was *Th1* gene. DNA in the gel was transferred onto Hybond-N^+ membrane, and analyzed by the same probes used in colony hybridization. In this analysis, positive signal bands at the location of 90 kb were observed in all 11 clones (Figure 3B). From these results, we concluded that *Th1* gene was successfully reconstructed from pBelloBAC into BIBAC2 vector. The *Th1*-BIBAC2 recombinant plasmid was used for the next study.

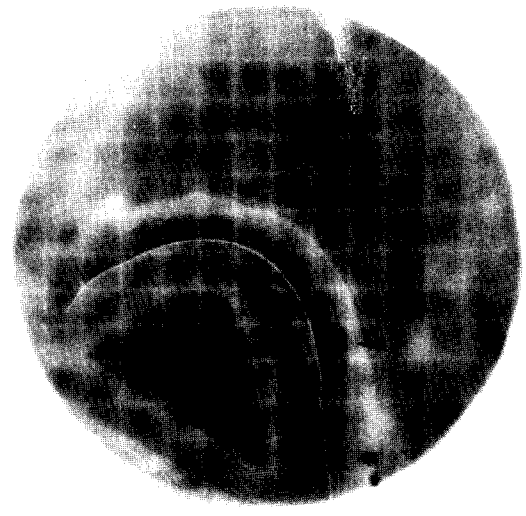

Figure 2. Colony hybridization for *Th1*-BIBAC2 recombinants selection. After colony was inoculated onto Hybond-N^+ membranes (Amersham) and incubated for overnight at 37℃, colony hybridization was performed by ECL™ direct nucleic acid labeling and detection systems. About 10 kb fragment of *Th1* gene digested by *Hin*dIII was used as probes. Total 24 out of 476 colonies exhibited positive signals. The arrow represents the detected positive signal patterns of nucleotide sequence homologous to the putative transforming clone of *Th1* gene.

Transformation of BIBAC2 containing Th1 gene from E. coli to Agrobacterium

Agrobacterium strain COR308 was provided from the Cornell University (Ithaca, NY, U.S.A.). Its genotype is UIA143, pMP90 and pCH32. UIA143 represents a *rec*A-deficient derivative of *A. tumefaciens* strain C58. The helper plasmid pCH32 carries *vir*E1 and *vir*E2 from *A. tumefaciens* strain A6. The conditions for *Agrobacterium* transformation were same as mentioned earlier in the reconstruction of *Th 1* gene from pBelloBAC to BIBAC2 in E. coli. For testing the stability of BIBAC2 vectors in *Agrobacterium*, DNA from *Agrobacterium* was isolated as the same methods mentioned in *E. coli* plasmid mini-preparation. Figure 4 shows that all *Th1*-BIBAC2 clones in *Agrobacterium* contain intact *Th1* gene because the band has been observed at the location of 90 kb.

Agrobacterium-mediated transformation by vacuum infiltration

Based on the hypothesis that if *Th1* gene in BIBAC2 vector is transferred successively into mutant plants, mutant plants will be recovered to wild type by genetic complementation. Genetic complementation test for the *th1*-201 mutants was conducted. The *th1*-201 mutant seeds were provided from AIMS (*Arabidopsis* Information Management System, *Arabidopsis* Biological Resource Center, OH, U.S.A.). The *th1*-201 mutant plants were grown in the growth chamber under 16 hrs light condition at 21℃, and treated with 1 mM thiamin for maintaining normal growth. When primary inflorescence reached to 5 to 10 cm, transformation of COR308 containing *Th1*-BIBAC2 plasmid into plants was conducted by the vacuum infiltration method as mentioned by Bechtold et al. (1993). Briefly, small scale *Agrobacterium* cultures were grown in liquid YEP medium with 40 mg/L kanamycin for 2 days at 28℃. Then, 6 mL of the small-scale cultures were inoculated into 3 liter YEP medium for overnight until OD_{600} reached to 0.8. Cells were then harvested by centrifugation at 5,000 rpm for 15 mins, and gently resuspended with 1/3 of the initial volume of the infiltration medium for vacuum infiltration. Infiltration medium was consisted of 1x MS salts, 1x B5 vitamins (Gamborg et al., 1968), 0.5 g/L 2-(N-morpholono) ethanesulfonic acid (MES), 5% sucrose, 0.44 mM benzylaminopurine (BAP), and 0.02% Silwet L-77 (Osi Specialties, Inc., NY, U.S.A.) at pH 5.7. Pots were inverted and placed inside the vacuum desiccator that had a container filled with *Agrobacterium* suspension. Vacuum pressure was applied for 10 mins, and then released quickly. After that, pots were transferred into the growth chamber, placed them on the side, and covered with clear plastic bags. Plastic bag was removed one day later. Then, pots were returned to upright position. Seeds were harvested 5-6 weeks later.

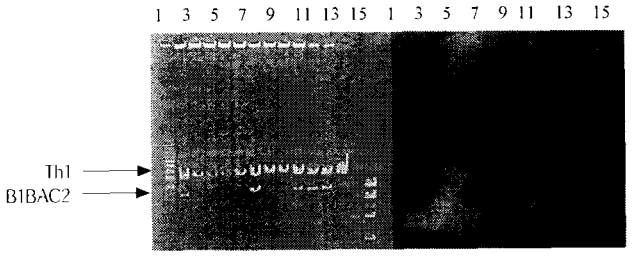

Figure 3. Pulsed-field gel electrophoresis and Southern hybridization patterns of the reconstructed *Th1*-BIBAC2 recombinants. (A) Ethidium bromide-stained DNA analyzed by Pulsed-field gel electrophoresis. After plasmid DNA isolation from 24 candidate clones, *Not*I digested DNA was analyzed by Pulsed-field gel electrophoresis for 16 hrs on 1% Pulsed Field Certified Agarose gel under the conditions of 6 V/cm, initial switch time 50s, final switch time 90s, temperature 14℃, 120 degree angle, and 1 x TBE buffer. (B) Southern hybridization by ECL™ system. DNA in the gel was transferred onto Hybond-N⁺ membrane. About 10 kb fragment of *Th1* gene digested with *Hin*dIII was used as probes. Lane 1; λconcatemer, lanes 2-12; transformants, lane 13; Th1 gene, lane 14; *Th1* gene fragments digested with *Hin*dIII, lane 15; double digested λDNA by *Hin*dIII and PstI as a size marker.

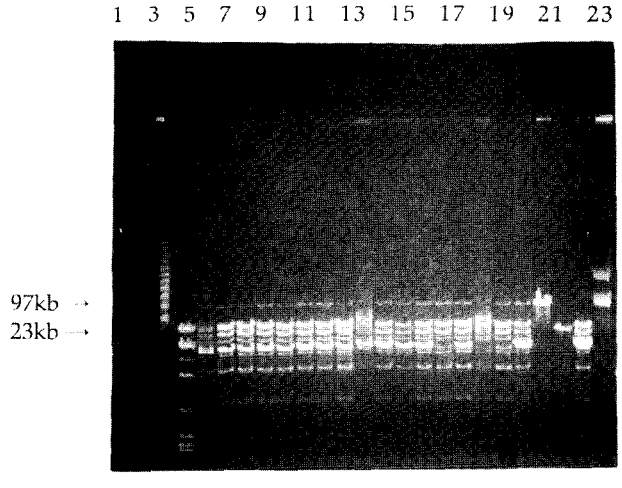

Figure 4. Pulse-field gel electrophoresis pattern of transformed *Th1*-BIBAC2 DNA in *Agrobacterium*. After plasmid DNA was isolated from 17 clones picked randomly. DNA was digested by *Not*I. Pulse-field gel electrophoresis analysis was performed for 16 hrs on 1% Pulsed Field Certified Agarose gel under the same conditions mentioned in Figure 3. Lane 1; λconcatemer, lane 2; double digested λ DNA by *Hin*dIII and *Pst*I as a size marker, lanes 3-19; transformants digested by *Not*I, lane 20; intact *Th1* gene fragments, lane 21; BIBAC2, lane 22; COR308 digested by *Not*I, and lane 23; COR308.

Germination assay of transgenic seeds by antibiotic and phenotypic selection

T_1 seeds were selected by *in vitro* germination test on thiamin-free MS medium containing 0.5 g/L MES, 1% sucrose, 10 mg/L hygromycin, 0.7% agar and pH 5.7. Until now, 12,000 seeds were obtained. Germination test is being conducted on selection medium (1x MS salts, 1x B5 vitamins, 0.5 g/L MES, 1% sucrose, at pH 5.7) containing 10 mg/L hygromycin.

Agrobacterium-mediated large DNA transformation in monocots.

In monocots, conventional gene transformation system using *Agrobacterium* has been well developed and documented in rice (Rhodora and Hodges, 1996; Zhang et al., 1997; Lee et al., 1998). However, large DNA transformation system using *Agrobacterium* has not been reported yet. Thus to establish large DNA transformation system for the monocots, japonica type rice cultivar Jinmi was used as a model plant. Target gene source originated from BIBAC2 containing 150 kb human genomic DNA was provided from Dr. Hamilton at the Cornell University (Ithaca, NY, U.S.A.). *Agrobacterium tumefaciens*, COR356 (UIA 143, pMP90, pCH32 and BIBAC2::H150), was used as bacterial sources for large DNA transformation into rice (*Oryza sativa* L.).

Callus induction

For large DNA transformation into rice, calli derived from scutellum were used for starting materials as mentioned by Hiei et al. (1994). Matured seeds were dehusked, sterilized with 70% ethanol for 1 min, and then double sterilized with 4 % sodium hypochlorite containing Tween 20 (200 μL/mL) for 20 mins on the reciprocal shaker (Vision Scientific Co., Korea). Seeds were washed 3 times with sterilized deionized water, and then placed on N6 callus induction medium for 3 weeks at 25℃ under 3,000 Lux light condition (Chu et al., 1975). After callus induction (Figure 6A), calli turned brown color were removed, and fresh yellowish calli were pre-cultured for 3 days at dark condition in the same supplements before large DNA transformation.

Agrobacterium inoculation

Agrobacterium strain COR356, which contains the 150 kb human genomic DNA in BIBAC2, was used for a bacterial source (Figure 5). COR356 was prepared for 3 days on solid AB medium (Chilton et al., 1974) containing 50 mg/L hygromycin, 50 mg/L kanamycin and 5 mg/L tetracycline at 28℃. This strain propagated on solid AB medium was suspended in liquid AA medium (Toriyama and Hinata, 1985) containing 30 mg/L acetosyringone. After final cell concentration was adjusted to 10^7 cell/mL, calli were completely soaked into COR356 suspension for 3-5 mins, and then excess solution was removed from

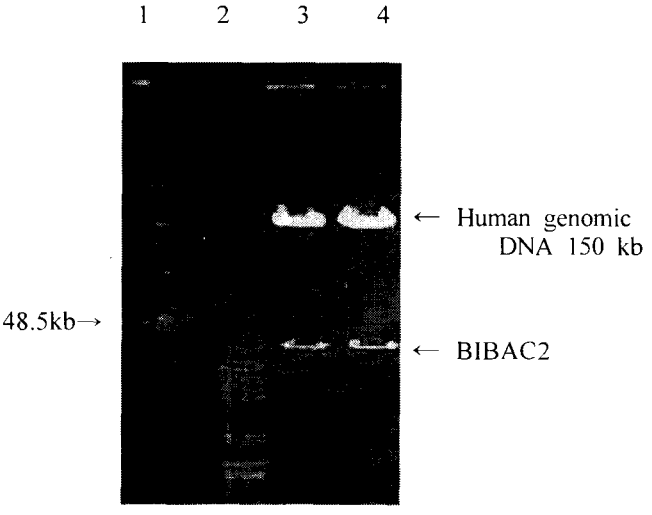

Figure 5. Digested pattern of 150 kb human genomic DNA in BIBAC2 by restriction enzyme. BIBAC2:: H150 DNA in *Agrobacterium* strain COR356 was digested by *Not*I. Pulse-field gel electrophoresis was conducted under the same conditions mentioned in Figure 3. Lane 1; λconcatemer, lane 2; double digested λDNA by *Hin*dIII and *Pst*I as a size marker, lanes 3 and 4 represent BIBAC2 and 150 kb human genomic DNA in E. coli., respectively.

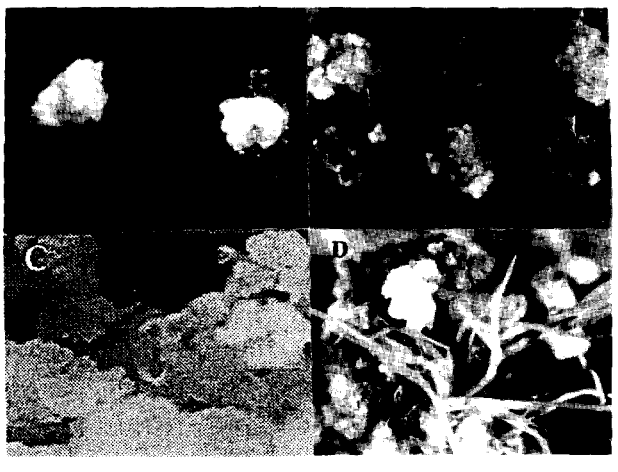

Figure 6. Developmental stages for inducing transgenic plants. (A) Three-week-old calli co-cultivated with COR356. (B) Selection of resistant calli on medium containing 5 mg/L hygromycin; (a) brown calli represent dying cells, and (b) yellow calli represent regenerating cells. (C) Green spot calli appeared in regenerating medium after 3 weeks of the culture. (D) Shoot formation after 5 weeks of the culture in regenerating medium.

Table 2. Transformation frequency using *Agrobacterium* COR356 in rice.

Experiment repeat	Number of calli derived from scutellum			
	Co-cultivated calli (A)	Produced [a]Hyg^R calli	Produced Hyg^R plants (B)	T.F. (%) (B/A × 100)
1	120	84	26	21.6
2	90	67	19	21.1

[a]Hyg^R : Hygromycin resistant

calli on the sterilized filter paper for a few seconds. Soaked calli were transferred onto a piece of filter paper which was covered on N6 co-culture medium (10 calli/petri dish), and co-cultured under dark condition for 3 days at 28℃. After co-culture, calli were washed three times with the sterilized deionized water containing 500 mg/L carbenicillin to suppress the growth of *Agrobacterium*. Washed calli were transferred onto N6 selection medium, and cultured under 3,000 Lux light condition at 25℃. After 30 days of the culture, survived calli were selected for regeneration. Hygromycin resistant cells were proliferated in medium containing 5 mg/L hygromycin after 30 days of the culture. Figure 6B shows that fresh calli are coming out from the transferred calli. The growth of most calli was delayed, and they turned brown color on N6 selection medium at the beginning of the culture. Two weeks later, the growth of calli was rapidly activated as soon as white calli started to appear from brown calli.

Regeneration process and antibiotic selection of rice transformants.

For shoot induction, selected calli were transferred onto shoot induction medium (Table 1) supplemented with 2 mg/L BAP and 5 mg/L hygromycin. Hygromycin resistant calli began to exhibit green spots after 3 weeks of the culture (Figure 6C). Shoots were formed after 6 weeks of the culture (Figure 6D). When shoot length reached to about 3 cm, regenerated shoots were transferred onto MS hormone-free root induction medium containing 5 mg/L hygromycin. The ratio of root induction was over 90%. After 10 weeks of the culture, hardening process was conducted to transgenic plants, and grown to maturity in pots (Figure 7). From these experimental protocols, 151 from 210 co-cultivated calli were survived in medium containing 5 mg/L hygromycin. Finally, 45 out of survived 151 calli were regenerated into plants. Transformation efficiency was 21.6%. Interestingly, this is comparable to the result of Lee et al. (1998) that showed 28-38% transformation frequency in herbicide resistant gene transformation in rice. Probably, it is thought that this low transformation efficiency observed in the present study may be related to the size of DNA transferred. Now, progeny analysis using T_1 seeds is being investigated to confirm whether large DNA fragments are correctly transformed into rice.

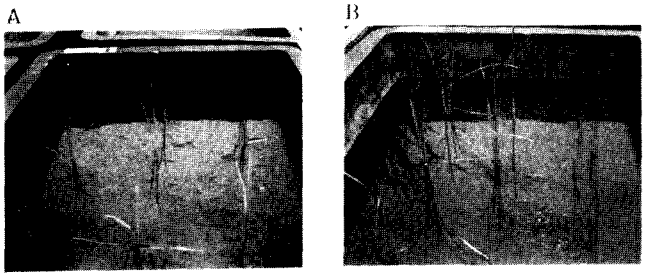

Figure 7. Transformed rice in the middle of hardening process. (A) transformed rice with COR314 containing only BIBAC2 vector, and (B) transformed rice with COR356 containing BIBAC2 vector and 150 kb human genomic DNA.

Conclusion

With increasing concerns on the functional genomic study, large DNA fragment transformation system has been highlighted in plants. Thus, in the present study, large DNA fragment transformation using BIBAC2 vector was attempted to *Arabidopsis* and rice as model plants. Taken all the results together, it is thought that large DNA fragment transformation technique can be applied to introduce agriculturally important gene clusters related to quantitative traits into plants. And it also can be used as a useful tool for studying functional genomics, or genetic dissection. The results of progeny analysis on *Arabidopsis* and rice transformants will be reported in the near future.

Acknowledgments

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References

Barton KA, Chilton MD (1983) *Agrobacterium* Ti plasmid as vectors for plant genetic engineering. Methods Enzymol 101: 527-539.

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci Paris Sci La Vie/Life Sci 316: 1194-1199.

Birnboim HC, Doly J (1979) A rapid alkaline procedure for screening recombinant plasmid DNA. Nucleic Acid Res 7: 1513-1523.

Burke DT, Carle GF, Olson MY (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 236: 806-812.

Capecchi M (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell 22: 479-488.

Chan MT, Chang HH, Ho SL, Tong WF, Yu SM (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/-glucuronidase gene. Plant Mol Biol 22: 491-506.

Chilton MD, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc Natl Acad Sci USA 71: 3672-3676.

Choi SD, Creelman RA, Mullet JE, Wing RA (1995) Construction and characterization of a bacterial artificial chromosome library from *Arabidopsis thaliana*. Weeds World 2: 17-20, and Plant Mol Biol Rep 13: 124-128.

Choi SD, Begum D, Koshinsky H, Ow DW, Wing RA (2000) A new approach for the identification and cloning of genes: the pBACwich system using Cre/lox site-specific recombination Nucleic Acids Research 28(7): e19.

Chu CC, Wang CS, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Scientia Sinica 18: 659-668.

Chu G, Vollrath D, Davis RW (1986) Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science 234: 1582-1585.

Cristou P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electronic discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technol 9: 957-962.

Fischhoff DA, Bowdish KS, Perlak FJ, Marrone PG, McCormick SM, Niedermeyer JG, Dean DA, Kusano-Kretzmer K, Mayer EJ, Rochester DE, Rogers SG, Fraley RT (1987) Insect tolerant transgenic tomato plants. Bio/Technology 5: 807-813.

Fraley RT, Rogers SC, Horsch RB, Sanders PR, Flick JS, Fink C, Hoffman N, Sanders P (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci 80: 4803-4807.

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158.

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, OBrien JV, Chambers SA, Adams Jr WR, Willetts NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 23: 603-618.

Hamilton CM, Frary A, Lewis C, Tanksley SD (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. Proc Natl Acad Sci USA 93: 9975-9979.

Hanahan D (1983) Studies on Transformation of *Escherichia coli* with plasmid. J Mol Biol 166: 557-580.

Hernalsteens JP (1980) The *Agrobacterium tumefaciens* Ti plasmid as a host vector system for inducing foreign DNA in plant cells. Nature 287: 654-657.

Hiei Y, Ohia S, Toshihiro K, Komari T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6: 271-282.

Kim YS, Nosaka K, Downs DM, Kwak JM, Park DH, Chung IK, Nam HG (1998) A *Brassica* cDNA clone encoding a bifunctional hydroxymethylpyrimidine kinase/thiamin-phosphate pyrophosphorylase involved in thiamin biosynthesis. Plant Molecular Biology 37: 955-966.

Klee HJ, Yanofsky MF, Nester EW (1985) Vectors for transformation of higher plants. Bio/Technology 3: 634-642.

Klein TM, Wolt ED, Wu R, Sanford JC (1987) High-velocity microprojectile for delivering nucleic acids into living cells. Nature 327: 70-73.

Komeda Y, Tanaka M, Nishimune T (1988) A *th1* mutant of *Arabidopsis thaliana* is defective for a thiamin-phosphate-synthesizing enzyme: Thiamin Phosphate Pyrophosphorylase. Plant Physiol 88: 248-250.

Lee HY, Lee CH, Kim HI, Han WD, Choi JE, Kim JH, Lim YP (1998) Development of bialaphos-resistant transgenic rice using *Agrobacterium tumefaciens*. Kor J Plant Tissue Culture 25: 283-288.

Marton L, Browse J (1991) Facile transformation of *Arabidopsis*. Plant Cell Reports 10: 235-239.

Neuhaus G, Spangeberg G, Mittelsten-Scheid O, Schweizer HG (1987) Transgenic rape seed plants obtained by the microinjection of DNA into microspore derived embryoids. Theor Appl Genet 75: 30-36.

Rhodora RA, Thomas KH (1996) *Agrobacterium*-mediated transformation of *japonica* and *indica* rice varieties. Planta 199: 612-617.

Shah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA, Tumer NE, Hironaka CM, Sanders PR, Gasser CS, Aykent S, Siegel NR, Rogers SG, Fraley RT (1986) Engineering Herbicide Tolerance in Transgenic Plants. Science 233: 478-481.

Songstaad DD, Lowe KL, Betz SR , Cabrera-Ponce JL (1992) Callus cultures as alternative target tissues in microprojectile-mediated transformation of maize. Agronomy Abstract 198.

Shizuya H, Birren B, Kim UJ, Mancino V, Slepak T, Tachiiri Y, Simon M (1992) Cloning and stable maintenance of 300-kilobase-pair fragment of human DNA in *E. coli* using an F-factor-based vector. Proc Natl Acad Sci USA 89: 8794-8797.

Toriyama K, Hinata K (1985) Cell suspension and protoplast culture in rice. Plant Sci 41: 179-183.

Willetts N, Skurray R (1987) Structure and function of the F factor and mechanism of conjugation. *Escherichia coli* and *Salmonela typhimurium:* Cellular and molecular biology (ed., Neidhart et al.). American Society for Microbiology, Washington, D. C. 2: 1110-1133.

Zhang J, Xu RJ, Elliott MC, Chen DF (1997) *Agrobacterium tumefaciens*-mediated transformation of elite indica and japonica rice cultivars. Molecular Biotechnology 8: 223-231.