J Plant Res (2004) 117:95–99
Digital Object Identifier (DOI) 10.1007/s10265-003-0141-3

ORIGINAL ARTICLE

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Obtaining transgenic plants using the bio-active beads method

Received: September 17, 2003 / Accepted: November 4, 2003 / Published online: February 13, 2004

Abstract Several methods of transformation are currently available for delivering exogenous DNA into animal and plant cells. In this study, a novel and efficient transformation system for DNA delivery/expression with a capacity to transport DNA of high molecular weight was developed. This system can overcome the shortcomings of traditional transformation methods such as *Agrobacterium*-mediated transformation, particle bombardment, and the electroporation method. The method developed in this study uses calcium alginate micro beads to immobilize DNA molecules in combination with polyethylene glycol treatment. In addition, it is simple and low-cost, and requires limited equipment. Using this method, we have successfully transformed tobacco plants, screening by kanamycin resistance. The transformed genes in the transformants were confirmed by PCR and Southern hybridization.

Key words Bio-active beads · Plant regeneration · Polyethylene glycol treatment · Transformation

Introduction

The genetic transformation of plants is a crucial step in gene manipulation. Several transformation methods are currently available for delivering exogenous DNA into plant cells (Rakoczy-Trojanowaka 2002); *Agrobacterium*-mediated transformation, particle bombardment, and electroporation are routinely used, for example. Some alternative systems such as infiltration (Chung et al. 2002) and silicon carbide-mediated transformation (Kaeppler et al. 1990) are also frequently used. However, each of these systems has certain disadvantages, for example, low transformation efficiency, plant species limitations, difficulties in the acquisition of regenerated plants, and unprofitable results because of the presence of multiple copies of introduced genes. Thus, there was a need for the development of a new transformation method for plant genetic manipulation research.

In this study, we developed an efficient gene-delivery system for plants using calcium alginate bio-active beads to immobilize high-density DNA molecules, in combination with polyethylene glycol (PEG) treatment. Alginate is a hydrophilic polysaccharide that gels in the presence of Ca^{2+} ions. Since calcium alginate is harmless to both animal and plant cells, it has been used to immobilize bacteria in bioreactors and to encapsulate plant somatic embryos as artificial seeds (Kersulec et al. 1993). The mean size and variation of particle size can be regulated by carefully controlling reaction time and concentrations of alginate (Sone et al. 2002).

The efficiency of transient expression with bio-active beads was reported as being 5- to 10-fold higher than PEG treatment with naked plasmids (Sone et al. 2002). Using this method, chromosomal DNA of up to 450 kb has been successfully transformed into yeast cells (Mizukami et al. 2003). In this paper, the regeneration of transformed tobacco plants using the bio-active beads system is reported.

Materials and methods

Plasmid construction

Two different plasmids were used in this study: the pUC18-sGFP (4.1-kb) plasmid containing a cauliflower mosaic virus 35S promoter, green fluorescent protein (*GFP*) coding sequence, and nopaline synthase 3 terminal was used for observation of transient expression to confirm the transformation procedure; and the pTN80 (5-kb) plasmid with a Pm35S promoter (a modified cauliflower mosaic virus 35S

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promoter), neomycin phosphotransferase II (*NPTII*) coding sequence, and the terminating cauliflower mosaic virus polyadenylation signal was used for selection by kanamycin resistance to produce transgenic plants.

Preparation of bio-active beads for immobilizing plasmid DNA

The procedure for bio-active bead production has been described previously (Sone et al. 2002). Firstly, 50 µl plasmid DNA solution (1 µg/µl) was mixed with 450 µl of 100 mM $CaCl_2$ solution. Next, to form a water/oil emulsion, 900 µl isoamyl alcohol was added to a 1.5 ml micro-tube containing an aqueous phase of 100 µl of 0.5% sodium alginate solution. An ultrasonic disrupter (UR-20P; Tomy Seiko, Tokyo, Japan) was used for emulsification. After emulsification for 10 s, 500 µl $CaCl_2$ solution containing plasmid DNA was added immediately. The solution was then mixed using a micro-tube mixer (CST-040; Asahi Technoglass, Tokyo, Japan) for 10 s at the maximum speed of 3,000 rpm. To harvest the produced bio-active beads, the micro-tube was centrifuged at 5,000 rpm for 3 min. Bio-active beads made from low concentrations of alginate cannot be precipitated completely by centrifugation; they persist around the interface of the organic and aqueous phases. The upper isoamyl alcohol phase was removed, taking care not to remove the bio-active beads located around the interface. After adding 100 mM $CaCl_2$, the solution was mixed using the micro-tube mixer until the precipitated bio-active beads were re-suspended. Centrifugation was conducted at 5,000 rpm for 3 min to eliminate the isoamyl alcohol completely. This washing step was repeated at least twice, and the final volume was adjusted to 50 µl.

Co-transformation is a simple alternation method for generating transgenic plants carrying active genes. In this study, two types of plasmid DNA were used for co-transformation. The different DNA amount immobilized in the beads had been checked before the co-transformation. The pTN80 plasmid DNA and pUC18-sGFP DNA solution was mixed with $CaCl_2$ solution for preparation of bio-active beads.

Plant material and culture conditions

Tobacco SR-1 (*Nicotiana tabacum* L. including the sterol reductase 1 gene) shoots were cultured on a Murashige and Skoog (MS) medium containing 1% agar. The leaves were excised and soaked in a culture medium with an enzyme mixture (0.25% cellulase [Onozuka R-10], 0.1% macerozyme R-10, 0.5 M mannitol, 2.5 mM 2-[morpholino]ethane sulfonic acid [MES] [pH 5.7] and 0.5×K3 medium) for at least 16 h in the dark. The protoplast suspension was passed through a nylon mesh with a pore size of 100 µm to remove any large debris. To stimulate protoplast precipitation, the protoplast suspension was mixed with a equivalent volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, pH 5.6). The protoplasts were then harvested by centrifugation at 800 rpm for 5 min, using round-bottom glass centrifugation tubes with screw caps.

Sterile eggplant (*Solanum integrifolium*) seedlings were cultured in 0.5×MS medium containing 1% agar. After 10 days, the cotyledons were excised and treated with an enzyme mixture (0.5% cellulase, 0.1% pectinase, 0.5 M mannitol, and 0.5×MS medium, 1% sucrose, pH 5.6), overnight at 24°C. The protoplast suspension was passed through a nylon mesh with a pore size of 100 µm to remove any large debris. Protoplasts were then harvested by centrifugation at 800 rpm for 5 min, using round-bottom glass centrifugation tubes with screw caps.

Sterile carrot (*Daucus carota*) seedlings were cultured in an MS medium containing 1% agar. Friable calli were induced with 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/l kinetin in MS liquid medium for at least 3 weeks, after which they were transferred to a suspension culture. Calli in the log phase were transferred to a mixture of enzymes (1.0% cellulase [Onozuka RS], 0.1% pectolyase Y-23, 0.5 M mannitol, and 5 mM MES, pH 5.7), and digested at 30°C for 4 h, mixing gently. The protoplast suspension was filtered through a 100-µm cell strainer (Becton Dickinson, N.J., USA) and centrifuged for 5 min at 900 rpm.

Transformation of the tobacco protoplasts using bio-active beads

The transformation materials were combined with PEG treatment according to the protocol described by Negrutiu et al. (1987) with several modifications. Protoplasts were re-suspended in MaMg solution (15 mM $MgCl_2$, 0.4 M mannitol, 0.1% MES, pH 5.6) to increase competency. Protoplast suspension (500 µl) with 5×10^5 cells/ml was then transferred to a 15-ml glass centrifugation tube. Bio-active beads were gently mixed with the protoplast suspension, after which 825 µl of 40% (w/v) PEG CMS6 solution (40% PEG 6000, 0.4 M mannitol, 0.1 M $Ca(NO_3)_2.4H_2O$, pH 7–9, sterilized by passage through a 0.45-µm pore filter) was added, and the final concentration of PEG was adjusted to 24%. As a negative control, protoplast suspension mixed with 50 µl distilled water was treated with PEG. After 20 min of PEG treatment in the dark, 815 µl of 0.2 M $CaCl_2$ solution was added to dilute the PEG, and mixed to disperse the protoplasts. This was repeated 3 times after which the centrifugation tube was filled with W5 solution. The suspension was centrifuged for 5 min at 800 rpm, and the precipitated protoplasts were re-suspended in a 10 ml mixture of 5 ml W5 solution and 5 ml 2× medium (2×MS, 0.2 ml/l 2,4D, 2 mg/l α-naphthaleneacetic acid [NAA], 0.4 mg/l 6-benzyladenine, 2× vitamins, 1% sugar and 0.4 M mannitol). The suspension was centrifuged again to eliminate the PEG completely.

Finally, the protoplasts were re-suspended in 1.5 ml of protoplast 2× medium, and solidified with 1.5 ml of gellangum solution (0.4% gellangum, 0.4 M mannitol, 1% sucrose) in a 35- or 60-mm dish. The dishes were cultured in the dark for at least 16 h to observe transient expression of GFP under an inverted fluorescence microscope, IX-70

(Olympus, Tokyo, Japan). The number of cells expressing GFP was counted through a fluorescence filter WIB/GFP, and fluorescent images were captured through another filter WIBA/GFP.

Regeneration of transformed tobacco SR-1

Following confirmation of GFP transient expression, subculturing was continued. The dishes were incubated at 25°C in the dark. Culture medium A (1×MS, 1% sucrose, 1 mg/ml 6-benzyladenine, 0.1 mg/ml NAA, B5 vitamins, 0.2 M mannitol) (3 ml) was supplemented for regeneration after 1 week's incubation. Meanwhile, 3 µl of 20 mg/ml kanamycin solution was added to the medium for selection of transformed plants by the *NPTII* gene. Culture medium A (6 ml) and 6 µl of 20 mg/ml kanamycin solution were supplemented again after 2-weeks incubation, after which the protoplasts were transferred to 90-mm dishes. Most protoplasts died after addition of the kanamycin solution but some protoplasts continued to actively divide even when kanamycin concentrations elevated. Nine colonies formed 16 days after co-transformation.

After 18 days the dishes were put under illumination. Culture medium B (medium A without the mannitol) (12 ml) was used for regeneration, and 12 µl kanamycin solution was supplemented after 26 days. The cultured protoplasts were then divided into two dishes. When the calli reached 1 cm in diameter, they were transferred to an agar medium, which contained medium C (medium B without NAA). Two months after co-transformation, the larger calli turned green and were transferred to an MS 104 plate (10×MS, 100×Fe-EDTA, 200×B5 vitamins, 500 mg/l 6-benzyladenine, 500 mg/l NAA, 3% sugar and 0.8% agar [Vasil 1984]) to induce shoot growth. Following shoot induction, the large calli were transferred to hormone-free MS medium (10×MS, 100×Fe-EDTA, 200×MS vitamins, 3% sugar, 0.8% agar) to induce the roots. Thin roots were observed 5 days after transfer of the shoots to the hormone-free medium.

Molecular analyses of transgenic plants

Total DNAs were isolated from leaves of 4 transgenic plants (T1, T2, T3 and T4) using the cetyltrimethylammonium bromide method (Weising 1991). PCRs were then performed with the primers of the *GFP* and *NPTII* genes. The 5′ primer (5′-TTCAAGGACGACGGCAACTA-3′) and 3′ primer (5′-CGCTTCTCGTTGGGGTCTTT-3′) were chosen to amplify the 328-bp fragment of the *GFP* gene. The 5′ primer (5′-GGCTATGACGCACACCA-3′) and 3′ primer (5′-GCGATACCGTAAACCACGAG-3′) were chosen to amplify the 680-bp fragment of the *NPTII* gene. Conditions of the thermal cycle were: 95°C for 30 s, 55°C for 30 s, and 74°C for 30 s, for the first cycle; and 95°C for 15 s, 60.3°C for 15 s and 74°C for 30 s, for the following 30 cycles with KOD Dash polymerase (Toyobo, Osaka, Japan).

The total genome DNAs were digested with *Hin*dIII for 20 h and separated on 0.8% agarose gels. Electrophoresis was conducted for 15 h at 20 V in 1×Tris-acetate-EDTA (TAE) buffer. The DNA was then transferred to Hybond N^+ membranes (Amersham, N.J., USA) using the alkaline transfer method. The 328-bp fragment of *GFP* and the 680-bp fragment of *NPTII* were used for Southern hybridization. These probes were labeled with thermostable alkaline phosphatase. Southern hybridization and wash conditions were as those previously described (Sambrook et al. 1989).

Results and discussion

The transient expression of GFP in tobacco BY-2 cells was observed after application of the bio-active beads method (Sone et al. 2002). To analyze the capability of this method on other plant species, pUC18-sGFP plasmids were transformed into eggplant, tobacco SR-1 and carrot protoplasts using the bio-active beads method. Observations of transient expression in the protoplasts indicated that this method had been successful (Fig. 1). This suggests that this method has a wide application in many plant species, not only tobacco. To obtain the transformed plants, the pTN80 and pUC18-sGFP plasmids were used with co-transformation to select the transformed protoplasts. Table 1 shows the co-transformation results, with and without bio-active bead treatment in tobacco SR-1. Bio-active beads with pUC18-sGFP and pUC18-sGFP plus pTN80 showed similar transient GFP expression efficiencies. This indicates that if another type of plasmid is added during co-transformation it has no effect on transformation efficiency. After 5 days co-transformation of the tobacco SR-1 plants, the protoplasts began to divide (Fig. 2A). When the regenerated plants grew to a height of more than 5 cm (Fig. 2B), they were transferred from the culture bottles to pots and maintained in the incubation room at 26°C under illumination for 16 h per day. The tobacco plants began flowering 5.5 months after transformation (Fig. 2C).

Four independent kanamycin resistant transgenic tobacco SR-1 plants (T1, T2, T3 and T4) were randomly selected from the 9 regenerated plants, for molecular analyses to confirm the presence of the *GFP* and *NPTII* genes in the genomes. Total genomic DNAs were isolated from the leaves of the transformed and wild type plants. PCR analyses revealed that all transformed plants possessed the *NPTII* gene (Fig. 3B), and one plant (T1) possessed both the *NPTII* and *GFP* genes (Fig. 3A). No PCR amplification was observed in the wild type under identical reaction conditions. Southern hybridization experiments also confirmed the integration of the *NPTII* gene in each genome. Three bands in the T1, two bands in the T3 and only one band in the T2 and T4 plants were observed (Fig. 3D). These hybridizing patterns indicate that the copy number of transformed DNA is different in each transgenic plant. One clear band (Fig. 3C) was observed when the genomic DNA of T1 was hybridized with *GFP* probe, indicating that this plant was successfully co-transformed with both the *GFP* and *NPTII* genes. Compared with the particle bombardment method

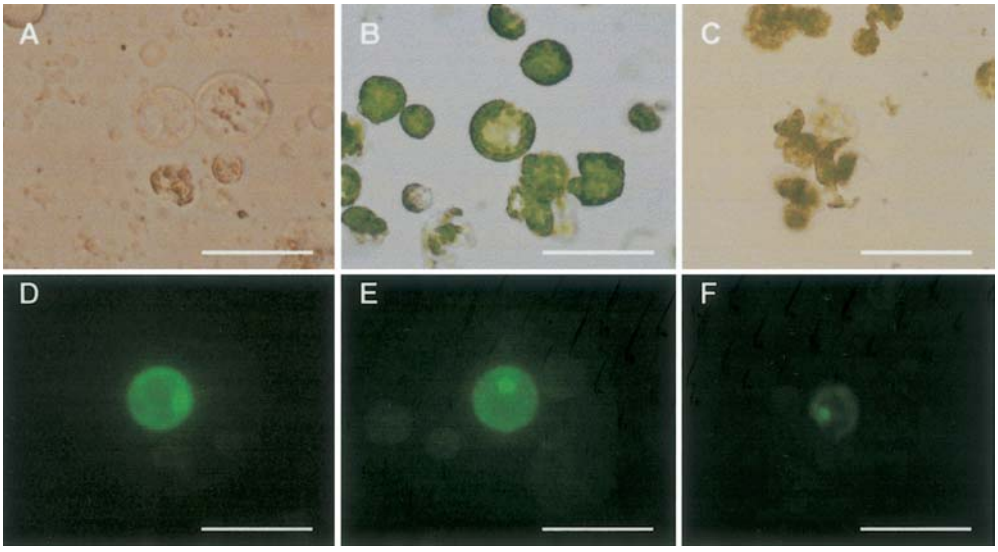

Fig. 1A–F Green fluorescent protein (GFP) expression of several plant species. **A–C** Phase contrast images of the protoplasts. **D–F** Fluorescent images of the transient GFP expression in eggplant (*Solanum integrifolium*), tobacco (*Nicotiana tabacum* SR-1) and carrot (*Daucus carota*) protoplasts, respectively. *Bars* 50 μm

Table 1 Comparison of transformations with and without treatment with bio-active beads

Experiment[a]	Amount of DNA (μg)		GFP[b]	Kanamycin[c]
	pUC18-sGFP	pTN80		
Naked DNA	50	–	2	–
Naked CoT	50	25	1	0
Bio-active beads	50	–	18	–
Bio-active beads CoT-1	50	25	23	4
Bio-active beads CoT-2	50	25	11	3
Bio-active beads CoT-3	50	25	19	2

[a]*Naked* DNA without treatment with bio-active beads, *CoT* co-transformation with two plasmids including pUC18-sGFP and pTN80
[b]Number of protoplasts with transient expression of green fluorescent protein per 10^5 protoplasts
[c]Number of calli surviving in selected culture medium 2 months following co-transformation

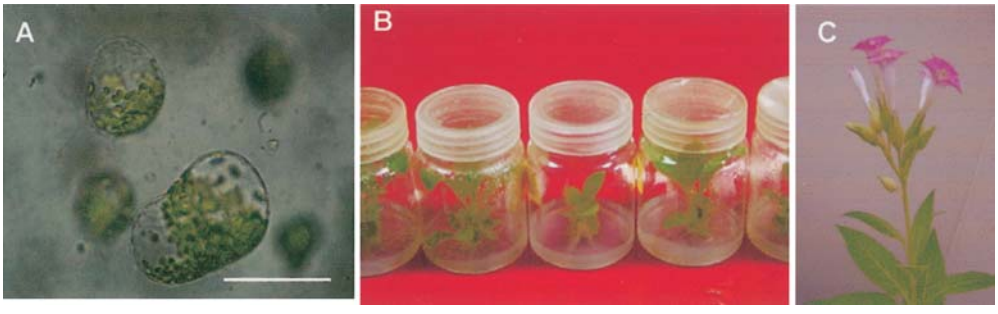

Fig. 2A–C Transgenic tobacco SR-1. **A** Cell division of protoplast 5 days after co-transformation. **B** Transgenic tobacco SR-1 regenerated plants 4 months after being transformed. **C** Flowering transgenic tobacco SR-1 5.5 months after transformation

(Kohli et al. 2003), this system produced low-copy-number transgenic loci.

In this study, a novel and efficient transformation method for plants was demonstrated. Results of the transformation of tobacco SR-1 showed a more efficient transient expression than by PEG treatment alone (Table 1). The results clearly demonstrate that the transformation efficiency of the bio-active beads method is higher than that obtained using the naked DNA and PEG treatment methods. Only one flowering transgenic plant was obtained with both the *GFP* and *NPTII* genes although a high degree of GFP transient expression was detected. The main reason

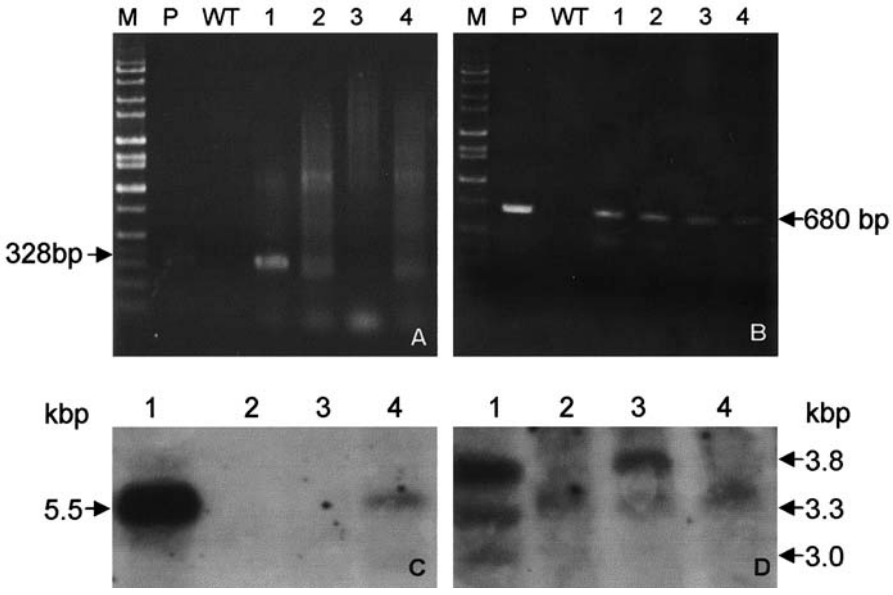

Fig. 3A–D PCR and Southern hybridization analyses of transgenic tobacco SR-1 plants. **A** PCR amplification with a pair of *GFP* gene primers. **B** PCR amplification with a pair of *NPTII* gene primers. *Lane M* marker (Hi-Lo DNA marker), *lane P* positive control, *lane WT* wild type, *lanes 1–4* 4 transgenic plants. **C** Southern hybridization with a *GFP* probe. **D** Southern hybridization with a *NPTII* probe

that *GFP* was not detected after selection with kanamycin is that the transformants were not selected using GFP-fluorescence but using kanamycin resistance. This method was applied to eggplant and carrot plants, and subsequent transient expression was observed (Fig. 1). Comparison with the *Agrobacterium*-mediated transformation method suggests that the bio-active beads system is applicable on a wider number of plant species. Transformation efficiency is approximately 10^{-5}/cell with the bio-active beads method. Although this is lower than that of the *Agrobacterium*-medium transformation (Jackson and Linskens 2003) and electroporation methods (Shimamoto et al. 1989), the bio-active beads method is an alternative transformation system for the generation of transgenic plants, and has a wide applicability, low-copy-number of transgenic loci, and is easy in practice.

Another possible advantage of the bio-active beads method is its capacity for huge DNA delivery. This was demonstrated in the transformation of yeast with chromosomal DNA of up to 450 kb (Mizukami at al. 2003). The next step is to transform plant species with larger DNA molecules using the bio-active beads method successfully.

Acknowledgements We would like to thank Dr. Yasuo Niwa (Shizuoka University) and Dr. Mitsuyasa Hasebe (NIBB) for kindly providing us with the pUC18-sGFP and pTN80 plasmids, respectively. This study was supported by Basic Research Activities for Innovative Biosciences, Japan.

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