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Tissue Culture Note

Improvement of bioactive bead-mediated transformation by concomitant application of electroporation

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Abstract Techniques of exogenous gene introduction in plant cells have been actively applied for producing transgenic cultured cells that produce high levels of useful biological substances and are used for the molecular breeding of plants. A novel gene transfer technique has been developed using calcium alginate micro-beads (bioactive beads) to entrap genetic material. Although this technique has several advantages such as high transformation efficiency and the ability to introduce large DNA molecules, the precise optimal application conditions remain to be determined. Here, the optimal conditions for the concomitant use of bioactive beads and electroporation, such as polyethylene glycol (PEG) concentration, alginate concentration, electric field intensity, pulse duration, and pulse numbers, were determined for improved gene transfer efficiency of bioactive bead-mediated plant transformation. Consequently, highly efficient transient transgene expression (up to 4.7%) was achieved by applying electrical pulses (0.75 kV cm^{-1}, 30 μs, 3×) to a protoplast suspension in 12% polyethylene glycol (PEG).

Key words: Bioactive bead, electroporation, polyethylene glycol, protoplast.

Genetic engineering techniques now permit the transfer the genes across species. They have been used for the production of cultured cells that produce high levels of useful biological substances and for effective molecular breeding. Several gene transfer methods have been developed till date, and among these, electroporation has primarily been used for direct gene transfer in plant protoplasts. Electroporation is easy and has high gene transfer efficiency. Thus far, the transfer of not only DNA but also RNA (Nishiguchi et al. 1986) and antibody (Maccarrone et al. 1995) in protoplasts has been attempted. By this method, the gene transfer efficiency increased with DNA concentration and was affected by the amplitude and duration of the electrical pulses as well as by the composition of the electroporation medium (Fromm et al. 1985; Bates 1994; Gallois et al. 1995). In some studies, electroporation is performed in the presence of polyethylene glycol (PEG). Moreover, a simple chemical technique based on the synergistic interaction magnesium chloride ($MgCl_2$) and PEG was reported to be extremely efficient for protoplast transformation by electroporation (Tyagi et al. 1989; Shillito et al. 1985; Negrutiu et al. 1987).

Recently, a novel gene transfer method for plant protoplasts termed bioactive bead- mediated transformation has been developed. In this method, calcium alginate beads that are several microns in size that can entrap genetic material are added to a protoplast suspension; subsequently, this mixture is treated with PEG solution. This method has several significant features such as high transformation efficiency (10× that of the PEG method) and the capacity to introduce large DNA fragments such as yeast artificial chromosomes (YACs) in protoplasts (Sone et al. 2002; Mizukami et al. 2003; Liu et al. 2004). More recently, the efficiency of bioactive bead-mediated plant transformation was further improved by using DNA-lipofectin complex as the entrapped genetic material instead of naked DNA that was used in the original bioactive bead method. In the improved method, transformation efficiency increased 4-fold as compared to the original method (Murakawa et al. 2008). However, one of the limitations of this method is that the presence of PEG in high concentrations of up to 24% could destroy the structure of the cell membrane and decrease the viability of the protoplasts, resulting in lower gene transfer efficiency. PEG is a water-soluble poly alcohol that acts as a molecular bridge between the surfaces of a protoplast and adjacent beads. We considered that the concomitant use of electroporation with bioactive beads would be effective in decreasing the

Abbreviations: PEG, polyethylene glycol.
This article can be found at http://www.jspcmb.jp/

required PEG concentration.

In the present study, we investigated the application conditions such as PEG concentration, alginate concentration, electric field intensity, pulse duration, and number of pulses applied and found that when the bioactive bead method was used in combination with electroporation, it resulted in a very high level of transient transgene expression (4.7%) in tobacco SR-1 cells.

As a transgene, the synthetic mutant GFP gene that was inserted downstream of the CaMV35S promoter in pUC18 vector (pUC18-sGFP) was used. Plasmid DNA was purified using the QIAfilter Maxi kit (QIAGEN) and stored in TE buffer at 1 g l^{-1}. The gene transfer method is as follows. Tobacco SR-1 cotyledons cultivated in MS medium were treated with 0.25% Cellulase Onozuka R10 (Yakult Co., Tokyo, Japan) and 0.1% Macerozyme R10 (Kyowa Chemical Products Co., Osaka, Japan) in a solution containing 500 mM mannitol, 2.5 mM MES, 1.5% sucrose and 1/2MS for 16 hr at 25°C to obtain protoplasts. The protoplasts were washed twice with 400 mM mannitol solution and suspended in MaMg solution (400 mM mannitol, 15 mM $MgCl_2$, 0.1% MES, pH 5.6) at a cell density of 5×10^5 cells ml^{-1}.

Bioactive beads with entrapped plasmid DNA were prepared as described previously (Sone et al. 2002). Briefly, 0.5% sodium alginate solution (100 μl) was mixed with isoamyl alcohol (900 μl) in a microtube and then emulsified by sonication. Plasmid DNA (25 μl) and 100 mM calcium chloride solution (475 μl) were added to the emulsion, and vigorously stirred using a tube mixer. Following centrifugation at 900×*g* for 3 min, the emulsion was resuspended in calcium chloride solution. Centrifugation was repeated 3 times.

Bioactive beads with entrapped plasmid DNA (50 μl) were mixed with a protoplast suspension (250 μl). PEG solution (24% PEG6000, 400 mM mannitol, 30 mM $MgCl_2$, pH 7–9) was further added to this suspension to achieve final concentrations of 8%, 12%, and 16%. After incubating for 10 min at room temperature, the suspension was transferred to a 60-mm petri dish. Subsequently, electrical pulses (0.75 kV cm^{-1}, 30 μs, 3×) were applied in an electroporation chamber with a 2-mm interelectrode gap by using a cell fusion apparatus (Shimazu, Tokyo, Japan). Following the application of the electrical pulses, 2 ml of 200 mM calcium chloride ($CaCl_2$) solution (200 mM $CaCl_2$, 0.4 M mannitol, pH 5.8) was added to the suspension and centrifuged at 85×*g* for 3 min. After additional centrifugation, the protoplasts were resuspended in 5 ml of liquid medium (0.45 M mannitol containing K3S medium, pH 5.8). The protoplasts were cultured for 24 hr in the dark, and the number of protoplasts that expressed green fluorescent protein (GFP) was counted under a fluorescence microscope (Zeiss, Oberkochen, Germany). Transgene expression efficiency was determined by calculating the ratio of the number of GFP-expressing protoplasts to the total number of protoplasts (*i.e.*, 12.5×10^4).

Figure 1 shows the effect of PEG concentration on the gene transfer efficiency of electroporation. In the absence of PEG, only weak transgene expression levels were obtained with both naked plasmid DNA and plasmid DNA that entrapped bioactive beads. Transgene expression was promoted by the addition of PEG; the expression efficiency of the bioactive beads as DNA sources was the highest when the final concentration of PEG was 12%. According to the PEG treatment protocol, the optimal concentration of PEG was lower when using electroporation. The main function of the PEG could probably be to promote interaction between the bioactive beads and the cell membrane. Low concentrations of PEG are less toxic to protoplasts and improve the survival rates of protoplasts.

Further, we examined the parameters of electrical pulse application. Among the various parameters for electroporation, the most important factor is the pulse intensity that induces transient damage to the lipid bilayer of the cell membrane. Pulse intensity is generally represented as a combination of electrical field intensity and pulse width. Thus, we determined the transgene expression efficiency of electroporation under various combinations of electrical field intensity and pulse width. Figure 2 shows the effects of electrical field intensity and pulse width on the transgene expression efficiency of electroporation at a fixed PEG concentration of 12%. For all the tested electrical field intensities, the expression efficiency was high when the pulse width was 30 μs. Among the various electrical field intensities, the highest expression efficiency was obtained at 0.75 kV cm^{-1}. The

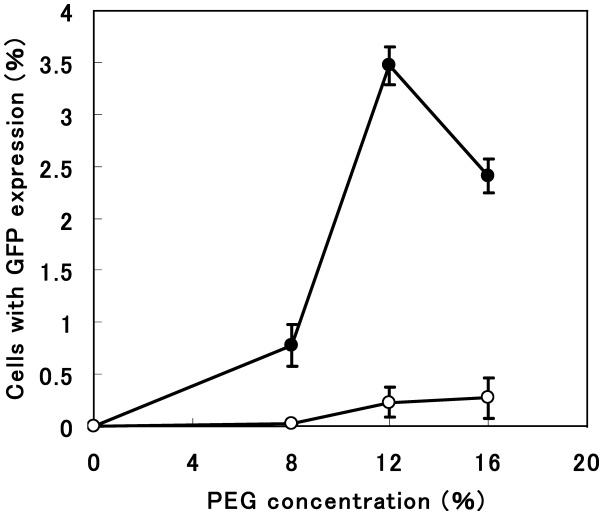

Figure 1. Effect of PEG concentration. The efficiency of transfection reached 3.5% at the highest. Open circles show treatment using naked plasmid DNA. Closed circles show bioactive beads-mediated treatment. Results are expressed as the mean±SD for N=3.

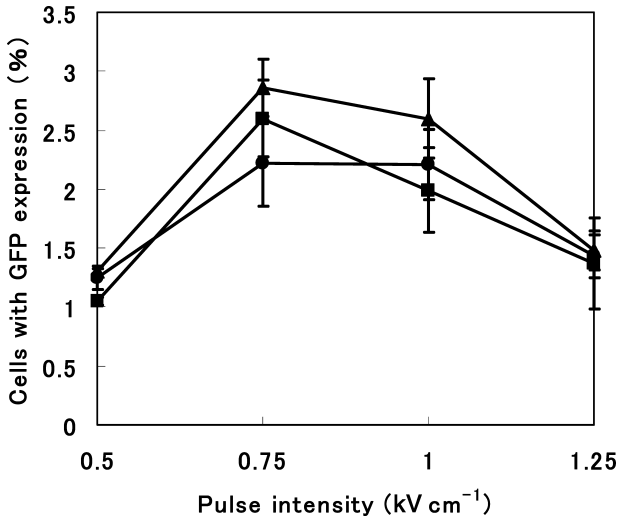

Figure 2. Effect of field strength. When the time of pulse is 30 μs and the concentration of PEG is 12% respectively, the efficiency of transfection reached highest. Closed circles show 20 μs pulse, closed triangles show 30 μs pulse, and closed squares show 40 μs pulse. Results are expressed as the mean±SD for N=3.

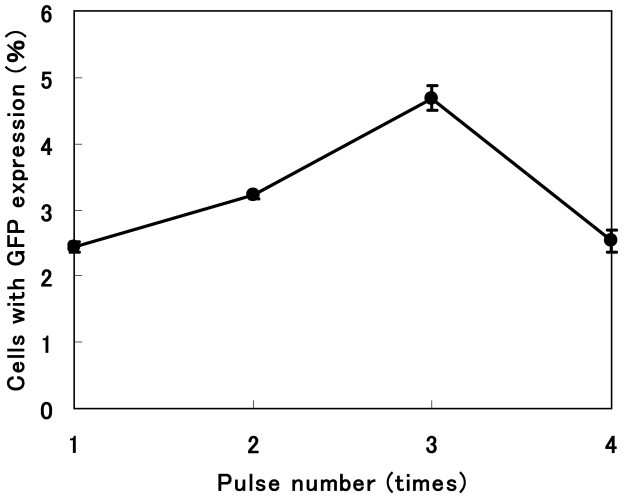

Figure 3. Effect of pulse numbers. When the time of pulse and the field strength of pulse are fixed to 30 μs and 0.75 kV cm^{-1} respectively, the efficiency of transfection reached 4.7% at the highest. Results are expressed as the mean±SD for N=3.

expression efficiency was low when the electrical field intensity was beyond this level. This suggests that relatively strong electrical pulses adversely affect the survival and viability of protoplasts. The optimal combination of electrical field intensity (0.75 kV cm^{-1}) and pulse width (30 μs) obtained from these results was used as the standard condition during the remaining part of the study.

Figure 3 shows the effects of the number of pulses on transgene expression efficiency. Transgene expression efficiency increased with the number of pulses; it was highest when the 3 pulses were applied, and decreased with more than 3 pulses.

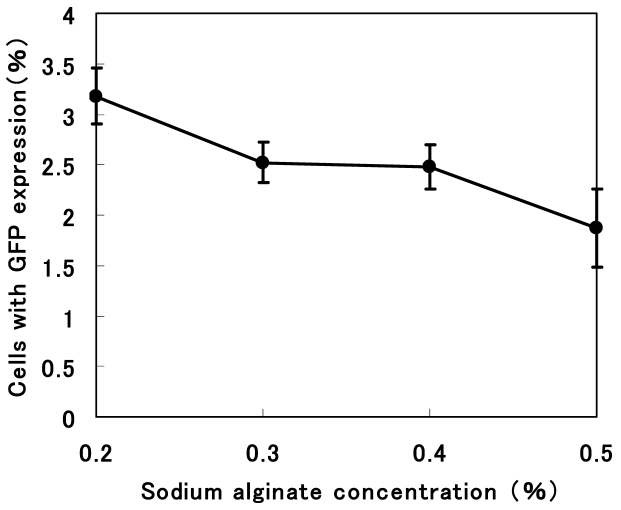

Figure 4. Effect of sodium alginate concentration. When the time of pulse and the field strength of pulse are fixed to 30 μs and 0.75 kV cm^{-1}, 3 times, the efficiency of transfection reached highest. Results are expressed as the mean±SD for N=3.

Previous studies of bioactive beads have shown that gene transfer efficiency is dependent on the concentration of sodium alginate, a component of the beads (Higashi et al. 2004; Murakawa et al. 2008). Figure 4 shows the effect of the concentration of sodium alginate on transgene expression efficiency. Gene transfer efficiency was maximum at a sodium alginate concentration of 0.2% and decreased at higher concentrations. This suggests that transgene expression efficiency is affected by the physical properties of the beads, such as viscosity. It should be noted that preparing the beads with a sodium alginate concentration of less than 0.2% was difficult.

Due to the successful development of gene transfer vectors and efficient methods for DNA transfection, the use of exogenous gene transfer methods improved the transformation efficiency to protoplasts. In addition, the use of effective selection markers such as the kanamycin resistance and GFP genes made the investigation of transient gene expression easy. The gene transfer efficiency to tobacco protoplasts with chemical (*e.g.*, PEG treatment) and physical (*e.g.*, electroporation) methods is summarized and compared in Table 1. Bates demonstrated successful transgene expression (efficiency, 0.14%) following the transfer of the kanamycin resistance gene to tobacco mesophyll cells by electroporation (Bates 1994). Subsequently, Negrutiu et al. reported that the gene transfer efficiency of electroporation could be improved to 0.46% by the addition of magnesium ion (Mg^{2+}) and PEG that induce a supposed competent state in the targeted cells immediately after addition to the cell suspension using kanamycin resistance gene as a reporter (Negrutiu et al. 1987). Furthermore, Sone et al. succeeded in obtaining

Table 1. Comparison of transformation efficiency in various transfection principles.

Variety of tobacco	Transfection principle	Reporter gene	Transformation efficiency (%)	Reference
- (mesophyll)	Electroporation	Kanamycin resistance gene	0.14	Bates (1994)
SR-1	PEG- electroporation	Kanamycin resistance gene	0.46	Negrutiu et al. (1987)
BY-2	PEG-bioactive beads	Synthetic GFP gene	0.22	Sone et al. (2002)
BY-2	PEG-liposome-bioactive beads	Synthetic GFP gene	0.81	Murakawa et al. (2008)
SR-1	PEG- electroporation- bioactive beads	Synthetic GFP gene	4.7	This study

0.22% transgene expression efficiency in suspension-cultured tobacco cells by gene transfer using bioactive beads that entrap GFP-encoding DNA and subsequent PEG treatment resulted in 10 times higher efficiency than that obtained with naked DNA (Sone et al. 2002). GFP transgene expression efficiency was also improved when the bioactive bead method was used in combination with liposomes; the enhanced gene transfer was probably due to the formation of bioactive bead-aggregated complexes prior to incubation of the bioactive beads and liposomes and may have strengthened the interaction of the bioactive beads with the target cell membrane (Murakawa et al. 2008).

In the present study, we developed a modified gene transfer method using bioactive beads, during which PEG is added to the cell suspension prior to electroporation. With this method, *i.e.*, a combination of chemical techniques and electroporation, the GFP transgene expression efficiency in protoplasts could be increased to up to 4.7%. The improved efficiency was presumably due to the fact that the surviving population of protoplasts increased as the concentration of PEG used in this method was lower than that used in conventional PEG-based gene transfer methods (24%). The main function of PEG could probably be to promote interactions between DNA and the cell membrane rather than the promotion of DNA uptake. Lower concentrations of PEG are less toxic to the protoplasts (Shillito 1985).

Our method increases the gene transfer efficiency as compared to conventional bioactive beads methods. Also up to now, it is the most efficient transient gene transfer method for plant protoplasts, though reporter genes and host plant cells used are different. Taken together, these results reveal that the bioactive bead method with concomitant applications of both a low concentration of PEG and electroporation is very effective for gene transfer in plant protoplasts. Transformants using the bioactive bead method have thus far been obtained from the protoplasts of dicotyledonous species (Liu et al. 2004). It is expected that our novel technique can be applied to monocotyledonous plants also and for the transfer of macromolecules such as genomic DNA and chromosomes in the near future.

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