

Application of the Bio-Active Beads Method in Rice Transformation

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Abstract

The bio-active beads system has been verified as a novel transformation method. To enhance its versatility, we applied this method to the transformation of a monocot plant, rice. The transient GFP expression was observed 24 h after transformation. This indicates that the method can also be applied to monocotyledons.

Key words: Bio-active beads system, Monocotyledons, Transformation.

The production of genetically transformed plant tissues and plants is the focus of experimental plant science and the basis of agricultural biotechnology (Taylor and Fauquet 2002). Genetic engineering of monocotyledons has always been of great interest due to its potential benefits to crop improvement and subsequently to human nutrition (Dale *et al.*, 1993). Rice is not only an important food crop but also a model plant among monocotyledons because of its small genome size (Kurata and Fukui, 2003). The transfer of useful genes into rice is still a potential research area to enhance agricultural production. However, monocotyledonous plants are generally more recalcitrant to genetic transformation than dicotyledonous species because of the absence of reliable *Agrobacterium*-mediated transformation methods and the difficulties associated with the culture of monocotyledonous tissues *in vitro* (Vain *et al.*, 1995). Cereals are not natural hosts for *Agrobacterium* species and only recently has it been possible to achieve cereal transformation by this method (Kohli *et al.*, 2003). Rice is the first species to be transformed by *Agrobacterium* among cereals (Chan *et al.*, 1992). Successful transformation was successively reported for maize (Ishida *et al.*, 1996), barley (Tingay *et al.*, 1997), wheat (Cheng *et al.*, 1997), and sorghum (Zhao *et al.*, 2000). Despite these promising results, several difficulties such as host specificity and low efficiency remain associated with this strategy. A number of direct DNA transformation methods have also been attempted to transform the monocot-

yledon. Rice transformation by electroporation (Shimamoto *et al.*, 1989) and microinjection (Saito *et al.*, 2003) methods has been reported. However, the same problems including low transformation efficiency and difficulty in manipulation still exist. Particle bombardment has solved the problem of efficiency. However, it results in the integration of multiple, often rearranged, copies of the transgene (Wisniewski *et al.*, 2002). The further evolution of monocotyledon transformation technologies would therefore appear to depend on the development of techniques that allow controlled integration and expression of foreign DNA into the plant genome.

A novel and efficient transformation system, the bio-active beads system for DNA delivery/expression with a capacity to transport DNA of high molecular weight was previously reported (Sone *et al.*, 2002). This method had been applied to the transformation of yeast, and the physical stabilization of the yeast chromosomal DNA immobilized on bio-active beads had been evaluated (Mizukami *et al.*, 2003). This method was also applied for transforming mammalian cells (Higashi *et al.*, 2004). By means of this method, transformed tobacco SR-1 plants, the transformation being confirmed by screening for Kanamycin resistance were successfully obtained (Liu *et al.*, 2004a). Additionally, a 124-kbYAC had been transferred into tobacco BY-2 cells for finding common promoter (Liu *et al.*, 2004b). The transformation capability of this new system has been noticed recently. In order to understand the extent of the application of this new

transformation system, we applied it to rice transformation, to confirm whether the bio-active beads method can be applied to monocotyledons.

The procedure for the production of bio-active beads including pUC18-sGFP (Niwa *et al.*, 1999) has been described previously (Sone *et al.*, 2002; Mizukami *et al.*, 2003; Higashi *et al.*, 2004; Liu *et al.*, 2004a, b). The pUC18-sGFP (4.1 kb) of plasmid contains CaMV35S promoter, sGFP coding sequence, and NOS3 terminal was used for observation of transient expression. The rice seeds (*Oryza sativa* L. ssp. Japonica cv. Nipponbare) were surface-sterilized with 70% ethanol for 10 min and sodium hypochlorite solution for 10 min. The procedure for callus induction, and preparation of the callus suspension culture was similar to a previous protocol (Lee *et al.*, 1991). Rice protoplasts were prepared according to the previously specified procedure with several modifications (Shimamoto *et al.*, 1989). Since the commercial cellulose Onozuka RS and the commercial pectate lyase Pectolyase Y-23 are widely used as enzymes for protoplast formation, we also used both these enzymes, however, the enzyme concentration was modified. A 40 ml enzyme mixture solution consisting of 0.1% (w v⁻¹) pectolyase Y-23 (Kikkoman, Tokyo), 4% (w v⁻¹) Cellulase Onozuka RS (Yakult, Tokyo), 0.02% (w v⁻¹) CaCl₂, 0.2% (w v⁻¹) k-dextran sulfate, and 18% (w v⁻¹) mannitol was kept for 3 h at 30°C with shaking. The protoplasts were then harvested by centrifugation at 800 rpm for 5 min. Twenty-five milliliters rice washing medium and 25 ml W5 solution was used to separate the enzyme after the centrifugation. The protoplasts and the bio-active beads were mixed with polyethylene-glycol (PEG) according to the protocol described (Sone *et al.*, 2002; Liu *et al.*, 2004a). The PEG treatment time was about 30 min in the dark.

After the 24 h transformation, we observed transient green fluorescent protein (GFP) expression under an inverted fluorescence microscope (Fig. 1).

The signal intensity was almost similar to the transient GFP expression observed in tobacco BY-2 (Liu *et al.*, 2004b). This indicates that the expressed GFP gene coincides with the gene in transformed tobacco cells. The transformation efficiency is about 2.2×10^{-5} after repeating the experiment four times (Table 1). When the transformation was performed using only the PEG treatment without bio-active beads, no transient expression was observed in the tobacco cells. Treatment with twice the concentration of the enzyme would result in the formation of approximately 5-7 times the number of protoplasts. However, there was no obvious increase in the transformation efficiency with an increase in the number of protoplasts. The treatment by high concentration of enzyme maybe brings some damages to the protoplast and effects the transformation efficiency. Compared with the traditional method, the efficiency of our method is not higher than the bombardment and electroporation methods (Saito *et al.*, 2003; Lee *et al.*, 1991). Nevertheless, the transient GFP expression indicates that we had successfully transferred plasmid pUC18-sGFP into rice protoplasts by the bio-active beads method.

The bio-active beads can protect the DNA molecule and keep it intact from damage during the transfer into the target cells (Mizukami *et al.*, 2003). This transfer mechanism is more suitable for the delivery of large size molecules. For example, it will realize the YAC or BAC carrying multiple gene transfer into plant cells collectively. As most agronomic characteristics are polygenic in nature, plant genetic engineering will require the manipulation of complex metabolic or regulatory pathways involving multiple genes or gene complexes (Chen *et al.*, 1998). Therefore, applying the bio-active beads system to the monocotyledons will prove to be tremendously advantageous to agronomic transgene studies.

Although the transformation efficiency was not

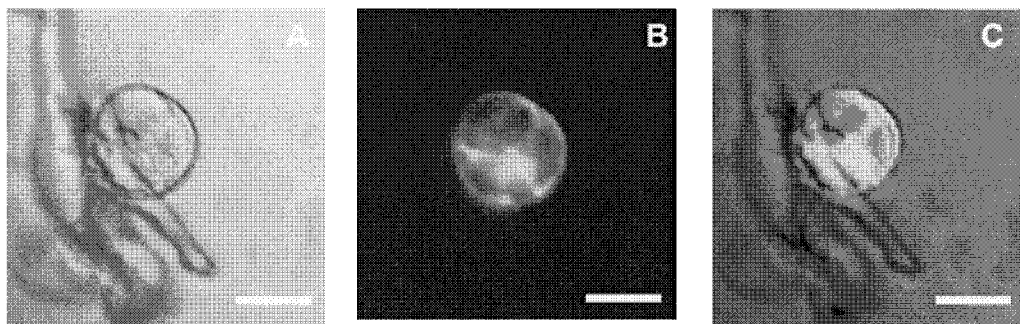


Fig. 1 Transient GFP expression 24 h after transformation of rice protoplasts. (A) Phase contrast image of the protoplast; (B) Fluorescent image of the transient expression of GFP after the transformation; (C) Merged image. The bars=10 μ m.

Table 1 Transient GFP expression of rice protoplasts.

Experiment	Concentration of enzyme ¹⁾	Transient GFP expression	Total number of protoplasts	Efficiency
1-A	1 ×	2	1 × 10 ⁵	2.0 × 10 ⁻⁵
1-B	1 ×	1	1 × 10 ⁵	1.0 × 10 ⁻⁵
2-A	2 ×	22	5 × 10 ⁵	4.4 × 10 ⁻⁵
2-B	2 ×	8	5 × 10 ⁵	1.6 × 10 ⁻⁵
3-A	2 ×	13	5 × 10 ⁵	2.6 × 10 ⁻⁵
3-B	2 ×	9	5 × 10 ⁵	1.8 × 10 ⁻⁵
4-A	2 ×	19	7 × 10 ⁵	2.7 × 10 ⁻⁵
4-B	2 ×	12	7 × 10 ⁵	1.7 × 10 ⁻⁵
Total	-	106	36 × 10 ⁵	-
Average	-	-	-	2.2 × 10 ⁻⁵

¹⁾ 1 × : enzyme concentration: 0.05% (w v⁻¹) pectolyase Y-23, 2% (w v⁻¹) Cellulase Onozuka RS, 0.01% (w v⁻¹) CaCl₂, 0.1% (w v⁻¹) k-dextran sulfate, and 9% (w v⁻¹) mannitol;

2 × : enzyme concentration: 0.1% (w v⁻¹) pectolyase Y-23, 4% (w v⁻¹) Cellulase Onozuka RS, 0.02% (w v⁻¹) CaCl₂, 0.2% (w v⁻¹) k-dextran sulfate, and 18% (w v⁻¹) mannitol.

sufficiently high when the bio-active beads method was used in the rice transformation, the characteristics of this method, such as a high carrying capability of large DNA molecules and extensive application to different species still bestow a potential benefit to studies in gene delivery.

We attempted to apply the bio-active beads method to monocotyledons for the first time, and the extensive application capability was reconfirmed. The expected advantage of this method will be challenged while attempting the transfer of wheat genes into the rice genome for crop evolution.

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References

- Chan, M. T., Lee, T. M. and Chang, H. H., 1992. Transformation of indica rice (*Oryza sativa*) mediated by *Agrobacterium tumefaciens*. *Plant Cell Physiol.*, **33**: 577-583.
- Chen, L., Marmey, P., Taylor, N. J., Brizard, J. P., Espinoza, C., D'Cruz, P., Huet, H., Zhang, S., Kochko, A., Beachy, R. N., and Fauquet, C., 1998. Expression and inheritance of multiple transgenes in rice plants. *Nature Biotechnol.*, **16**: 1060-1064.
- Cheng, M., Fry, J.E., Pang, S.Z., Zhou, H.P., Hironaka, C. M., Duncan, D.R., Conner, T.W. and Wan, Y.C., 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol.*, **115**: 971-980.
- Dale, P. J., Irwin, J. A. and Scheffler, J. A., 1993. The experimental and commercial release of transgenic crop plants. *Plant breeding*, **111**: 1-22.
- Higashi, T., Nagamori, E., Sone, T., Matsunaga, S. and Fukui, K., 2004. A novel transfection method for mammalian cells using calcium alginate microbeads. *J. Biosci. Bioeng.*, **97**: 191-195.
- Ishida, Y., Saito, H., Ohta, Y., Komari, T., and Kumashiro, T., 1996. High efficiency transformation of maize (*Zea mays*) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnol.*, **14**: 745-750.
- Kohli, A., Twyman, R. M., Abranches, R., Wegel, E., Stoger, E., and Christou, P., 2003. Transgene integration, organization and interaction in plants. *Plant Mol. Biol.*, **52**: 247-258.
- Kurata, N. and Fukui, K., 2003. Chromosome research in genus *Oryza*. In J. S. Nanda and S. D. Sharma (Eds.) *Monograph in genus Oryza*, pp. 213-261. Science publishers, New Hampshire.
- Lee, N., Wang, Y., Yang, J., Ge, K., Huang, S., and Tan J., 1991. Efficient transformation and regeneration of rice small cell groups. *Proc. Natl. Acad. Sci. USA*, **88**: 6389-6393.
- Liu, H., Kawabe, A., Matsunaga, S., Murakawa, T., Mizukami, A., Yanagisawa, M., Nagamori, E., Harashima, S., Kobayashi, A., and Fukui, K., 2004(a). Obtaining transgenic plants using the bio-active beads method. *J. Plant Res.*, **117**: 95-99.
- Liu, H., Kawabe, A., Matsunaga, S., Kim, Y. H., Higashi, T., Uchiyama, S., Harashima, S., Kobayashi, A., and Fukui, K., 2004(b). An *Arabidopsis thaliana* gene on the yeast artificial chromosome can be transcribed in tobacco cells. *Cytologia*, **69**: 235-240.

- Mizukami, A., Nagamori, E., Takakura, Y., Matsunaga, S., Kaneko, Y., Kajiyama, S., Harashima, S., Kobayashi, A., and Fukui, K., 2003. Transformation of yeast using calcium alginate microbeads with surface-immobilized chromosomal DNA. *BioTechniques*, **35**: 734-740.
- Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M., and Kobayashi, H., 1999. Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. *Plant J.*, **18**: 455-463.
- Saito, M., Mukai, Y., Komazaki, T., Oh, K. B., Nishizawa, Y., Tomiyama, M., Shibuya, N., and Matsuoka, H., 2003. Expression of rice chitinase gene triggered by the direct injection of Ca^{2+} . *J. Biotechnol.*, **105**: 41-49.
- Shimamoto, K., Terada, R., Izawa, T., and Fujimoto, H., 1989. Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature*, **338**: 274-276.
- Sone, T., Nagamori, E., Ikeuchi, T., Mizukami, A., Takakura, Y., Kajiyama, S., Fukusaki, E., Harashima, S., Kobayashi, A., and Fukui, K., 2002. A novel gene delivery system in plant with calcium alginate microbeads. *J. Biosci. Bioeng.*, **94**: 87-91.
- Taylor, N. J. and Fauquet, C. M., 2002. Microparticle bombardment as a tool in plant science and agricultural biotechnology. *DNA and Cell Biol.*, **21**: 963-977.
- Tingay, S., Mcelroy, D., Kalla, R., Fieg, S., Wang, M. B., Thornton, S. and Brettell, R., 1997. *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J.*, **11**: 1369-1376.
- Vain, P., De Buyser, J., Bui Trang, V., Haicour, R., and Henry, Y., 1995. Foreign gene delivery into monocotyledonous species. *Biotechnol. Adv.*, **13**: 653-671.
- Wisniewski, J. P., Frangne, N., Massonneau, A., and Dumas, C., 2002. Between myth and reality: genetically modified maize, an example of a sizeable scientific controversy. *Biochimie*, **84**: 1095-1103.
- Zhao, Z. Y., Cai, T. S., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, M., Schroeder, S., Hondred, D., Seltzer, J. and Pierce, D., 2000. *Agrobacterium*-mediated sorghum transformation. *Plant Mol. Biol.*, **44**: 789-798.