Improvement of the Aluminum Borate Whisker-Mediated Method of DNA Delivery into Rice Callus

Kiyotaka Mizuno, Wataru Takahashi*, Takashi Ohyama, Takiko Shimada** and Osamu Tanaka

(Department of Biology, Faculty of Science and Engineering, Konan University, Okamoto, Higashinada-ku, Kobe 658-8501, Japan; **Research Institute of Agricultural Resource, Ishikawa Agricultural College, 1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan)

Abstract: We improved the aluminum borate whisker-mediated method of DNA delivery into rice callus (Oryza sativa L., cv. Notohikari). The following factors were examined: amount of whiskers, the kind of apparatus for agitation, the type of whiskers, duration of agitation, and agitation speed. Twenty callused scutellar tissues were agitated in a 1.5 ml microtube containing aluminum borate whiskers (ABW), liquid medium, and the plasmid pAct1-F carrying the β-glucuronidase (GUS) gene. After the agitation, the scutellar tissues were subjected to transient GUS expression assay, which visualizes GUS-expressing cells as blue spots. Efficiencies of DNA delivery were evaluated as the number of blue spots resulting from the assay. In the present study, we succeeded in improving the efficiency of DNA delivery by changing the apparatus for agitation from rotary (Vortex Genie 2) to multidirectional (MT-360), and the type of ABW from Alborex Y to an aminosilane-coated Alborex (Alborex YS3A).

Key words: Aluminum borate whiskers, β-glucuronidase gene, Rice callus, Silicon carbide whiskers, Transgenic plant.

Kaeppler et al. (1990) first reported the method of silicon carbide whisker (SCW)-mediated DNA delivery into plant cells. In their method, plant cells were vigorously agitated together with silicon carbide "whiskers" in liquid medium containing DNA. During agitation, SCW, which consists of needle-like microfibers about 0.5 µm in diameter and 10-80 µm long, acted as needles, facilitating DNA delivery into the cells. The whisker method is simpler and less expensive than other gene transfer methods, such as those involving Agrobacterium, particle bombardment, polyethylene glycol, and electroporation.

To date, SCW-mediated DNA delivery into plant cells has been reported for maize (Kaeppler et al., 1990, 1992), tobacco (Kaeppler et al., 1990, 1992), Agrostis alba (Asano et al., 1991), wheat (Omirulleh et al., 1996; Brisibe et al., 2000), and rice (Nagatani et al., 1997). Furthermore, transgenic plants produced by the SCW method include those of maize (Frame et al., 1994; Petolino et al., 2000), Lolium multiflorum, Lolium perenne, Festuca arundinacea, Agrostis stolonifera (Dalton et al., 1998), and rice (Matsushita et al., 1999). However, SCW can be toxic and mutagenic to organisms, including humans (Vaughan et al., 1991; Svensson et al., 1997). Therefore, we recently developed a safer aluminum borate whisker (ABW)-mediated method of producing transgenic rice plants (Takahashi et al., 2000). Although many characteristics of ABW are similar to those of SCW, to the best of our knowledge, there have been no reports of mutagenicity of ABW to organisms. Here we report the improved efficiency of the ABW-mediated method of DNA delivery into rice callus.

Materials and Methods

1. Callus culture

Calli were induced from the culture of mature seeds of a japonica rice (Oryza sativa L., cv. Notohikari) as previously reported (Takahashi et al., 2000). Mature seeds were sterilized with 10% (v/v) sodium hypochlorite for 60 min and rinsed three times with sterile distilled water. They were then cultured on filter paper in a Petri dish containing liquid Linsmaier-Skoog (LS) medium (Linsmaier and Skoog, 1965) that was supplemented with 2 mg L⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D). After 7 days of culture, the callused scutellar tissues (hereafter referred to as scutellar tissues) of embryos were isolated from the seeds and used as target tissues for experiments of DNA delivery. Cultures were incubated in the light (40
\( \mu \text{mol m}^{-2} \text{s}^{-1} \) at 25°C.

2. Plasmid DNA

The plasmid pAct1-F (McElroy et al., 1990) harboring the \( \beta \)-glucuronidase (GUS) gene driven by a rice actin-1 gene promoter was kindly provided by Dr. R. Wu (Cornell University, Ithaca, NY, USA). The plasmid was amplified in liquid culture of \( E. \text{coli} \) for isolation with a QIAGEN\textsuperscript{TM} Maxi Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions.

3. Whisker preparation

Required amount of aluminum borate whisker (ABW) (Alborex Y and Alborex YS3A, kindly provided by Shikoku Chemicals Co., Kagawa, Japan) in a 1.5-ml microtube was sterilized by autoclaving at 121°C for 20 min immediately before use.

4. DNA delivery

Twenty scutellar tissues (approx. 250 mg) were transferred into a 1.5-mL microtube that contained the designated amount of ABW and 290 \( \mu \text{L} \) liquid LS medium supplemented with 2 mg \( L^{-1} \) 2,4-D, 0.25 M sorbitol, and 0.25 M mannitol (hereafter referred to as LS2SM medium). The plasmid pAct1-F (25 \( \mu \text{g} \)) were added to the microtube. After being tapped, the tube was kept at room temperature for 30 min and then the mixture was agitated with either a Vortex Genie 2 (Scientific Industries Inc., Bohemia, NY, USA) or Microtube mixer (model MT-360, Tomy, Tokyo, Japan) at various speeds and for various durations. After the agitation, the scutellar tissues were rinsed with liquid LS2SM medium and transferred onto solid LS medium containing 2 mg \( L^{-1} \) 2,4-D and 0.32% (w v\(^{-1}\)) Gelrite (Wako, Osaka, Japan). Cultures were incubated in the light (40 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) at 25°C.

![Image of graphs and diagrams](image-url)

Fig. 1. The efficiency of aluminum borate whisker (ABW)-mediated DNA delivery under various conditions.

A: Effect of the agitation device on DNA delivery. Various amounts of Alborex Y were added to microtubes containing the scutellar tissues, and agitated with either a Vortex Genie 2 (open circles) or MT-360 (solid circles) at maximum speed for 30 min. B: Effect of the types of ABW (Alborex Y [open circles] and Alborex YS3A [solid circles]) on DNA delivery. Various amounts of each type of ABW were added to the microtubes, and were agitated with MT-360 at maximum speed for 30 min. C: Effect of the duration of agitation on DNA delivery. Alborex YS3A (16 mg) was added to each microtube, then agitated with MT-360 at maximum speed for various durations. D: Effect of agitation speed on DNA delivery. Alborex YS3A (16 mg) was added to each microtube, then mixed with the MT-360 at various speeds for 30 min. Data were expressed as the number of blue spots per 20 scutellar tissues in each treatment.
5. **Transient GUS expression assay**

After incubation for 2 days, the treated scutellar tissues were subjected to transient GUS expression assays for evaluation of the efficiency of DNA delivery (Jefferson et al., 1987). To develop the GUS-expressing cells, we incubated the treated tissues at 37°C for 24 h in X-Gluc solution [1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt (X-Gluc), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 100 mM phosphate buffer (pH 7), 20% methanol, and 0.3% Triton X-100]. The number of GUS-expressing loci (blue spots) on each of the 20 scutellar tissues per treatment was counted under a stereomicroscope. Data were expressed as the number of blue spots per 20 scutellar tissues for each treatment.

### Results

1. **Effect of the kind of apparatus for agitation**

   The mixtures of scutellar tissues and various amounts of Alborex Y (0 to 100 mg per microtube) in microtubes were agitated using either a Vortex Genie 2 or MT-360 at maximum speed for 30 min. As shown in Fig. 1A, the efficiency of DNA delivery was influenced by the kind of agitating device: MT-360 was more effective than Vortex Genie 2. In particular, the efficiency of DNA delivery achieved using MT-360 and 32 mg of Alborex Y was approximately twice that of Vortex Genie 2 and 64 mg of Alborex Y (conditions previously found to be optimal [Takahashi et al., 2000]). Therefore, we decided to use MT-360 for agitation in the remaining experiments.

2. **Effect of the type of whiskers**

   We compared the effect of agitation with two types of ABW Alborex Y and Alborex YS3A (0 to 100 mg per microtube) at maximum speed for 30 min on the DNA delivery into scutellar tissues. Fig. 1B indicates that for DNA delivery, Alborex YS3A is clearly superior to Alborex Y. The optimal amount of Alborex YS3A was 16 mg, which is half the optimal amount of Alborex Y (32 mg). Therefore, we used 16 mg of Alborex YS3A in the remaining experiments.

3. **Effect of duration of agitation**

   We then tested the effect of the duration of agitation with an Alborex YS3A and MT-360 at maximum speed. Fig. 1C indicates that a 30-min agitation is the optimum for DNA delivery. Prolonged (>30 min) agitation decreased the efficiency.

4. **Effect of agitation speed**

   Using the optimal conditions defined by the preceding experiments, we evaluated the effect of the agitation speed on DNA delivery. Figure 1D shows that the efficiency of DNA delivery was increased markedly by increasing the agitation speed of the MT-360 up to setting 8 (2400 rpm). However, agitation at speed 10 (maximum speed 2445 rpm) resulted in a slightly lower efficiency of DNA delivery than that for setting 8.

   The optimal conditions for DNA delivery in the present study were as follows: mixing apparatus for agitation, MT-360; type of ABW, Alborex YS3A; amount of Alborex YS3A, 16 mg; duration of agitation, 30 min; and agitation speed, setting 8 of MT-360. Under these conditions, the efficiency of DNA delivery was significantly improved compared to previous methods.
conditions, as shown in Fig. 2, the efficiency of DNA delivery was increased approximately 3-fold compared with previous results (Takahashi et al., 2000).

Discussion

The ABW-mediated method of DNA delivery into rice callus reported previously (Takahashi et al., 2000) was clearly improved in this study. Our success in enhancing DNA delivery owes to the use of MT-360 for agitation instead of Vortex Genie 2, and ABW Alborex YS3A instead of Alborex Y.

Previous studies (Frame et al., 1994; Petolino et al., 2000) showed that the method of agitation obviously influences the efficiency of DNA delivery. Petolino et al. (2000) stated that rapid multidirectional oscillation is efficient in the SCW method. We also obtained similar results in the present study by comparing Vortex Genie 2 and MT-360, which agitate the mixture quite differently. That is, Vortex Genie 2 agitates the liquid content of a microtube by spinning it along the inside wall of the tube, indicating rotary agitation. MT-360, on the other hand, vibrates and stirs the liquid content of a microtube, indicating multidirectional agitation. The transient GUS expression assay in the present experiment also showed that multidirectional agitation with MT-360 is more efficient for DNA delivery compared with rotary agitation with Vortex Genie 2.

Wang et al. (1995) reported that transient GUS expression differs significantly with the source of SCW. They surmised that the surface characteristics of the whiskers and the possible presence of toxic byproducts of the manufacturing process may affect the transformation efficiency. We found that Alborex YS3A, which was produced by coating Alborex Y with aminosilane to strengthen the whiskers, was more suitable than Alborex Y. Therefore, as previously mentioned (Takahashi et al., 2000), we feel that the physical strength of the whiskers is one of the most important factors in any whisker-based method.

The present study and published report (Frame et al., 1994; Matsushita et al., 1999; Petolino et al., 2000; Takahashi et al., 2000) showed that the efficiency of DNA delivery in the whisker method is increased by increasing the duration of agitation, agitation speed, and/or amount of whiskers. However, an extremely prolonged duration of agitation, excessive agitation speed, and too large an amount of whiskers reduces the efficiency. This is likely due to severe cell damage, thereby triggering the loss of target cell viability (Frame et al., 1994; Petolino et al., 2000). Thus, it is obvious that, in the whisker method, the physical parameters, which may relate to the efficiency of collisions between the whiskers and target cells, have ambivalent effects. Accordingly, for further improvement of the whisker method, findings of the optimal balance among many physical characters are necessary.

Although we did not produce transformed plants in the present study, Brisibe et al. (2000) reported that whisker-mediated gene transfer is better for stable gene expression than the particle-bombardment method. This further improvement of the whisker method may lead to higher efficiency of stable transformation of target cells. Thus, we believe that the information obtained in this study will be helpful for the production of transgenic plants, including rice, by the ABW method.

References

Asano, Y., Otsuki, Y. and Ugaki, M. 1991. Electroporation-mediated and silicon carbide fiber-mediated DNA delivery in *Agrostis alba* L. (Red top). Plant Sci. 79 : 247-252.

Brisibe, A.E., Gajdosova, A., Olesen, A. and Andersen, S.B. 2000. Cytodifferentiation and transformation of embryogenic callus lines derived from anther culture of wheat. J. Exp. Bot. 51 : 187-196.

Dalton, S.J., Bettany, A.J.E., Timms, E. and Morris, P. 1998. Transgenic plants of *Lolium multiflorum*, *Lolium perenne*, *Festuca arundinacea* and *Agrostis stolonifera* by silicon carbide fibre-mediated transformation of cell suspension cultures. Plant Sci. 132 : 31-43.

Frame, B.R., Drayton, P.R., Bagnall, S.V., Lewnau, C.J., Bullock, W.P., Wilson, H.M., Dunwell, J.M., Thompson, J.A. and Wang, K. 1994. Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation. Plant J. 6 : 941-948.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6 : 3901-3907.

Kaeppler, H.F., Gu, W., Somers, D.A., Rines, H.W. and Cockburn, A.F. 1990. Silicon carbide fiber-mediated DNA delivery into plant cells. Plant Cell Rep. 9 : 415-418.

Kaeppler, H.F., Somers, D.A., Rines, H.W. and Cockburn, A.F. 1992. Silicon carbide fiber-mediated stable transformation of plant cells. Theor. Appl. Genet. 84 : 560-566.

Linssmaier, E.M. and Skoog, F. 1965. Organic growth factor requirement of tobacco tissue culture. Physiol. Plant. 18 : 100-127.

Matsushita, J., Otani, M., Wakita, Y., Tanaka, O. and Shimada, T. 1999. Transgenic plant regeneration through silicon carbide whisker-mediated transformation of rice (*Oryza sativa* L.). Breed. Sci. 49 : 21-26.

McElroy, D., Zhang, W., Cao, J. and Wu, R. 1990. Isolation of an efficient actin promoter for use in rice transformation. The Plant Cell. 2 : 163-171.

Nagatani, N., Honda, H., Shimada, T. and Kobayashi, T. 1997. DNA delivery into rice cells and transformation using silicon carbide whisker whiskers. Biotechnol. Tech. 11 : 471-473.

Omirulleh, S., Ismagulova, A., Karabaev, M., Meshi, T. and Iwabuchi, M. 1996. Silicon carbide fiber-mediated DNA delivery into cells of wheat (*Triticum aestivum* L.) mature embryos. Plant Cell Rep. 16 : 133-136.

Petolino, J.F., Hopkins, N.L., Kosegi, B.D. and Skokut, M. 2000. Whisker-mediated transformation of embryogenic callus of maize. Plant Cell Rep. 19 : 781-786.
Svensson, I., Artursson, E., Leanderson, P., Berglind, R. and Lindgren, F. 1997. Toxicity in vitro of some silicon carbides and silicon nitrides: whiskers and powders. Am. J. Ind. Med. 31: 335-343.

Takahashi, W., Shimada, T., Matsushita, J. and Tanaka, O. 2000. Aluminium borate whisker-mediated DNA delivery into callus of rice and production of transgenic rice plant. Plant Prod. Sci. 3: 219-224.

Vaughan, G.L., Jordan, J. and Karr, S. 1991. The toxicity, in vitro, of silicon carbide whiskers. Environ. Res. 56: 57-67.

Wang, K., Drayton, P., Frame, B., Dunwell, J. and Thompson, J. 1995. Whisker-mediated plant transformation: an alternative technology. In Vitro Cell. Dev. Biol. 31: 101-104.