Chapter 25
Direct Gene Transfer into Plant Mature Seeds via Electroporation After Vacuum Treatment

Takashi Hagio

1 Introduction

A number of direct gene transfer methods have been used successfully in plant genetic engineering, providing powerful tools to investigate fundamental and applied problems in plant biology (Chowrira et al., 1996; D'halluin et al., 1992; Morandini and Salamini, 2003; Rakoczy-Trojanowska, 2002; Songstad et al., 1995). In cereals, several methods have been found to be suitable for obtaining transgenic plant; these include bombardment of scutellum (Hagio et al., 1995) and inflorescence cultures (He et al., 2001), and silicon carbide fiber-mediated DNA delivery (Asano et al., 1991) and *Agrobacterium tumefaciens* transformation (Potrykus, 1990). Electroporation of cereal protoplasts also has proved successful but it involves prolonged cell treatments and generally is limited by the difficulties of regeneration from cereal protoplast cultures (Fromm et al., 1987). Many laboratories worldwide are now using *Agrobacterium* as a vehicle for routine production of transgenic crop plants. The primary application of the particle system (Klein et al., 1987) has been for transformation of species recalcitrant to conventional *Agrobacterium* (Binns, 1990) or protoplast methods. But these conventional methods can be applied to the species and varieties that are amenable to tissue culture (Machii et al., 1998). Mature seeds are readily available and free from the seasonal limits that immature embryo, inflorescence, and anther have. This method enables us to produce transgenic plants without time-consuming tissue culture process.

T. Hagio
National Institute of Agrobiological Sciences, Tsukuba,
Ibaraki 305-8602, Japan
e-mail: hagio94@nias.affrc.go.jp

H. Nakamura (ed.), *Electroporation and Sonoporation in Developmental Biology*,
DOI: 10.1007/978-4-431-09427-2_25, © Springer 2009

2 Procedure

Day 1

1. Mature seeds are precultured one day prior to gene transfer in germination buffer, which is composed of:

   - 0.2% Polyvinylpyrrolidone (PVP)
   - 0.2% Sodium hypochlorite solution (active chlorine: approx. 0.001%)

Day 2

2. The seeds are washed with sterile distilled water several times.
3. The seeds are soaked in 2 ml electroporation buffer in 6-cm petri dish.

Electroporation buffer composition:

- 910 µl Sterile distilled water
- 500 µl 1% Polyvinylpyrrolidone (PVP) solution
- 30 µl 10% Tween 20 solution
- 100 µl 0.1 M spermidine
- 200 µl Plasmid DNA solution(1 µg/µl TE buffer)
- 100 µl 10% Cellulase Onozuka RS(Yakult Honsha. Minato-ku Tokyo Japan)
- 160 µl 2.5 M Calcium chloride dihydrate

The total amount is 2000 µl (2 ml).

4. The petri dish containing the seeds soaked in the electroporation buffer is placed in a vacuum chamber (Fig. 25.1). The petri dish is placed on ice in the vacuum chamber.
5. The vacuum pump is turned on for about 10 min. The vacuum pump is turned off and the valves of the vacuum chamber are closed. The vacuum degree is about 0.09 MPa. The sample is kept under the vacuum condition for at least 1 h.
6. The valves of the vacuum chamber are opened and the petri dish is taken out. The seed and buffer are transferred into the electroporation chamber (Fig. 25.2). The width of the chamber is 1.0 cm and contains 1.0 ml of electroporation buffer. The electroporation chamber is placed on ice and connected with the electroporation device using the designated cables.
7. Electrical pulses are added (Figs. 25.1 and 25.3).

Electrical conditions:

- Voltage: 50–100 V/cm
- Pulse duration: 50–99 ms
- Pulse interval: 50–99 ms
- Pulse number: 50–99 times

8. After the electroporation, the chamber is placed on ice for at least 2 min. The seeds and buffer are transferred into a 6-cm petri dish. The sample is incubated under an appropriate condition depending on the plant species. For the stable transformation of wheat, the sample is incubated at 10°C in dark.

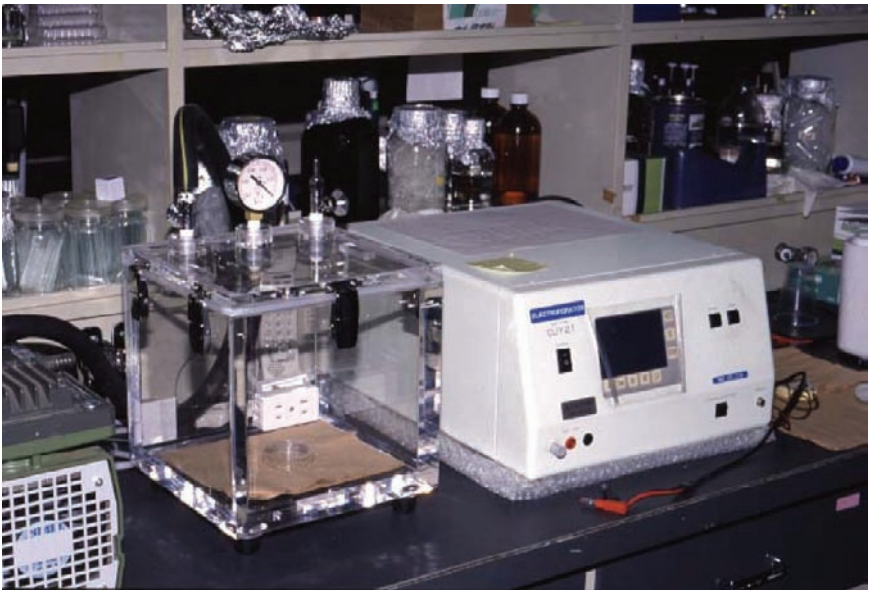

Fig. 25.1 A power supply for electroporation (*right*) and a vacuum chamber (*left*)

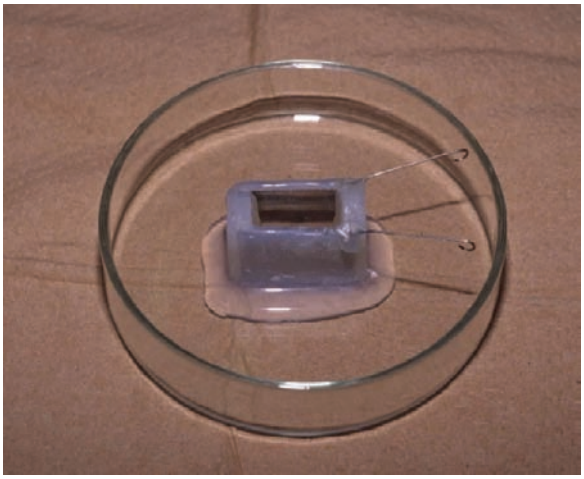

Fig. 25.2 Electroporation chamber. Electrodes are made of platinum. Approximately 20 wheat seeds and 30 rice seeds can be treated

Day 3

9. The incubation of the sample is continued.

Day 4

10. The assay of the transient gene expression is started by adding X-Gluc solution if GUS gene is introduced (Jefferson, 1987; Jefferson et al., 1987). If NPT

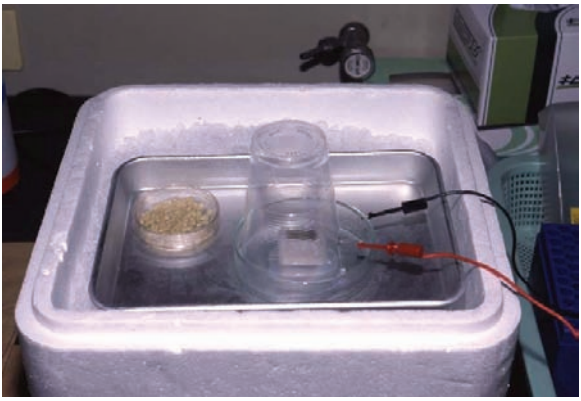

Fig. 25.3 Electroporation chamber placed on ice

II gene is introduced to obtain stably transformed plants, seeds are incubated in the selection medium containing approximately 200 ppm G418 (Chawla, 2002). A toxicity test is recommended to determine the optimum concentration of G418. The strength of G418 varies depending on the manufacturer and/or manufacturing lot. Stably transformed wheat plants were produced using this procedure.

3 Application and Results

To determine the physical parameter, plasmid pWI-GUS, which was fully described by Ugaki et al. (1991), was used. It has a β-glucuronidase (GUS) gene as a reporter. Typically, 20 mature seeds of wheat were soaked in germination buffer and incubated at 10°C in the dark overnight. Then the seeds were incubated in an electroporation buffer containing pWI-GUS plasmid for 3 h with or without the condition of reduced air pressure. Electroporation was carried out with an electroporation device (CUY-21, NEPA GENE, Chiba Japan) (Figs. 25.2 and 25.3) that generates rectangular electric pulses, in a 1.0-cm-wide cuvette containing 1.0 ml of electroporation buffer. Rectangular wave pulses with various field strength, pulse length, and pulse numbers were applied to samples. The optimal electrical conditions for DNA delivery into wheat was estimated to be around 50 V/cm, 50 ms pulse duration, and 99 pulses. After electroporation, the seeds were incubated in a germination buffer for 2 days, after which they were transferred to a GUS assay buffer (Jefferson, 1987). When the seeds were electroporated after depressurization in a vacuum chamber, significantly higher GUS activity was detected (Fig. 25.4). Depressurization treatment prior to electroporation is crucial because it may enhance transport of the buffer into the intercellular space (Figs. 25.4 and 25.5).

To obtain stably transformed wheat and rice plant, pWI-H5K plasmid (Ugaki et al., 1994) was used. The plasmid was derived from pWI-GUS, which is fully

Fig. 25.4 GUS gene expression on wheat mature seeds. pWI-GUS Plasmid was introduced. (**a**) Depressurization pretreatment was made prior to electroporation. (**b**) Depressurization pretreatment was not made prior to electroporation. (**c**) Nontransformed control

described by Ugaki et al. (1991). The GUS gene cassette was removed from pWI-GUS and neomycin phosphotransferase NPT II gene was inserted into the plasmid as a selective marker. One hundred mature seeds of wheat were soaked in germination buffer and incubated at 10°C in the dark for 2 days. Then the seeds were incubated in electroporation buffer for 3 h under the condition of reduced air pressure. In one treatment 20 seeds were placed in an electroporation cuvette and electroporation was performed. Buffer composition and the physical parameters generally were the same as in the transient expression experiment mentioned above. After electroporation, the seeds were incubated in germination buffer for 2 days at 10°C Then the seeds were transferred to a selection medium containing 200 ppm geneticin sulfate (GIBCO) and incubated for 2 weeks in a growth cabinet. After the selection culture, germinated seeds were transplanted into pots with soil and were grown in growth chambers. During the heading period, fresh leaves were collected and genome DNA was extracted for PCR and Southern analysis to detect the introduced NPT II gene. In the wheat experiment, approximately 10,000 seeds were treated and 53 geneticin-resistant plants were regenerated. PCR analysis showed that 32 plants showed positive. Of these, 32 transformants, DNA samples from eight plants, to date were tested in Southern hybridization (Fig. 25.7) . There are high copy numbers of transgene integration in some plants. This may indicate that multiple copies of the transgene were present in the integration locus. The eight plants normally reached maturity and self-fertilized seeds have been obtained (Fig. 25.6). Two independent T_1 lines were examined for the transmission of NPT II gene by PCR analysis (Fig. 25.8). For each line, ten seeds were randomly chosen and seeded in a pot. Genome DNA was extracted from the seedlings and PCR analysis was made. Positive response was observed (Fig. 25.8) in all 20 samples. This confirmed the transmission of the introduced NPT II gene. These experiments have demonstrated that DNA has been delivered into the wheat mature embryos of mature seeds via electroporation with the help of depressurization treatment, and transgenic plants have been recovered. In rice experiments we obtained similar results (data not shown).

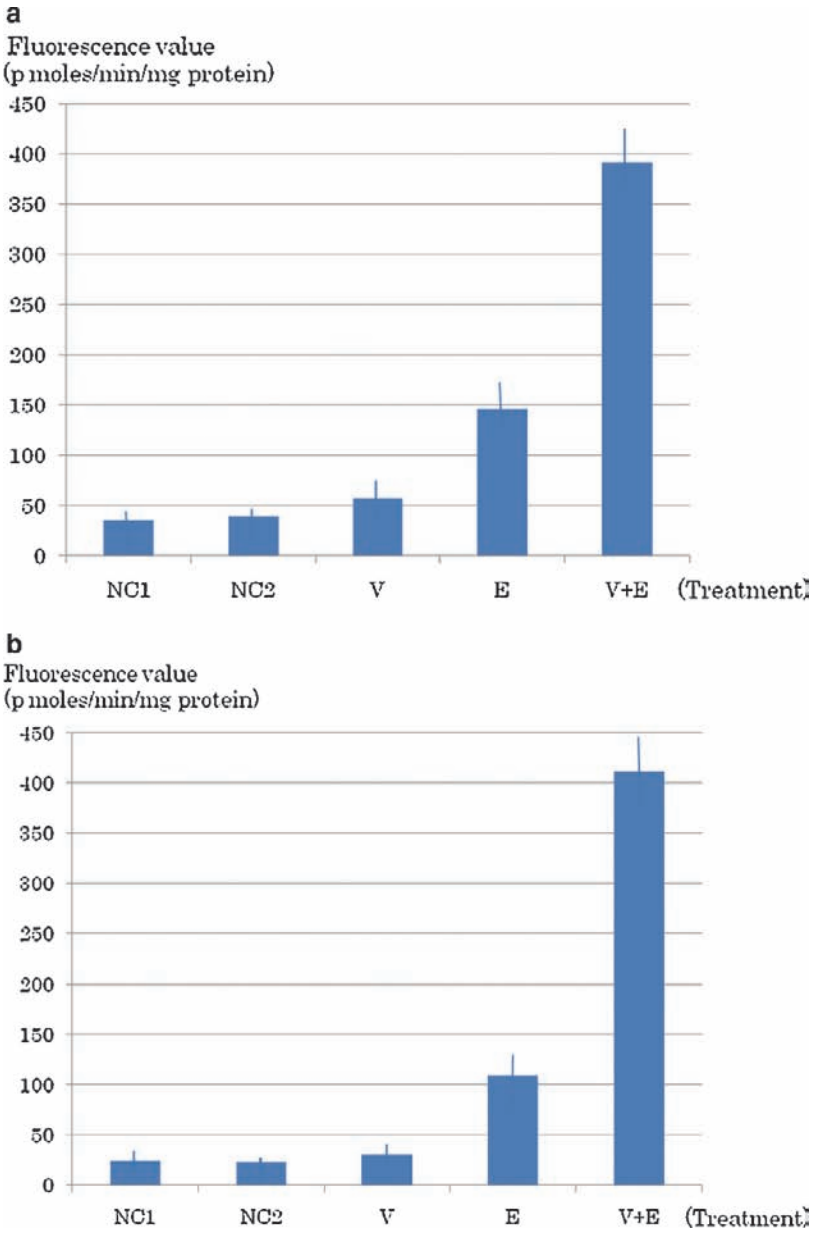

Fig. 25.5 Fluorometric assay of GUS gene expression in wheat (**a**) and rice (**b**). NC1: Negative control No.1 (the seeds were soaked in the electroporation buffer without containing plasmid DNA). NC2: Negative control No.2 (the seeds were soaked in the electroporation buffer containing plasmid DNA). V: The seeds were soaked in the electroporation buffer containing plasmid DNA and the vacuum treatment was made. E: The seeds were soaked in the electroporation buffer containing plasmid DNA before electroporation. V + E: The seeds were soaked in the electroporation buffer containing plasmid DNA and the vacuum treatment was performed before electroporation. Bar indicates standard error

Fig. 25.6 Fertile transgenic wheat

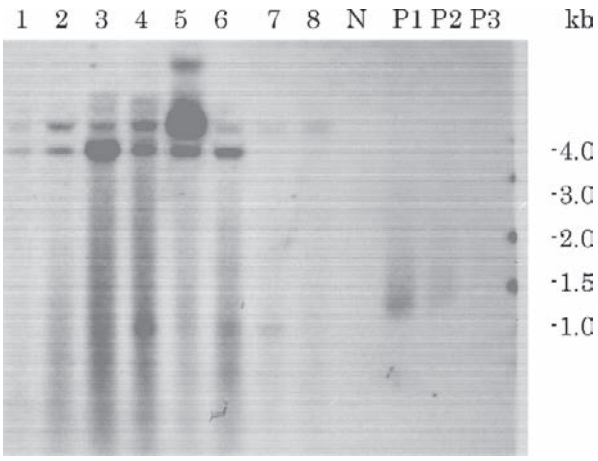

Fig. 25.7 Southern hybridization analysis of T_0 plants. Lane 1–8: DNA of T_0 plants digested with Xho I N: DNA of nontransgenic plant digested with Xho I ; P1–P3: fragment of NPT II (approx 1.0 kb) gene derived from the plasmid pWI H5K; P1: approximately equivalent to 10 copies of NPT II fragment; P2: approximately equivalent to two copies of NPT II fragment; P3: approximately equivalent to 1 copy NPT II fragment

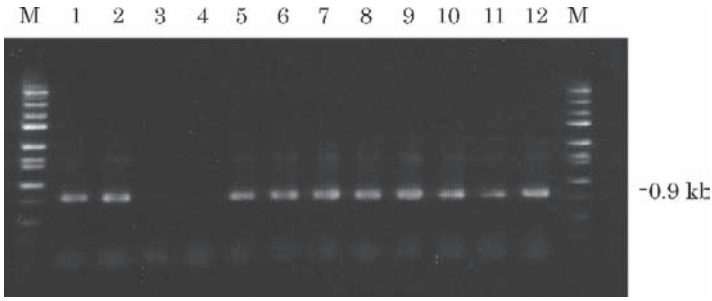

Fig. 25.8 PCR analysis of NPT II gene in the presence of T_1 plants. M: marker, lane1: positive control with plasmid pWI-H5K; lane 2: positive control with plasmid pWI-H5K and nontransgenic plant DNA; lane 3: negative control with water; lane 4: negative control with nontransgenic plant DNA; lane 5–8: T_1 plants from T_0 line No.1; lane 9–12: T_1 plants from T_0 line No.2

We also observed transient GUS gene expression in seeds of soybean, tomato, and *Brassica*, using pUC-derived plasmids (Chawla et al., 2002). Conditions of electroporation and the buffer contents were the same as in the case of wheat and rice. In about 10% of soybean seeds and 15% of *Brassica* and tomato seeds, blue loci, which were the indicative of transient GUS expression, were visually observed mainly in the shoot tip area.

This simple method of gene transfer in plants will be a promising direction for basic research and biotechnological applications. It has good potential for gene transfer to walled cells. A number of DNA delivery techniques have been developed for cereal transformation (Walden and Wingender, 1995). They include direct gene transfer by particle bombardment and *Agrobacterium*-mediated transformation. Successful results have been obtained by using these methods, but the methods are still not sufficiently reliable to be applied to a wide spectrum of plant species and/or varieties. Electroporation-mediated direct gene transfer has been developed and has been used in generating transgenic plants (Sorokin et al., 2000). So far, mostly protoplasts have been the target of direct gene transfer, and fewer reports on *in planta* transformation have been published (Sinclair et al., 2004). Target tissues of *in planta* transformation were immature embryos, nodal buds, inflorescences, and so forth, but using mature seeds with the help of depressurization treatment has yet to be reported.

Transgenic plant regeneration has been established using mature seeds of wheat and rice. Wheat and rice are the first- and/or second-ranking cereals by weight (FAO, 2002). Cereal breeding programs will benefit from new procedures for the generation of varieties with improved disease resistance, enhanced stress tolerance, and increased yield. Genetic manipulation will make significant contributions to these programs. The wheat transformation procedure presented here does not require the establishment of genotype-dependent tissue culture of immature embryos (Machii et al., 1998). To obtain immature embryos, plants need to be grown in fields or greenhouses for 5 to 8 months. This method may be especially useful for DNA delivery into the plant species that have poor tissue culture response. At present, this technique is dependent on a combination of factors including pretreatment of the targeted materials, plasmid constructs, and optimization of electroporation conditions. This method is simpler

and more rapid than conventional techniques, and it can be applied to a wide range of commercial rice and wheat varieties. These results also indicate that this technique has a potentiality of transferring foreign DNA into tissues of a wide range of organisms. Further study is in progress to improve this method in our laboratory.

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