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## **Production of transgenic rice by introducing rice dwarf virus genome segment 10 cDNA.**

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### **Introduction**

Recent work has demonstrated that transformation of plant cells by viral genome cDNAs induces resistance to virus infection. *Agrobacterium tumefaciens* Ti-plasmid-mediated DNA transfer is routinely employed in these experiments. Unfortunately, use of *A. tumefaciens* infection has been limited to dicots and it is very difficult to use in monocots. Most of transgenic plants to which virus resistance was introduced are dicots plants so far.

Rice dwarf virus (RDV) belongs to *Phytoreovirus* and has a genome comprising 12 segmented double-stranded RNAs. Because RDV infects only monocot plants, it is difficult to produce transgenic plants resistant to virus infections by using the technique of Ti-plasmid-mediated DNA transfer. It has been shown that foreign DNAs can be introduced into a plant genome via direct gene transfer into protoplasts without help of *A. tumefaciens* and that this DNA is stably incorporated into the nuclear genome. But this technique requires plant regeneration at high frequency from protoplasts. A method for isolating protoplasts from rice callus has already been developed in our laboratory.

We attempted to introduce the cDNA to the genome segment 10 (S10) of RDV into rice protoplasts by an electroporation technique for the direct gene transfer. And this is the first report of producing transgenic rice by introducing RDV genome.

### **Materials and Methods**

#### **Protoplasts isolation**

Protoplasts were isolated from suspension culture of *Oryza sativa* L., cv. Yûkara. The suspension culture was derived from mature embryo callus. It was used 3-5 days after transfer and resuspended in an enzyme solution consisting of 4% Cellulase RS (Yakult), 1% Macerozyme R10 (Yakult), 0.5% MES pH 5.6 and

0.4 M mannitol according to the method by Kyojuka *et al.*<sup>2)</sup>. Protoplasts were resuspended in a 2 x concentrated electroporation buffer.

### Plasmids

Plasmid pCaRD10 contained the full-length cDNA of RDV genome S10 derived from pUpRD28<sup>3)</sup> down stream of the CaMV 35S promoter. A plasmid pUC19-35S-HPT was kindly provided by Dr. Hirano (National Institute of Genetics). This plasmid contained CaMV 35S promoter and hygromycin phosphotransferase (HPT) gene and was used as a selectable marker. These plasmids were linearized with *Dra* I before dissolved in an electroporation buffer.

### Electroporation

Protoplasts ( $4 \times 10^6$ /ml), plasmid DNAs (20  $\mu$ g/ml) and calf thymus DNA (50  $\mu$ g/ml) were resuspended in an electroporation buffer.

Three electroporation buffers were tested in this work. First one contained 70 mM KCl, 5 mM MgCl<sub>2</sub>, 0.1% MES pH 5.8, and 0.4 M mannitol (referred to as the MES buffer type I), according to Shimamoto *et al.*<sup>5)</sup>; second one 7 mM KCl, 4 mM CaCl<sub>2</sub>, 0.5 mM MES pH 5.8, and 6.5% mannitol (referred to as the MES buffer type II), according to Toriyama *et al.*<sup>7)</sup>; and the last one 70 mM aspartic acid monopotassium salt, 5 mM calcium gluconate, 5 mM MES pH 5.8 and 0.4 M mannitol (referred to as the ASP buffer), according to Tada *et al.*<sup>6)</sup>.

Samples were given an electrical pulse in the chamber of gene pulser (0.8 ml, 0.4 mm distance, Bio-Rad). Electrical pulses tested were at between 100 V/cm and 1000 V/cm (hand-made product). The time constants were tested for between 10 msec and 60 msec and they were measured by a storage scope (Iwatu Ds-8600). Protoplasts before and after electroporation were incubated on ice for 20 minutes.

### Selection of hygromycin resistant colonies

Electroporated protoplasts were cultured and regenerated according to the method by Kyojuka *et al.*,<sup>2)</sup>. Protoplasts were incubated for 2 weeks with hygromycin-free medium, then transferred to the liquid medium containing 20  $\mu$ g/ml hygromycin. After further incubation for 2 weeks, callus were transferred to an agar plate containing 30  $\mu$ g/ml hygromycin. Hygromycin resistant colonies were picked up onto an agar plate containing 50  $\mu$ g/ml hygromycin.

### DNA isolation and PCR detection

Rice genome DNA was isolated from one gram of leaves using a published procedure<sup>4)</sup>. These genome DNAs were digested with *Pst* I and 0.5  $\mu$ g of them were used for polymerase chain reaction (PCR). PCR was done using synthetic primers complementary to the terminal portions of RDV genome S10<sup>3)</sup> and a Gene Amplification kit (TaKaRa). The PCR products were electrophoresed on a 1.5%

agarose gel.

### Southern blot analysis

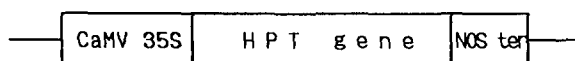
Fifteen  $\mu\text{g}$  of total DNA was digested with *Pst* I and electrophoresed on a 1.5% agarose gel and transferred to nylon membranes (Hybond N+, Amersham Co.). Hybridization was carried out using DIG Luminescent detection kit (Boehringer Mannheim). Plasmid pCaRD10 was used as a probe.

## Result

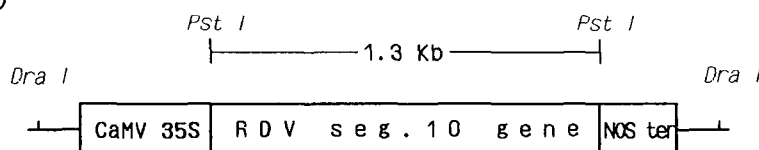
### Selection of hygromycin resistant colonies and transformation frequency

Hygromycin resistant colonies were obtained from both MES buffer type II and ASP buffer but no resistant callus was obtained from MES buffer type I (Table I). The ASP buffer was superior to MES buffer type II with respect to

(a)



(b)



**Fig. 1.** Diagram of pUC19-35S-HPT (a) and pCaRD10 (b). The restriction sites used in this work are indicated.

**Table 1.** Colony production and transformation frequency rice protoplast following electroporation with different buffers of rice protoplasts.

Buffers*	% of protoplasts dividing at day 10	Number of colonies from protoplasts 28 days after electroporation in the medium with 20 $\mu\text{g}/\text{ml}$ hygromycin	transformation frequency ( $\times 10^6$ )**
MES buffer type I	18.5	0	0
MES buffer type II	26.2	6	1.87
ASP buffer	14.2	55	17.1

Transformation frequency = number of colonies on medium containing hygromycin compared to the number of protoplasts plated.

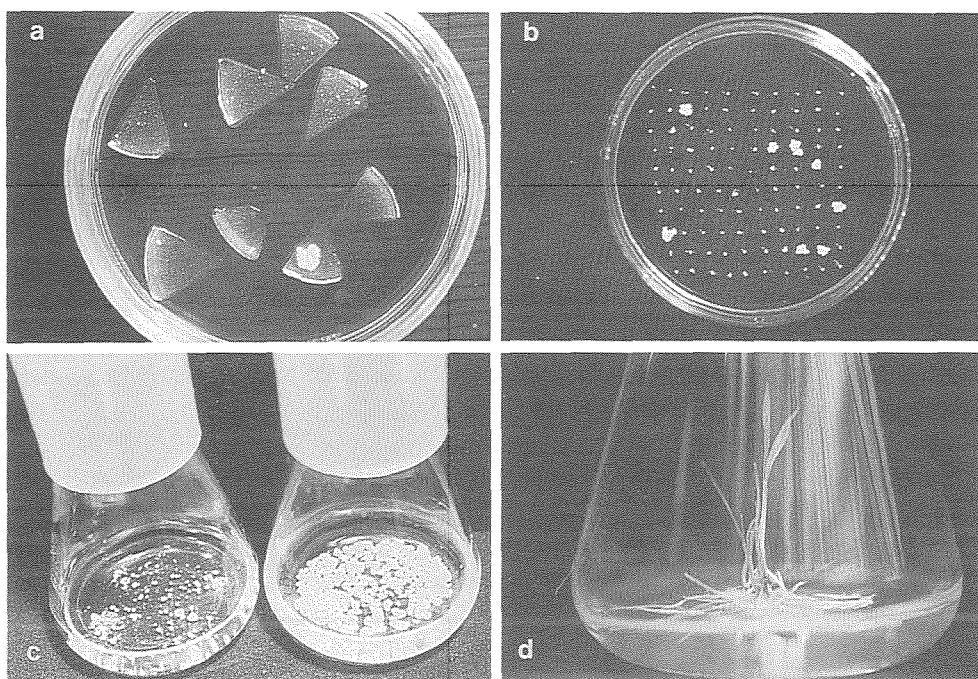
\* = buffer conditions were described in the text.

\*\* = (involved)  $3.2 \times 10^6$  protoplasts.

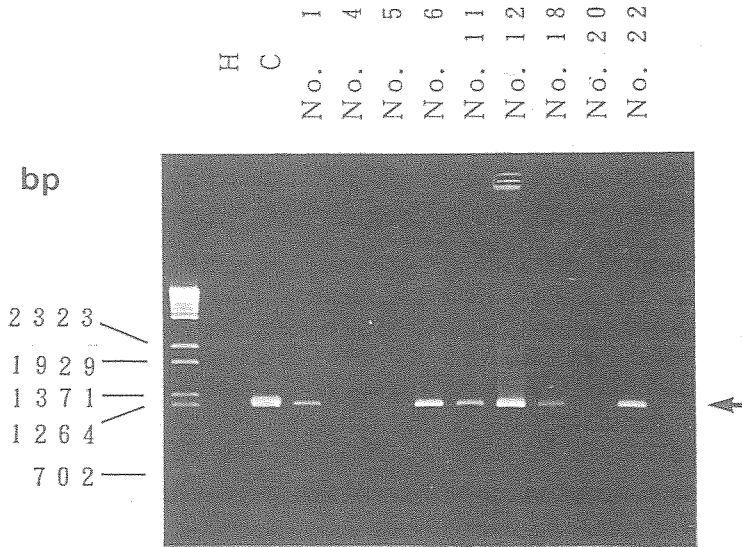
transformation efficiency. The most effective electrical condition was obtained by initial voltage at 600 V/cm and time constant for 28 msec with ASP buffer.

The use of hygromycin was found to be effective for selecting transformants in rice, because untransformed callus was unable to grow in the media containing antibiotics. Their cell division was inhibited by 20  $\mu\text{g/ml}$  of hygromycin when they were exposed to the antibiotic after 14 days of culturing (Fig. 2a). One month old callus was inhibited by hygromycin at a concentration of 50  $\mu\text{g/ml}$  (Fig. 2b). All of the hygromycin resistant colonies continued to grow when medium was replaced with a fresh liquid medium containing 100  $\mu\text{g/ml}$  hygromycin (Fig. 2c).

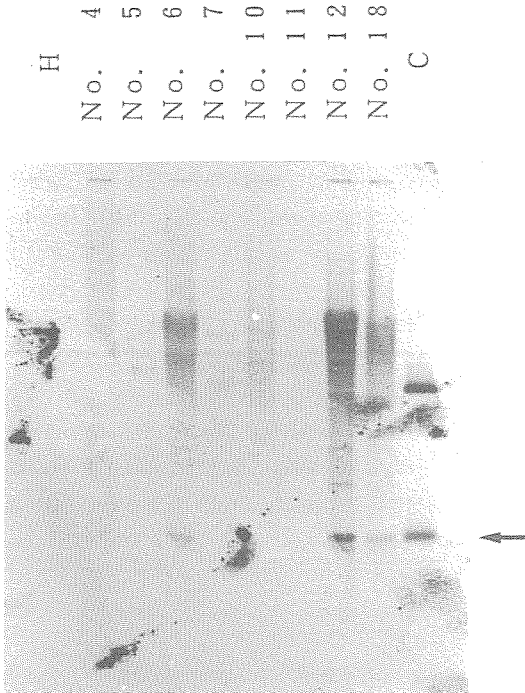
The colony formation in the medium containing hygromycin occurred following electroporation, producing 55 hygromycin resistant colonies from  $3.2 \times 10^6$  plasmid treated protoplasts. Twenty-four of 55 hygromycin resistant colonies were regenerated (Fig. 2d).



**Fig. 2.** Selection and regeneration of hygromycin resistant colonies. Protoplasts were electroporated in the presence of plasmid DNA (pCaRD10 and pUC19-35S-HPT) and selected with hygromycin as described in the text.  
a. Three weeks after electroporation and selected by 20  $\mu\text{g/ml}$  hygromycin.  
b. Callus growth on agar plate containing 30  $\mu\text{g/ml}$  hygromycin.  
c. Suspension culture of callus from electroporated (right) and unelectroporated (left) in the liquid medium containing 100  $\mu\text{g/ml}$  hygromycin.  
d. Regeneration from hygromycin resistant calli.



**Fig. 3.** Detection of amplified RDV genome segment 10 fragment from hygromycin resistant rice genome DNA. Lane C : PCR product from plasmid pCaRD10. Lane H : PCR product from untransformed rice total DNA. Numbers refer to individual regenerated plants. Arrow indicates the position of 1300 bp.



**Fig. 4.** Southern blot analysis of DNA extracted from plants regenerated from hygromycin-resistant calli. Lane H : Untransformed. Lane C : Plasmid pCaRD10 as a molecular weight standard. Numbers refer to individual regenerated plants. Arrow indicates the position of 1300 bp.

### Confirmation of transformation

Six of genomic DNAs from hygromycin resistant transgenic plants were amplified by polymerase chain reaction (Fig. 3). These amplified DNA fragments were comigrated with 1.3 Kb RDV genome S10 full length cDNA fragment after *Pst* I digestion. A signal was detected at the position corresponding to the 1.3 Kb *Pst* I digested RDV genome S10 cDNA fragment by genomic southern hybridization (Fig. 4).

### Discussion

The transgenic plants using gene transfer techniques are important for both investigation of gene function and genetic engineering of crops. Many plant viruses which infect dicot plants have been already used to introduce their genome cDNAs into host plant cells by using Ti plasmid of *A. tumefaciens*, and these transgenic plants have been shown to acquire resistance to the virus infection. But Ti-plasmid mediated transformation is applicable to most dicots and few monocots, so we tried direct gene transfer techniques for production of transformed rice plants by RDV genome cDNA to investigate genome functions.

Some experiments that produced transgenic rice have been already reported<sup>5,6,7,8)</sup>. There are two pulse forms of electroporation used. One is long square pulse with low voltage and the other is exponential pulse with high voltage. Preliminary experiments have suggested that the exponential pulse was commonly used for stable transformation. All reported conditions of exponential pulse were tried. The high transformation efficiency was obtained only by one condition (table 1).

Recent work on antibiotics used for selecting transformed rice cells showed that the high natural levels of resistance to kanamycin, which was used commonly as a selectable marker in dicots, existed in cultured cells of rice<sup>1)</sup>. And it may limit the use of this antibiotics for selecting transformed cells. In our experiments, hygromycin was used instead of kanamycin and it was effective for selecting transformed cells (Fig. 2).

A half of the hygromycin resistant colonies regenerated to plants. Regeneration efficiency of electroporated colonies was the same as that of unelectroporated colonies.

In this report, we established the condition of efficient transformation of rice and obtained transgenic rice plants by RDV genome S10. Investigating these transgenic plants will become an effective measure to make clear understanding of the function of RDV genome segments.

### Summary

We tried to transfer the RDV genome S10 to rice protoplasts by using electroporation system. Electroporation buffer, pulse voltage and the time constant during electroporation were found to be critical in obtaining the optimum transformation. The best condition of an electrical pulse, when used ASP electroporation buffer, was charging at 600 V/cm (hand-made product) and using the time constant of 28 msec ( $T=1/2$ ). A hygromycin resistance gene pUC19-35S-HPT was co-transformed as a selectable marker. We obtained 55 lines of hygromycin resistant calli from  $3.2 \times 10^6$  protoplasts. Two thirds of the regenerated rice plants from these hygromycin resistant cell lines were found to contain RDV genome S10 by a PCR method and genomic southern hybridization. We thus succeeded in establishing the condition of electroporation for transferring RDV genome segments to rice protoplasts and obtaining transgenic rice plants.

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