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Preparation of an Endosperm Protoplasts from a Dwarf Rice Variety and Transient Expression of Green-Fluorescent Protein

Hideyuki TAKAHASHI[1], Shigeto MORITA[1,2], Takehiro MASUMURA[1,2] and Kunisuke TANAKA[1,2]*

[1]*Laboratory of Genetic Engineering, Graduate School of Agriculture, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan*
[2]*Kyoto Prefectural Institute of Agricultural Biotechnology, Seika, Kyoto 619-0244, Japan*
* *Corresponding author:* E-mail address: ktanaka@kab.seika.kyoto.jp

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Abstract

Vital protoplasts were isolated from developing dwarf rice endosperms about 10 days post anthesis. A yield of $0.98 \times 10^5 \pm 0.12$ protoplasts was obtained from 10 developing grains. These protoplasts were viable for at least three days in the culture medium. Transient expression of the green-fluorescent protein (GFP) was detected after four hours of introduction of the GFP gene by the polyethylene glycol (PEG)-mediated DNA transfer. As expected, the fluorescence of GFP without the targeting sequence was observed diffusely in the cytoplasm. On the other hand, the fluorescence of the fusion protein for rice prolamin followed by the GFP was observed in the small particles and the ER-network. These endosperm protoplasts had no autofluorescence upon excitation with 490 nm (blue) light. The vital protoplasts obtained from this dwarf rice variety are very useful for the physiological, biochemical and molecular biology studies of rice endosperm.

Key words: endosperm, GFP, protoplasts.

Abbreviations

CaMV, cauliflower mosaic virus; CPW salts, protoplast washing media; GFP, green-fluorescent protein; NOS, nopaline synthase; PEG, polyethylene glycol; SOD, superoxide dismutase.

Introduction

Rice is the most important cereal crop because it serves as the staple food for a large population worldwide and is used as a model plant for research due to its small genome size. The information on the rice genome would be of useful for other important cereals, such as wheat, barley, corn and sorghum, considering the existence of the synteny among them. Thus rice has already been analyzed extensively and an enormous amount of information is available on the structure, the genetics and the physiology. However, rice is still required to be improved concerning the seed quality, especially the nutritional value and the taste. To improve the seed quality, we must study about the accumulation process of storage substances, especially in the endosperm, during the developmental stages. To study the mechanisms underlying the events that occur in the development of rice endosperm, use of developing endosperm protoplasts is preferable if the materials are available. The isolation of plant protoplasts has already been achieved in various tissues from numerous species. However, only a limited number of techniques to isolate cereal endosperm protoplasts have been reported, to develop maize (Schwall and Feix, 1988), wheat (Keeling *et al.*, 1989), and barley grain (Lee *et al.*, 1991; Diaz and Carbonero, 1992). However, no methods for isolation of protoplasts from the endosperm tissues of developing rice grains have yet been published. This might be due to the extremely fragile nature of the developing endosperm cells of rice. We report here a procedure by which to obtain vital protoplasts that would be useful for physiological, biochemical, and/or molecular biology researches of rice.

Transient expression techniques using protoplasts have been used to study gene expression in monocotyledonous as well as dicotyledonous plant species. The green-fluorescent protein (GFP) of the jellyfish *Aequorea victoria* is widely used in studies of protein translocation as a reporter that can be directly visualized in the living cells under a fluorescence microscope. The plant-adapted sGFP

(S65T) possesses a number of favorable traits as a universal reporter in living cells, and requires only 490 nm (blue) light for green fluorescence emission i.e. no exogenous substrate (Chiu *et al.,* 1996).

Here, we report the isolation of protoplasts from developing rice endosperms and the transient expression of the sGFP(S65T) reporter gene in the protoplasts. We also report the transient expression of the fusion gene for rice prolamin followed by the sGFP(S65T). Throughout this study, we used a rice dwarf mutant (*Oryza sativa* L. cv Hosetsu d.). The size of this rice plant is very small (about 20 cm in height, which is about one-fourth as tall as the ordinal rice variety, Nipponbare), and its life cycle is short (about 2.5 months). It can be grown easily and year-round in a growth chamber under fluorescent lamps of 170 μmol m^{-2} s^{-1} light intensity at 28℃ (Kurita *et al.,* 2002). These features make this variety very useful as an experimental rice model for studying the mechanisms of seed development. Furthermore, the transient expression of GFP in protoplasts from the endosperm of this variety would be a powerful tool for physiological, biochemical, and molecular biology studies of the events occurring inside the developing cells of rice endosperm.

Materials and Methods

Plant material

Rice dwarf mutant (*Oryza sativa* L. cv Hosetsu d.) was sown in plastic pots filled with soil from the experimental farm of Kyoto Prefectural Institute of Agricultural Biotechnology and commercial nutrients (HYPONeX; Hyponex Japan, Osaka, Japan). The plants were grown in a growth chamber under fluorescent lamps of 170 μmol m^{-2} s^{-1} under a 16 h light/8 h dark regime at a temperature of 28℃.

Isolation of protoplasts from developing rice endosperm

Rice grains at an early developmental stage (about 10 days post anthesis) grown under fluorescent light were washed with 70% (v/v) ethanol for 30 s, then surface-sterilized in 20% (v/v) antiformin for 30 min. After several washes in sterile distilled water, the hull, embryo, and pericarp were removed from the grains using a razor blade and forceps under aseptic conditions. This endosperm-rich tissue was sliced into transverse sections, and the sections were plasmolyzed for 1 h in protoplast washing media (CPW salts) [27.2 mg l^{-1} KH_2PO_4, 101 mg l^{-1} KNO_3, 1480 mg l^{-1} $CaCl_2 \cdot 2H_2O$, 246 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 0.16 mg l^{-1} KI, 0.025 mg l^{-1} $CuSO_4 \cdot 5H_2O$, (pH 5.8)] (Frearson *et al.,* 1973; Power and Chapman, 1985) with 11% (w/v) mannitol as osmoticum (CPW11M). After this treatment, endosperms were incubated in the dark at 28℃ for three hours in the same medium containing 1% (w/v) Cellulase Onozuka RS and 0.2% (w/v) Macerozyme R-10, pH 5.8, without shaking. The digested tissue was then filtered through a nylon sieve (250 μm pore size). The sediment in the filtrate was allowed to settle for 15 min. The supernatant was then transferred to a new tube and centrifuged at 40g for 3 min. After the sedimented protoplasts were removed, the supernatant was centrifuged again under the same condition. The two pelleted fractions by the consecutive centrifugation were combined to make the initially sedimented protoplast fraction. These protoplast fractions were resuspended in CPW11M and centrifuged (200*g*, 5 min) onto 70% Percoll cushion (CPW salts containing 70% (v/v) Percoll and 9.5% (w/v) mannitol) in order to purify the protoplasts. The protoplast fraction was carefully collected at the Percoll interface and resuspended in hormone-free R2 medium [150 mg l^{-1} $CaCl_2 \cdot 2H_2O$, 4,000 mg l^{-1} KNO_3, 218 mg l^{-1} KH_2PO_4, 250 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 3 mg l^{-1} H_3BO_3, 0.125 mg l^{-1} $Na_2MoO_4 \cdot 2H_2O$, 1.7 mg l^{-1} $MnSO_4 \cdot 4H_2O$, 0.125 mg l^{-1} $CuSO_4 \cdot 5H_2O$, 2 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 8.5 mg l^{-1} Fe-EDTA, 100 mg l^{-1} *myo*-Inositol, 1 mg l^{-1} Thiamin-HCl, 0.5 mg l^{-1} Nicotinic acid, 0.5 mg l^{-1} Pyridoxin-HCl, 2 mg l^{-1} L-Glycine, 9% (w/v) mannitol, 3% (w/v) sucrose, (pH 5.8)]. After centrifugation (40*g*, 3 min), the pellet was resuspended in the same medium. All procedures, especially the resuspending process, mixing, and pipetting of the material, were conducted very carefully. Shaking of the tube is significantly damaging, resulting in a decreased recovery.

PEG-mediated DNA transfer into protoplasts

In this process we followed basically Lee's method (1991). Plasmid uptake into protoplasts was mediated by polyethylene glycol (PEG). Aliquots of 250 μl of the protoplast suspension (containing 0.5-1.5x10^5 protoplasts) were mixed with 30 μg of plasmid DNA. A solution of 40% (w/v) PEG 3350 containing 0.4 M mannitol and 0.1 M $Ca(NO_3)_2 \cdot 4H_2O$ (pH 8.5) was added to the protoplast-DNA mixture to give a final concentration of 15% PEG and was mixed very gently. The mixture was allowed to stand for 20 min, then 1 ml of W_5 solution (154 mM NaCl, 5 mM KCl, 5 mM glucose, 125 mM $CaCl_2 \cdot 2H_2O$, pH 5.8) (Negrutiu *et al.,* 1987) was added at 20 min intervals to reach a final volume of 5 ml. The tubes were then centrifuged at 40*g* for 3 min, and the protoplast pellets were resuspended in

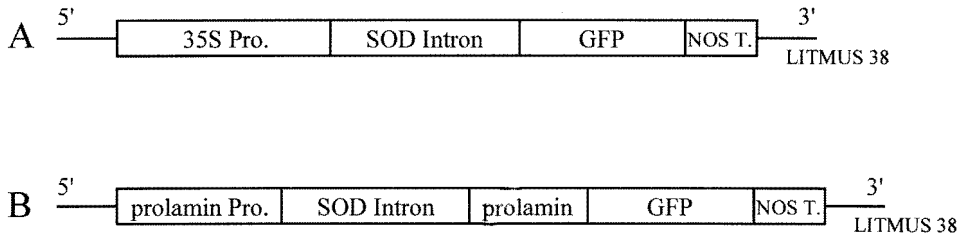

Fig. 1 Schematic illustrations of the structure of chimeric gene constructs used for the transient expression in rice endosperm protoplasts. (A) The plant-adapted sGFP(S65T) was inserted into pLITMUS 38 together with the CaMV 35S promoter and the rice cytosolic SOD first intron. (B) The plant-adapted sGFP(S65T) was inserted into pLITMUS 38 together with the rice prolamin promoter, the coding region for rice prolamin, and the rice cytosolic SOD first intron. 35S Pro., cauliflower mosaic virus 35S promoter; SOD intron, rice *SodCc2* first intron; GFP, sGFP(S65T); NOS T., nucleotide sequence for the polyadenylation signal in a nopaline synthase gene.

R2 medium and incubated in the dark at 28℃.

DNA construct

pLITMUS 38 contains the plant-adapted sGFP (S65T) reporter gene (Chiu *et al.,* 1996) under the control of the CaMV 35S promoter and the polyadenylation signal of the nopaline synthase (NOS) gene. The superoxide dismutase (SOD) *Cc2* first intron fragment (Sakamoto *et al.,* 1995) was inserted between the promoter and the sGFP(S65T) gene to enhance gene expression of GFP (**Fig. 1A**). Based on the plasmid, a fusion gene for rice prolamin followed by the sGFP(S65T) was constructed. To generate a fusion gene, the coding region for rice 13 kDa prolamin (λ *RM1*) (Mitsukawa *et al.,* 1999) was amplified by PCR using the primer set (forward, 5′-GCGGAGGGATCCATGAAGATCATTTTCGT ATTTGCTC-3′; reverse, 5′-GCGGAGGGATCCG CCGCCGCCGTACCAGACACCACCAACGG-3′).The fragment was inserted into the *Bam*HI site to the N terminus of the GFP-coding region. Furthermore, the CaMV 35S promoter was converted into the rice prolamin promoter. The 5′ flanking region from -840 to -44 of the genomic clone encoding rice 10 kDa prolamin polypeptides (Feng *et al.,* 1990) was used as the rice 10 kDa prolamin promoter (**Fig. 1B**).

Results and Discussion

Isolation of endosperm protoplasts

The procedure described here allows sufficiently high yields of metabolically active protoplasts from developing rice endosperm. The protoplast yield was dependent on the age of the endosperm tissue. Endosperm at about 10 days post anthesis was most suitable for the starting material of this procedure. A decrease in yield and viability of protoplasts obtained from later stage endosperm, due to starch increase, has been reported in maize (Schwall and Feix, 1988), wheat (Keeling *et al.,* 1989), and barley (Diaz and Carbonero, 1992).

The centrifugation of digested rice endosperm preparations on Percoll cushion provided an effective and rapid means to separate intact protoplasts from starch granules and other cell debris. Because the release of protoplasts starts from the central area of the endosperm tissue, as is the case for maize (Gallie and Young, 1994), contamination of the protoplasts by aleurone tissue was prevented by removing the endosperm slices before starting digestion of aleurone tissue. Actually, no release of protoplasts from the surface area (testa tissue) of the rice grain was observed, because the surface area of rice grain, in comparison with the internal area, is significantly resistant to the digestion treatment. A yield of $0.98 \times 10^5 \pm 0.12$ (mean of three different experiments) from 10 grains was obtained with this procedure. Freshly isolated protoplasts from grains of the same age varied considerably in size (20 to 40 μm in diameter) and starch content (**Fig. 2A**). The protoplasts were shown to be viable at above 80% using fluorescein diacetate (FDA) staining (Widholm, 1972) (**Fig. 2B**). These protoplasts were viable for at least three days in hormone-free R2 medium.

Transient expression assay of endosperm protoplasts

In order to establish a suitable transient expression procedure for the endosperm protoplasts, sGFP(S65T) was used as a reporter. The procedure for plasmid uptake into protoplasts was mediated by PEG, based on a report described that the electroporation procedure caused serious damage to the viability of barley and wheat endosperm protoplasts (Diaz *et al.,* 1993).

The green fluorescence of sGFP(S65T) under the control of the CaMV 35S promoter (**Fig. 1A**) could be seen four hours after PEG induced uptake of the

plasmid. The fluorescence was seen diffusely in the cytoplasm (**Fig. 3A**). It has been reported that sGFP(S65T) without the targeting sequence also accumulated in the cytoplasm and nucleus of leaves and roots of *Arabidopsis* (Chiu *et al.,* 1996). The endosperm protoplasts gave no autofluorescence upon excitation with 490 nm (blue) light (**Fig. 3A**). Transformation efficiency was up to 8% in the survivors. This efficiency would be sufficient to observe the movement of the gene expression product in the endosperm cells of model rice (Hosetsu d.).

We also transiently expressed the fusion gene for the coding region of rice 13 kDa prolamin (λ RM1) (Mitsukawa *et al.,* 1999) followed by the sGFP (S65T) under the control of the rice 10 kDa prolamin promoter (**Fig. 1B**). The 5′ flanking region from -840 to -44 of the genomic clone encoding rice 10 kDa prolamin polypeptides (Feng *et al.,* 1990) was used as the rice 10 kDa prolamin promoter. The 5′ flanking region could function as a promoter in the endosperm protoplasts (**Fig. 3B**). The fluorescence of the fusion protein was observed in the small particles and the ER-network (**Fig. 3B**). Rice prolamins aggregate within the rough endoplasmic reticulum (RER), and form protein body type I in rice endosperm cells (Yamagata *et al.,* 1982; Krishnan *et al.,* 1986). Probably, the small particles are the aggregates of the fusion protein for the rice 13 kDa protein followed by the GFP in the RER.

Establishment of transient transformation using the endosperm protoplasts from Hosetsu dwarf will provide a highly useful tool for investigating and understanding the physiological, biochemical and molecular biology mechanisms on working to develop cereal seeds. Although the procedure presented in this study was for the dwarf rice variety, an essentially identical procedure would be applicable to a wide variety of rice grains.

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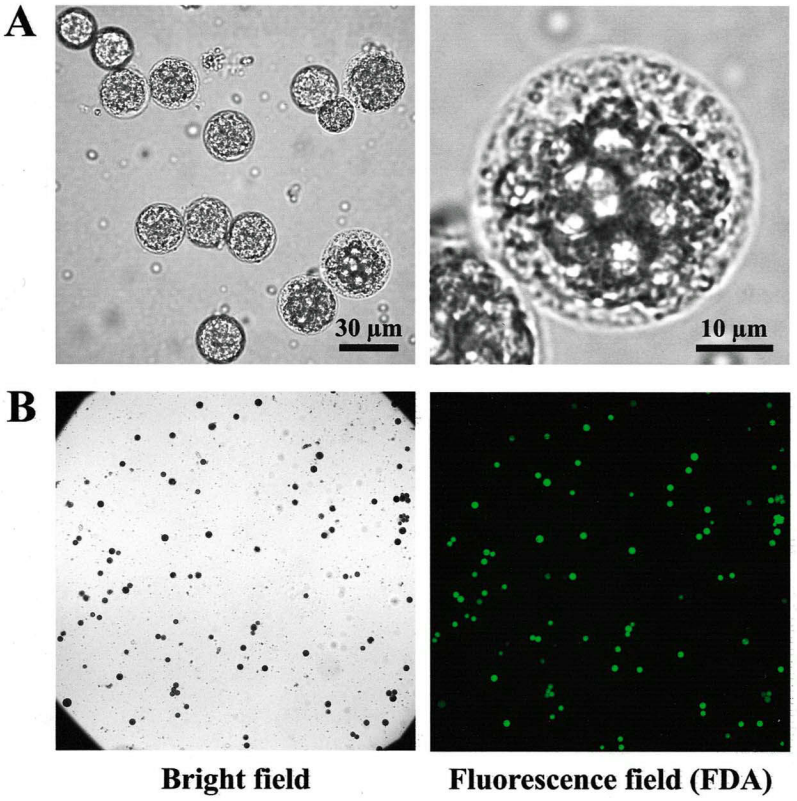

Fig. 2 Endosperm protoplasts from developing rice grains. (A) Light micrograph of freshly isolated endosperm protoplasts from developing rice grains. (B) Freshly prepared endosperm protoplasts were incubated with fluorescein diacetate (FDA) (5 μg ml^{-1}) for 10 min. The protoplasts were examined under a fluorescent microscope.

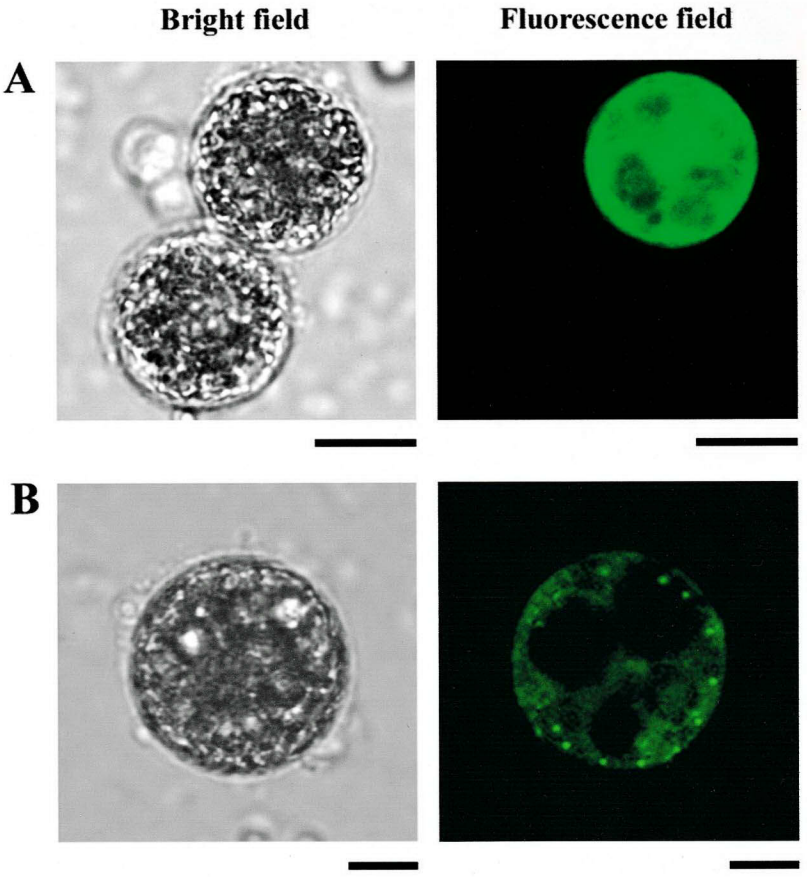

Fig. 3 The expression of sGFP(S65T) (A) and sGFP (S65T) fused to the rice prolamin. (B) in rice endosperm protoplasts. The left and right photographs were taken of the same field after 20 h incubation of DNA uptake. (A) A transformed protoplast is compared with a nontransformed protoplast. The sGFP signal is observed evenly throughout the cytoplasm. No autofluorescence is observed in the nontransformed protoplast under this condition. (B) The signal of the sGFP fused to the rice prolamin is observed in small particles and the ER-network. Bars = 10 μm.