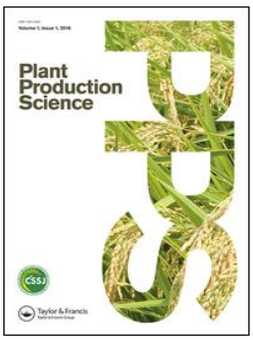

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Transient Expression of Green Fluorescent Protein in Rice Calluses : Optimization of Parameters for Helios Gene Gun Device

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Abstract : An optimized condition for particle bombardment is necessary for efficient genetic transformation. Parameters for Helios gene gun, the new system for nucleic acid delivery which is mainly consists of hand-held device sold by Bio-Rad Laboratories (California USA), were examined based on transient expression of synthetic green fluorescent protein (*sgfp*) in rice calluses of *indica* cv. Fatmawati and *japonica* cv. Nipponbare. In the experimental conditions that we examined, parameters found to be the most favorable conditions for transient expression of *sgfp* in rice callus cells were as follows: 200–250 psi helium pressure, 0.6 μm gold particle size, 0.25 mg gold particles per shot, and 1.5 μg plasmid-DNA per shot. Desiccation of callus cells for eight min was also found appropriate. The level of transient *sgfp* expression was not significantly influenced by the pre-culture for 4 to 12 d before bombardment or by callus age between 10 and 33 wk old in Fatmawati. These parameters for this particular device should improve the transient expression, thus enabling stable expression of introduced genes via Helios gene gun using callus as a target tissue.

Key words : Helios gene gun, Particle bombardment, Rice, Synthetic green fluorescent protein, Transient expression.

Among the well-known reporter genes, the green fluorescent protein (*gfp*) from the jellyfish (*Aequorea victoria*) is widely used visual reporter in biotechnology or molecular biology due to many advantages, such as no necessity of any additional co-factors (Heim et al., 1994), non-invasive detection (Chalfie et al., 1994), broad spectrum of host cells (Cubitt et al., 1995), and without toxicity to the host cells (Niwa, 2003). Moreover, in plant science, *gfp* has been extensively applied for testing the efficacy of the promoter (Newell et al., 2003), detection of virus activity (Oparka et al., 1997), protein localization (Hibberd et al., 1998; Jang et al., 1999) and optimization of transformation methods (Tee and Maziah, 2005). Recently, many types of *gfp* are available; one of them is a synthetic *gfp* [*sgfp* (S65T)], replacement of the serine in position 65 with a threonine) which is 100-fold brighter than wild-type *gfp* in plants (Chiu et al., 1996; Niwa et al., 1999). The *sgfp* has been used as an efficient reporter system for direct gene transfer in maize (Chiu et al., 1996), oat (Cho et al., 2003), soybean (El-Shemy et al., 2006) and others.

Transient expression of reporter genes is useful in both plant and animal sciences particularly for improving the efficiency of transformation technology. Physical and biological parameters of transformation technology including particle bombardment could be optimized for efficient genetic transformation. Schopke et al. (1997) pointed out that the establishment of optimal parameters in particle bombardment for any plant tissues is necessary.

Particle bombardment is now emerging as the method of choice for introduction of useful agricultural genes into rice and other cereal crops. However, most bombardment methods for rice transformation utilize the PDS-1000/He Biolistic Particle Delivery System (for example, Bec et al., 1998; Ghosh-Biswas et al., 1998; Jiang et al., 2000; Martinez-Trujillo et al., 2003), and there is no current protocol for rice callus cells bombarded using the Helios gene gun device (Bio-Rad Laboratories, USA) and transgenic plants obtained using this particular tool have not been reported yet. In this study, we used the Helios gene gun that differs from PDS-1000/He device. It does not use a vacuum chamber and thus can be used to deliver nucleic acids into tissues, organs, and even whole organisms (Taylor and Fauquet, 2002), thereby removing limitations to the target and its size. Other advantages of the Helios gene gun are portable in its size, wide application to plant and animal cells, and also the cartridge can be stored for several months.

Received 9 February 2007. Accepted 27 May 2007. Corresponding author: T. Yoshida (yoshidat@cc.utsunomiya-u.ac.jp, fax +81-28-649-5401).

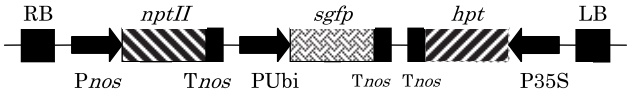

Fig. 1. Schematic map of plasmid pIG001. *hpt*, hygromycin phosphotransferase gene, conferring resistance to hygromycin B; *npt II*, neomycin phosphotransferase II gene, conferring resistance to kanamycin; *sgfp*, synthetic green fluorescent protein gene; PUbi, promoter of a maize Ubiquitin 1; P*nos*, promoter of *nos* (nopaline synthase) gene; P35S, the 35S promoter of Cauliflower mosaic virus; T*nos*, terminator of *nos*; RB, right border; LB, left border.

In this study, transient expression of *sgfp* (Chiu et al., 1996) was used as a reporter system for rapid assay to optimize the parameters of Helios gene gun for calluses of two distinct cultivars *indica* Fatmawati and *japonica* Nipponbare. These parameters are helium pressure, particle gold size, amount of gold particles per shot (MLQ), and amount of plasmid-DNA delivered per shot (DLR). In addition, the effects of desiccation treatment, pre-culture of calluses before bombardment, and callus age, which are considered as the biological parameters, were also evaluated. The objective of the current work was to optimize parameters for Helios gene gun bombardment by evaluating the transient expression of *sgfp* using calluses of two cultivars as target materials.

Materials and Methods

1. Plant material and proliferation of embryogenic callus cells

Embryogenic callus cells induced from scutellar tissues of mature seeds of two cvs. Fatmawati and Nipponbare and proliferated as described previously (Carsono and Yoshida, 2006), were used as target materials in bombardments.

2. Plasmid used for bombardment

Plasmid pIG001 with the *sgfp* gene and the hygromycin phosphotransferase (*hpt*) gene conferring resistant to hygromycin B was used in all bombardment experiments (Fig. 1). The *sgfp* gene was driven by a maize ubiquitin-1 promoter (Christensen and Quail, 1996), while the *hpt* gene under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter. The plasmid was amplified in *E. coli* DH5α cells and purified by the alkaline-SDS lysis method (Sambrook et al., 1989), followed by PEG/NaCl treatment. The concentration of plasmid-DNA was checked by Spectrophotometer (Gene Spec III, Naka Instruments Co., Ltd., Japan).

3. Helios gene gun bombardment procedures

The preparation of bombardment for Helios gene gun was done according to the manufacturer's instruction and method of Helenius et al. (2000) with a minor modification for rice callus. In brief, 40 μL of 50 mM spermidine was added into a microcentrifuge tube containing 12.5–25.0 mg gold particles, then sonicated for 5–8 s, and subsequently, 40 μL of plasmid DNA (50–100 μg) in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added and gently vortexed. While mixing, 40 μL of 1M $CaCl_2$ was added drop by drop, to associate the DNA with the gold particles. The suspension was allowed to settle at room temperature for 10 min.

The gold-DNA pellets were washed three times by vortexing in 1 mL of absolute ethanol, microcentrifuged at 12000 rpm for 1 min and the supernatant was removed. The pellet was then resuspended in 3 mL absolute ethanol-PVP (polyvinylpyrollidone; 0.05 mg mL^{-1}). By using a syringe attached to an adapter, this suspension was drawn into a 76.2 cm in length of the Tefzel tubing (Bio-Rad Laboratories, USA). The tubing was then transferred into a tubing preparation station. After gold beads were allowed to settle, ethanol was slowly drawn off and the turner was rotated for 30 s, smearing the gold-DNA around the inside of the tubing. The residual ethanol was removed by passing nitrogen through the tubing for 3–5 min. The tubing was cut into 2.54 cm long tubes and then loaded into the Helios gene gun device.

Eight embryogenic calluses, 4–5 mm in diameter, were placed in the center of Petri dish with filter papers (Fig. 2a), and covered with a plastic bowl with cavity that suites the spacer of Helios gene gun (Fig. 2b). These calluses were bombarded once, then immediately transferred to osmotic medium and incubated in the dark at 26°C. Osmotic treatment of callus cells with 0.4 M mannitol on medium NB_5 was applied for 4–6 h before and 16 h after bombardment.

4. GFP monitoring and transient assessment

sGFP-expressing cells were detected using a stereo-fluorescence microscope (MZFL III, Leica) assisted with the GFP2 filter to mask the red fluorescence of chlorophylls resulting from the non-transformed cells, thus allowing visualization of distinctive differences for the sGFP-expressing cells. The number of sGFP positive cells emitting green fluorescent spots was recorded, and always counted at the same magnification, i.e., 80X at the back and upper sides of the Petri dish, 2–3 days after bombardment. Digital images were then acquired using a CoolSNAP CCD camera with CoolSNAP software.

In this experiment, the Helios gene gun parameters were varied while all other parameters were maintained as standard procedure as follows: 200 psi (equal to 1,379 Kpa), 0.6 μm gold particle diameter, 0.250 μg gold particles per shot, 1.0 μg plasmid-DNA per shot, 4 d pre-culture, and 0.4 M mannitol of osmotic medium. Desiccation of callus cells was done using a vacuum drying oven (Yamato Scientific Co.,

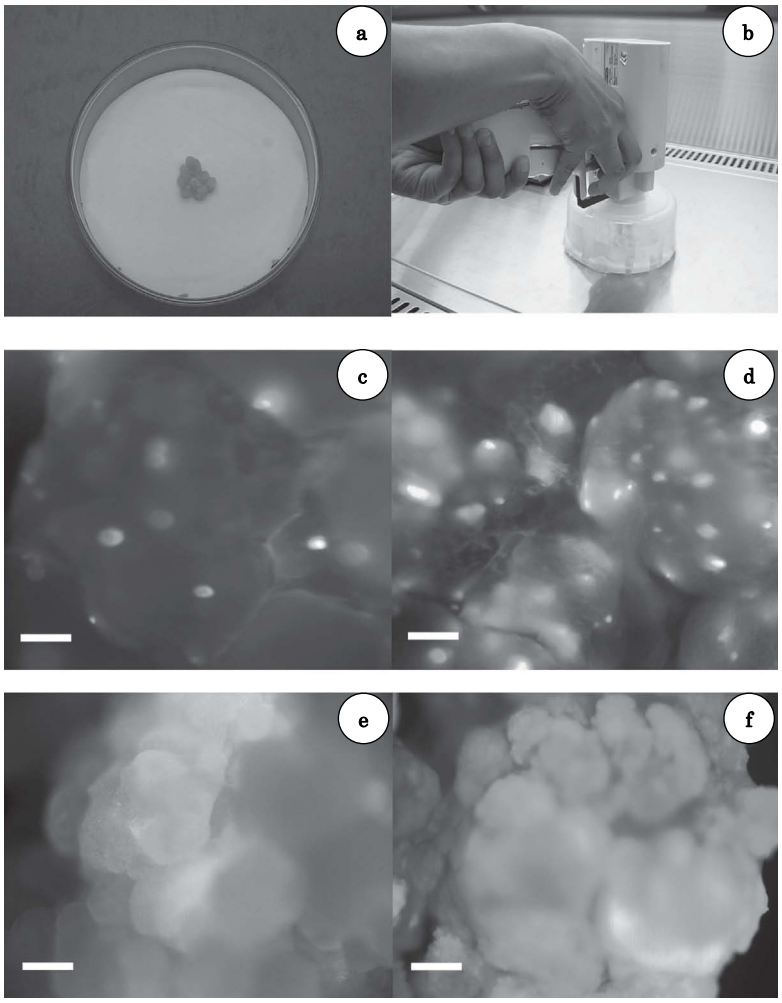

Fig. 2. (a) Eight embryogenic rice calluses ready for Helios gene gun bombardment. (b) Bombardment of rice calluses with a plastic bowl to avoid jumping the target tissues by bombardment. Transient *sgfp* expression in rice calluses cvs (c) Fatmawati and (d) Nipponbare (64X). The parameters applied were 250 psi, 0.6 μm gold size, 0.25 mg gold particles per shot, 1 μg plasmid-DNA delivered per shot, 0.4 M mannitol osmotic medium (4–6 h before and 16 h after bombardment), and 4-d pre-culture before bombardment. (e) Unbombarded calluses of cv. Fatmawati (control; 64X). (f) Calluses of cv. Fatmawati expressing *sgfp* after two consecutive selections (64X). Scale bars in c) to f) correspond to 50 μm.

Ltd., Japan) at 76 cm Hg without osmotic treatment. Callus cells in the same age were used for parameters tested. Because some calluses were broken into pieces after bombardment, data on the average of green fluorescent spots for eight calluses were not available, and only the total green fluorescent spots per bombardment was recorded.

Data were analyzed using one-way analysis of variance and the differences were analyzed using Duncan's multiple range test (DMRT). Standard errors of the means were calculated for four replications (bombardments).

Results and Discussion

Transient *sgfp* gene expression in rice calluses of two cultivars was observed within 48 to 72 h after bombardment with the Helios gene gun (Fig. 2c, d). In total, 162 bombardments were performed and 1,344 embryogenic calluses from two genotypes were bombarded. Selected callus lines expressing *sgfp* after two consecutive selections, which show the promising callus lines produced by Helios gene gun are presented in Fig. 2f, together with unbombarded calluses (Fig. 2e) as control. The highest level of transient expressions of *sgfp* was obtained with a helium acceleration pressure of 250 psi (1,724 Kpa) in both cultivars (Fig. 3). However, no significant difference was detected in the number of sGFP spots between 200 psi (1,379 Kpa) and 250 psi in Fatmawati. A low acceleration pressure resulted in low number

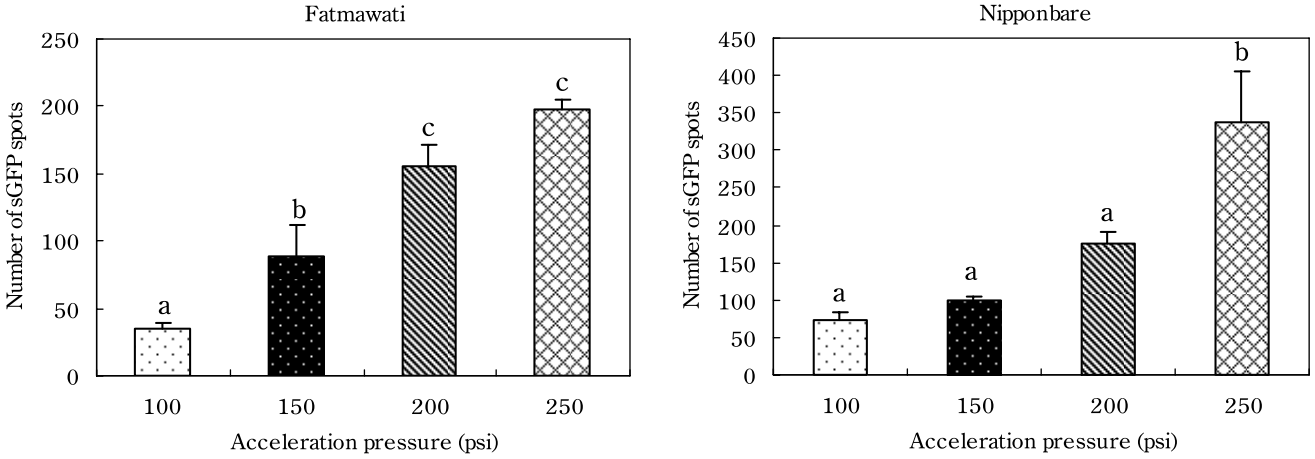

Fig. 3. Effect of acceleration pressure on *sgfp* expression in calluses of two cultivars. The bars represent the average with standard error of the means from 4 replicates, each with 8 embryogenic calluses. Same letters indicate values are not significantly different according to Duncan's multiple range test (DMRT, p=0.05).

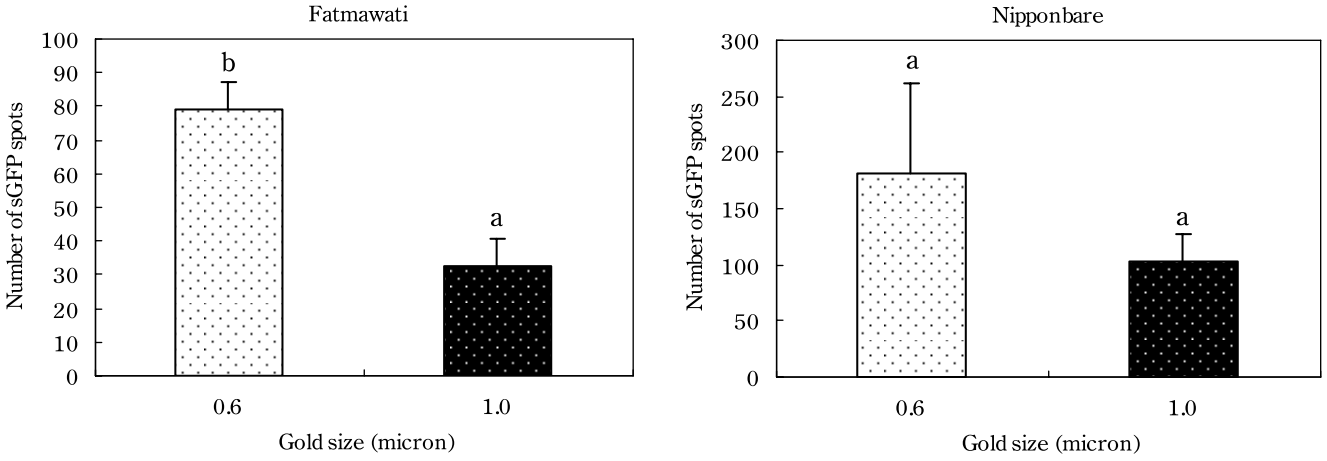

Fig. 4. Effect of gold particles size on *sgfp* expression in calluses of two cultivars. The bars represent the average with standard error of the means from 4 replicates, each with 8 embryogenic calluses. Same letters indicate values are not significantly different according to DMRT (p=0.05).

of sGFP spots. The high helium pressure seems to be very powerful to drive plasmid DNA from the inner surface of tubing, so that it can penetrate into deeper cell layers. It is presumed that acceleration pressure affects the depth of penetration and distribution of gold particles into callus cells as already reported by Rasco-Gaunt et al. (1999) and Tee and Maziah (2005) using a PDS-1000/He device. They assumed that a high acceleration pressure bombarded a smaller area than a low pressure. Moreover, Sanford et al. (1993) explained that the highest transient expression is generally achieved with rather violent treatments giving better particle penetration, but these conditions may affect the cell division or growth. Extra tissue damage or injury (Rasco-Gaunt et al., 1999; Tee and Maziah, 2005), more necrotic, no formation of new calluses or somatic embryos (Tadesse et al., 2003) are some adverse effects of bombarded tissues with high acceleration pressure due to high velocity of gold particles. Tissue damage may be due not only by helium acceleration pressure, but also by some other parameters such as gold particles size and amount of gold particles per shot.

Gold particles, 0.6 μm in diameter, produced significantly higher level of *sgfp* expression than 1.0 μm particles in Fatmawati, but not significant in Nipponbare (Fig. 4). It seems that a 0.6 μm gold particle is more suitable than a 1.0 μm particle to carry plasmid pIG001, which is around 18 kbp. This result is in agreement with a previous report that the 0.6 μm gold particle is best for transient expression of the *uidA* gene (β-glucuronidase, GUS) in the leaves of *Arabidopsis*, tobacco and birch bombarded when using a Helios gene gun (Helenius et al., 2000). A possible

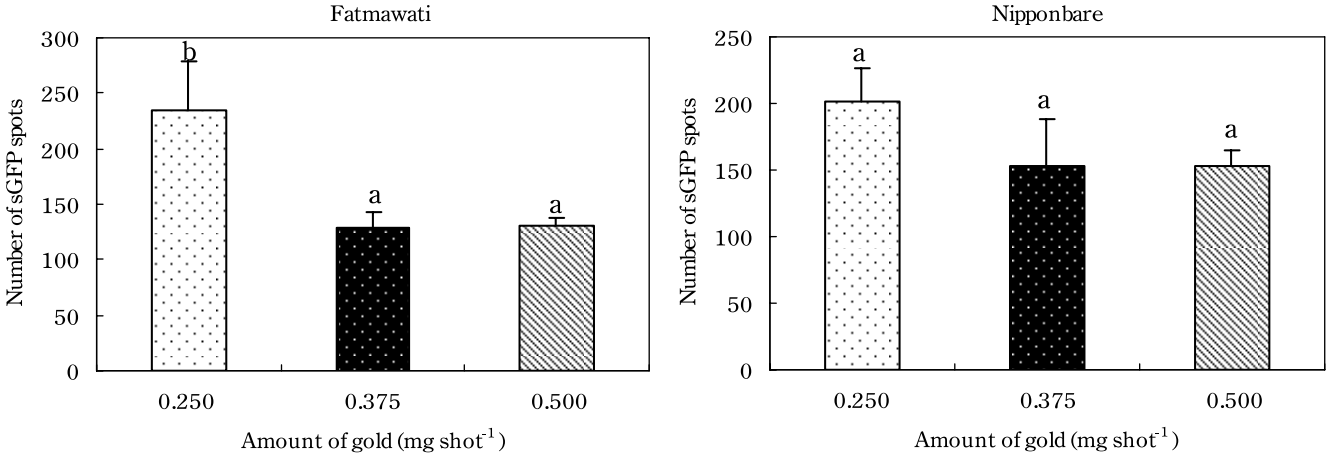

Fig. 5. Effect of the amount of gold particles delivered per shot on *sgfp* expression in calluses of two cultivars. The bars represent the average with standard error of the means from 4 replicates, each with 8 embryogenic calluses. Same letters indicate values are not significantly different according to DMRT (p=0.05).

explanation for this condition is that a small particle size (0.6 μm) may cause less cell damage of bombarded target tissues due to low particle mass, thus the cells may be capable of transiently expressing *sgfp* gene, even capable of undergoing further division or growth.

A small amount of gold particles or microcarriers delivered per shot (0.25 mg) led to a significant increase in transient expression of *sgfp*, particularly in Fatmawati (Fig. 5). No significant difference in the number of *sgfp* expression was found when Nipponbare calluses were bombarded with different amounts of gold particles. Gold particles at more than 0.25 mg per shot produced no improvement in *sgfp* expression in either cultivar. A large number of cells are probably damaged or killed by the large amount of gold particles delivered to the callus cells, resulting in a reduced amount of surviving cells, therefore undesirable for developing stable transformation. Hunold et al. (1994) found that the viability of particle-containing cells decreased within two days after bombardment, which supports our result. However, a minor difference was reported by Helenius et al. (2000) who found that 0.125 mg gold particles delivered per shot was effective for transient luciferase activity in *Arabidopsis* and tobacco leaves, but for *uid*A expression in birch leaves, 0.25 mg gold particles per shot were optimum.

The use of an appropriate concentration of DNA is important for efficient DNA-microcarrier binding (Parveez et al., 1998), thus in this experiment, we tested this concern. Plasmid-DNA delivered with 1.5 μg per shot produced the largest number of sGFP spots in Fatmawati, while in Nipponbare, 1.5 and 2.0 μg plasmid-DNA per shot were not significantly different in producing sGFP spots (Fig. 6). A similar observation has been observed in oil palm (Parveez et al., 1998), in which 1.5 μg plasmid-DNA per shot gave the highest level of the GUS expression in embryogenic callus. Increasing plasmid-DNA concentration improved the *sgfp* expression in Nipponbare, but not in Fatmawati calluses. It appears that the *sgfp* expression was genotype-dependent in terms of differences in callus cell types they produced, which has been described by Bec et al. (1998). Moreover, differences in the *sgfp* expression in calluses of the two cultivars bombarded using a Helios gene gun might be attributed to the difference in biological constraint such as the activity of endogenous nuclease (Barandiaran et al., 1998), local RNA silencing which blocks expression of transgene (Baulcombe, 2004; Miki et al., 2005) and physical constraint such as callus cell density, callus compactness or callus friability that might not be exactly identical in these cultivars.

The limitation of using Helios gene gun bombardment is the tubing that sometimes contains unequally distributed plasmid-DNA resulting in unequally spread plasmid-DNA in the target tissues. In the present experiment, the *sgfp* expression was mostly limited to 2 to 5 calluses out of 8 bombarded calluses. In addition, Harwood et al. (2000) found that this device was not suitable for barley transformation because the tissues jumped to other places by bombardment. However, by covering target tissues (callus cells) using a plastic bowl, this demerit could be overcome.

Bombarding target tissue at the right developmental stage is important because actively dividing cells are most receptive to the particle bombardment (Moore et al., 1994). Pre-culture of callus cells prior to bombardment did not affect the *sgfp* expression as shown in Fig. 7. However, it was reported that a 7 d pre-culture and 4 d pre-culture gave the highest GUS spots in immature embryo of oil palm (Parveez et al., 1998) and in microspores of wheat (Folling and Olesen,

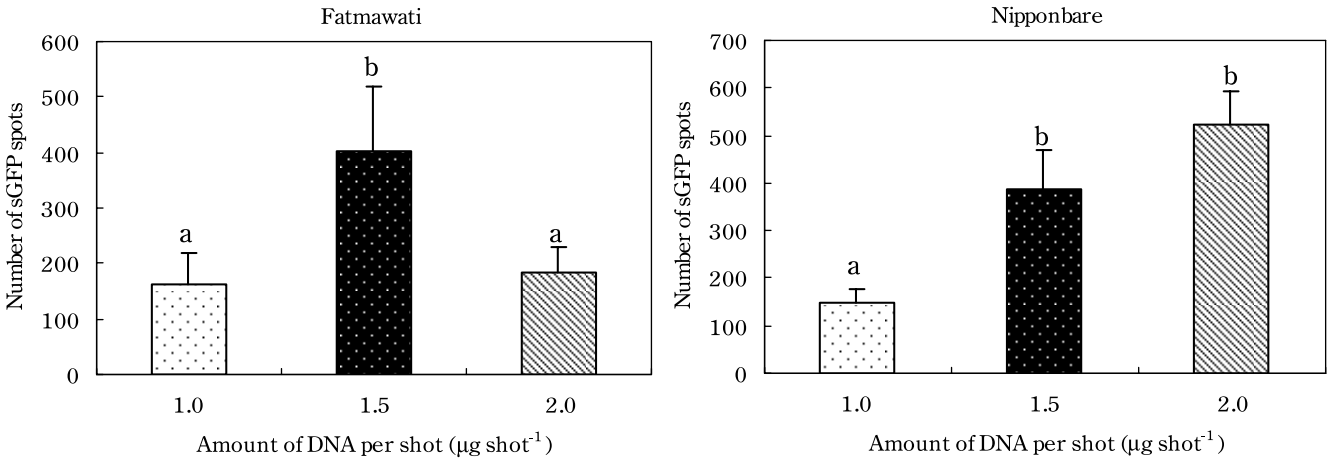

Fig. 6. Effect of the amount of plasmid-DNA delivered per shot on *sgfp* expression in calluses of two cultivars. The bars represent the average with standard error of the means from 4 replicates, each with 8 embryogenic calluses. Same letters indicate values are not significantly different according to DMRT (p=0.05).

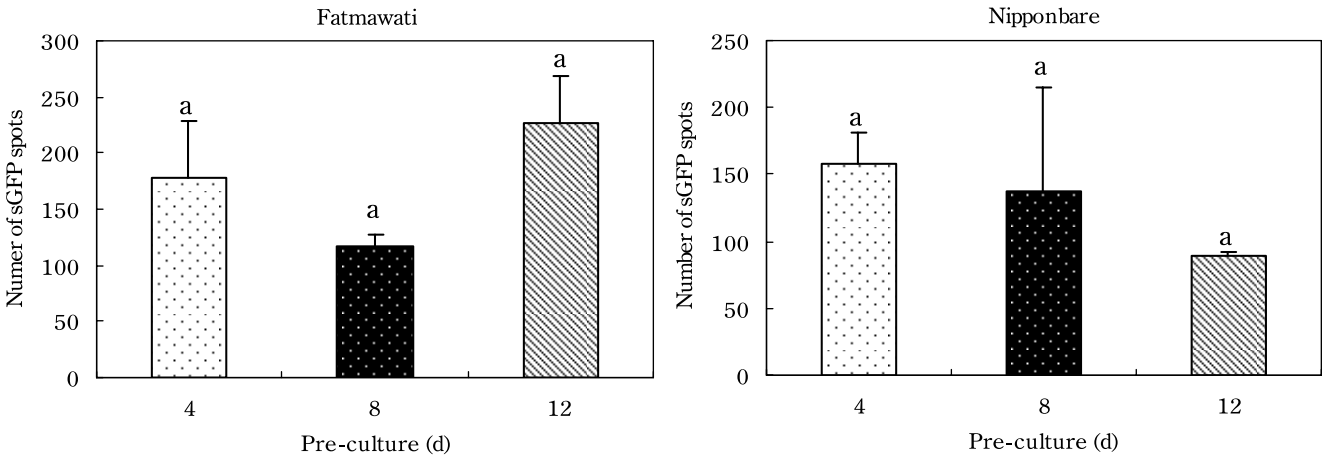

Fig. 7. Effect of pre-culture prior to bombardment on *sgfp* expression in calluses of two cultivars. The bars represent the average with standard error of the means from 4 replicates, each with 8 embryogenic calluses. Same letters indicate values are not significantly different according to DMRT (p=0.05).

2002). Moreover, in two types of orchid *Dendrobium* calluses, a 2 d pre-culture period was suitable for *sgfp* expression (Tee and Maziah, 2005), indicating that the effect of pre-culture duration for the efficiency of bombardment varies with the target tissue, though such variation was not significantly found in rice callus cells. However, in order to minimize the occurrence of somaclonal variations in the transgenic regenerants, less than or equal 4 d pre-culture would be beneficial.

Removing partial humidity of rice callus cells via desiccation treatment for 8 and 12 min prior to bombardment gave a larger number of sGFP spots in Fatmawati (Fig. 8), but not in Nipponbare. Desiccation treatment for more than 8 min was not effective due to a reduction in *sgfp* expression. It is apparent that prolonged exposure to desiccation leads to cell stress due to an excessive leakage of protoplasm. Optimal desiccation time makes callus cells to be properly plasmolyzed, which is apparently as the same effect of osmotic treatment, thus the cells may be less likely to extrude their protoplasm following penetration of the cell by gold particles (Vain et al., 1993).

The effect of age of target tissues for bombardment experiment should be considered for obtaining highly efficient transformation. Ramesh and Gupta (2005) discovered that maximum *uidA* expression in rice calluses was observed when calluses were bombarded after 44 d old (6.3 wk) and tended to decrease in older callus age (68 d old or 9.7 wk). However, in Fatmawati in this study, callus age did not significantly influence the number of sGFP spots, although the *sgfp* expression tended to decrease in older callus (Fig. 9), indicating the same tendency as previously observed. A decrease in *sgfp* expression in older callus cells might be

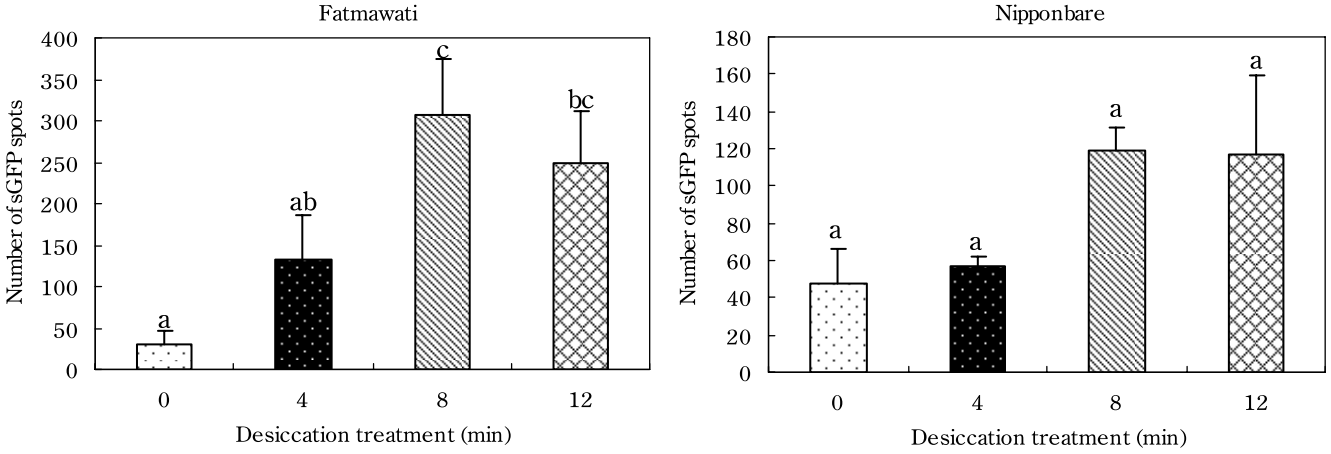

Fig. 8. Effect of desiccation treatment prior to bombardment on *sgfp* expression in calluses of two cultivars. The bars represent the average with standard error of the means from 4 replicates, each with 8 embryogenic calluses. Same letters indicate values are not significantly different according to DMRT (p=0.05).

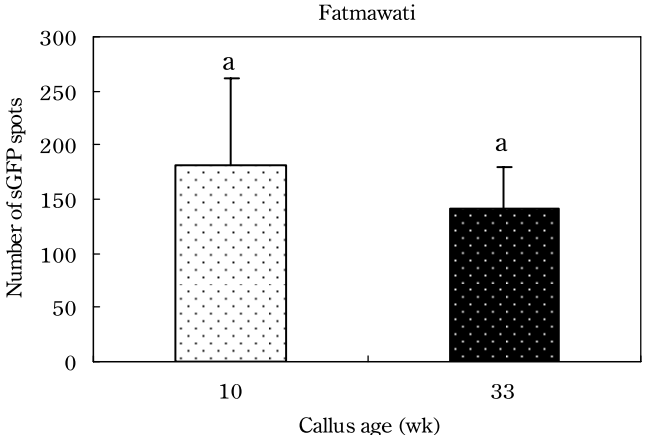

Fig. 9. Effect of callus age of Fatmawati on *sgfp* expression. The bars represent the average with standard error of the means from 4 replicates, each with 8 embryogenic calluses. Same letters indicate values are not significantly different according to DMRT (p=0.05).

associated with physiological state of deterioration of callus cells due to the genetic alteration, such as point or single-gene mutation, large-scale deletion, gross changes in chromosome structure/number (Kaeppler et al., 2000), transposable elements activation i.e. retrotransposons (Hirochika et al., 1996), chloroplast deletion (Abe et al., 2002), or epigenetic change, such as DNA methylation (Kaeppler et al., 2000; Joyce et al., 2003) which is most frequently observed when *in vitro* culture gets older.

For stable expression of inserted genes for rice callus bombarded using Helios gene gun, use of helium at a pressure of 200 to 250 psi with 0.6 μm gold particles size, 0.25 mg gold particles delivered per shot, 4 d pre-culture, desiccation for 8 min prior to bombardment, and younger callus age (3 to 6 wk), is therefore recommended. Optimized parameters for Helios gene gun that presented in this paper would be tremendously helpful for producing stable transformation of rice and other cereal crops.

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