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Research Information

Transient gene expression in the wheat leaf cells with the low-pressure gene delivery system

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Transformation with biolistic bombardment is a practical approach for understanding gene function in plants, of which stable transformants were not easily constructed, specially Triticeae species. Since the biolistic bombardment system is independent of plant species, it has been used as a transformation technique in wheat (Takumi and Shimada 1996, 1997). In addition, the transient expression assays with the biolistic bombardment has been applied to the studies of plant-pathogen interactions (Shirasu et al. 1999, Bryan et al. 2000, Allen et al. 2004, Douchokov et al. 2005). Recently, the novel hand-held gene delivery system GDS-80 (Wealtec Corporation, NV, USA) has been developed and applied to cultured cell lines (Chang et al. 2008), living animals (Lin et al. 2008) and plants (Kao et al. 2008). GDS-80 utilizes a supersonic gas flow for gene delivery, which enables to accelerate DNA-coated gold particles to 300 m/sec by low pressure Helium gas. This low pressure facilitates reduction of cell damages and enhances efficiency of transformation. Since the DNA-coated gold particles are directly injected into the hole of the sample loading sleeve of GDS-80, other equipment such as a rupture disc and extra preparations are not required, restraining running cost and time on the experiment. Here, efficiency of transient expression with GDS-80 was tested under various conditions: pressure, length, and ploidy level of *Triticum* species in order to find optimal conditions for the assay using the wheat leaf cells.

Triticum urartu KU199-1, *T. durum* cv. Langdon, and *T. aestivum* cv. Norin 4 were used in this study. The seeds were provided by the National BioResource Project (NBRP)-Wheat, Japan (https://www.nbrp.jp). These plants were grown on expanded vermiculite for 7 days under 12-h light/12-h dark cycle and 20˚C. A primary leaf of the one-week old seedling was used as a target of shooting with GDS-80. Gold particles stocks (60 mg/mL) were prepared according to the following procedure. One mL of 70% ethanol was added to 30 mg of 1.0 µm gold particles (GDS-G1.0, Wealtec Corporation, NV, USA) in a 1.5 mL micro-centrifuge tube. The 70% ethanol containing gold particles was vigorously mixed with a vortex mixer for 1 minute, and placed at room temperature for 5 minutes. Centrifuge at 3,381 x g for 30 seconds was performed with Centirfuge 5424 (Eppendorf AG, Hamburg, Germany), and the supernatant was discarded. After adding 1 mL of sterilized distilled water, the gold particles were vigorously mixed with a vortex mixer for 1 minute, and placed at room temperature for 1 minutes. Centrifuge at 3,381 x g for 5 seconds was performed, and the supernatant was removed. This washing process with sterilized distilled water was repeated two more times. To make 60 mg/mL gold particles stocks, 500 µl sterilized distilled water was added and mixed with the vortex mixer. From 500 µl of the mixture, 100 µl each was distributed into five micro-centrifuge tubes, and stored at 4 degrees Celsius.

Next, DNA-coated gold particles for 10 bombardments were prepared as follows. Of DNA solution (1 µg/µl or 5 µg/ul), 10 µl was added to a gold particles stock (60 mg/ml) in a 1.5 ml micro-centrifuge tube. While mixing with the vortex mixer, 40 µl of 0.1 M cold spermidine was added drop by drop into the tube, and then 100 µl

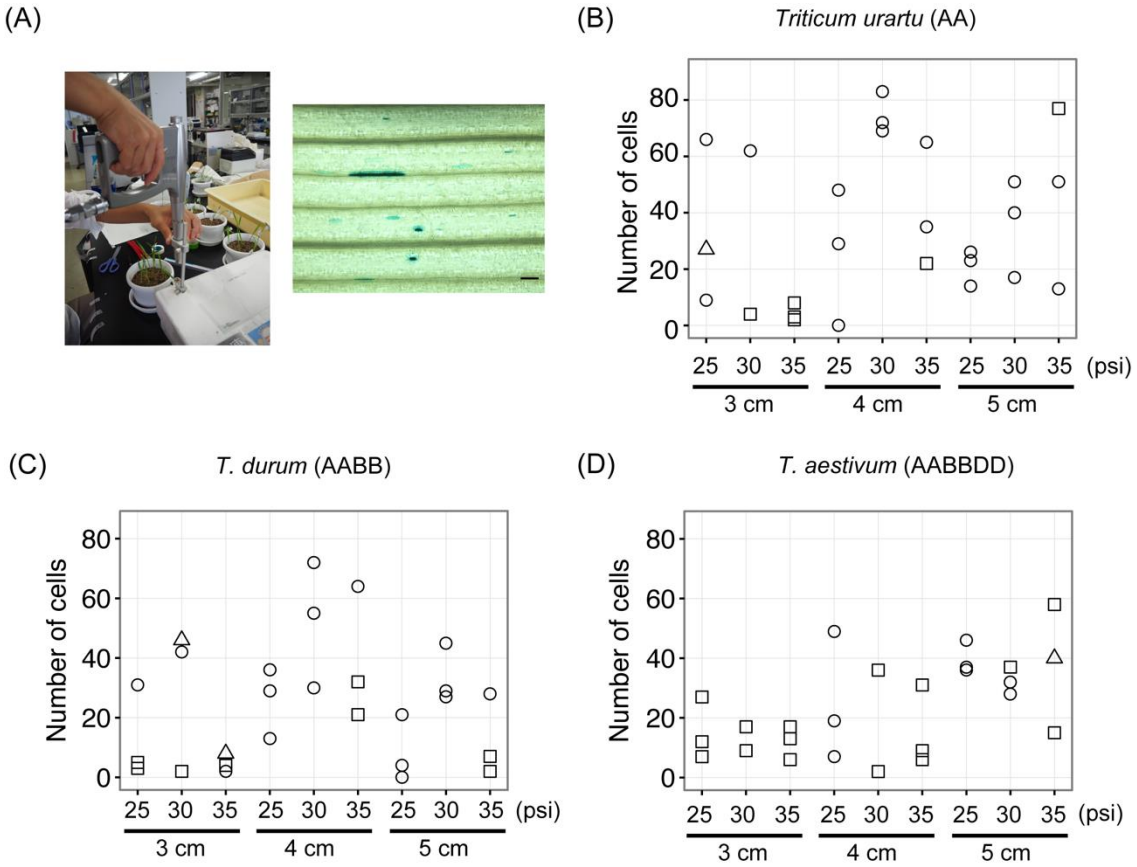

Fig. 1. Efficiency of transient expression under various pressures (psi) and distances (cm) between the leaf and the muzzle of GDS-80. The setting of GDS-80 for shooting and blue-staining cells in the primary leaf of *T. urartu* are shown (A). The scale bar on the epidermal cells is 80 µm. The numbers of blue-staining cells in the primary leaf of *T. urartu* KU199-1 (B), *T. durum* cv. Langdon (C) and *T. aestivum* cv. Norin 4 (D) are shown under the combinations of three pressures (25 psi, 30 psi and 35 psi) and three distances (3 cm, 4 cm and 5 cm). Circles, triangles and squares indicate non-damaged, little damaged (the wounded leaf surface was observed), and seriously damaged (the leaf was torn or pieced) leaf cells after shooting of GDS-80, respectively. One µg/µl of plasmid DNA was used for the preparation of DNA-coated gold particles.

of 2.5 M cold $CaCl_2$ was added drop by drop into the tube. The tube was vigorously mixed with the vortex mixer for another 1 minute. Centrifuge at 3,381 x g for 30 seconds was performed, and the supernatant was discarded. After adding 200 µl of 70% cold ethanol to the tube, the DNA-coated gold particles were mixed with the vortex mixer for 30 seconds. Centrifuge at 3,381 x g for 30 seconds was conducted, and the supernatant was discarded. To suspend the DNA-coated gold particles, 100 µl of 100% cold ethanol was added and mixed well. Since 10 µl of DNA-coated gold particles is necessary per one shot, 100 µl of the aliquot allows 10 bombardments. Ten µl of the aliquot was directly injected to the hole of the sample loading sleeve of GDS-80. The gun was shot after the adjustment of appropriate pressure of Helium gas and distance between a sample and the muzzle (Fig. 1A).

To optimize the bombardment conditions for transient gene expression in the leaf cells of *T. urartu* KU-199-1, *T. durum* cv. Langdon and *T. aestivum* cv. Norin 4 with GDS-80, efficiency of transient expression was evaluated by delivering the pAHC17 plasmid vector including a GUS reporter gene driven by the maize ubiquitin promoter (Christensen et al. 1992) to plant cells and counting blue-staining cells 2 days after the shot (Fig. 1A). GUS expression cells were stained with the GUS staining buffer (40 mM 5-bromo-4-chloro-3-indoxyl-D glucuronide, 50 mM phosphate buffer pH 7.2, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, and 0.3% Triton X). Vacuum infiltration was performed with a water aspirator for 25 minutes. The infiltrated leaves in the GUS staining buffer were incubated

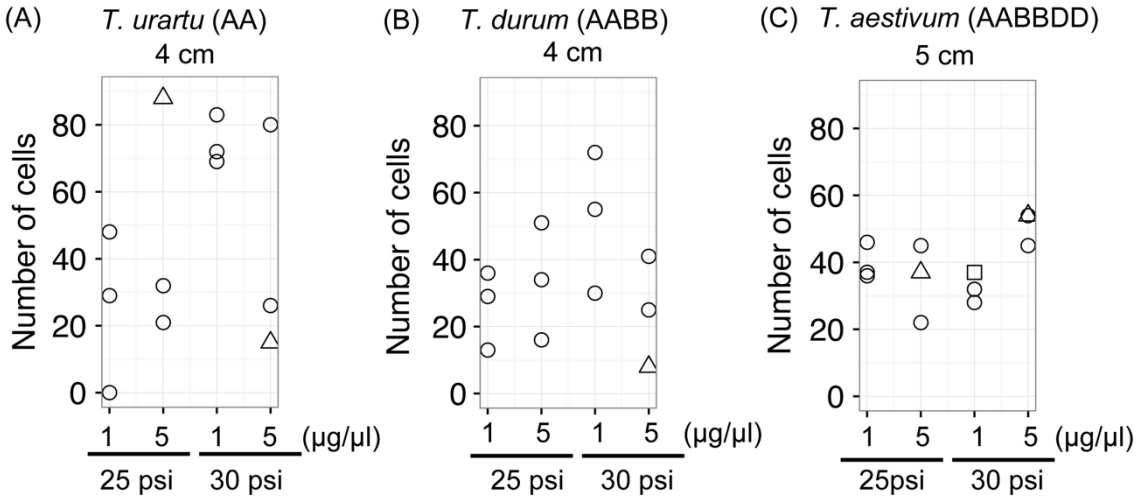

Fig. 2. Comparisons in the efficiency of transient GUS expression between 1 μg/μl and 5 μg/μl of plasmid DNA that were used for the preparation of DNA-coated gold particles. The distance between the leaf and the muzzle for *T. urartu* KU-199-1 (A) and *T. durum* cv. Langdon (B) were set to 4 cm. The distance for *T. aestivum* cv. Norin 4 (C) was set to 5 cm. Circles, triangles and squares indicate non-damaged, little damaged, and seriously damaged leaf cells after shooting of GDS-80, respectively.

at 37 ºC for 16 hours. After chlorophyll from the leaves was removed with 96 % ethanol, blue-staining cells were counted under the optical microscope.

At first, to narrow down pressure conditions, DNA-coated particles were shot to primary leaves of *T. urartu* with GDS-80 under the pressure from 25 psi to 60 psi. Under over 40 psi pressure, the leaves were damaged and a small number of cells gave a blue precipitate. Then, efficiency of transient expression in a primary leaf of *T. urartu* was more precisely examined under 25 psi, 30 psi and 35 psi, while changing the distance between the leaf and the muzzle of GDS-80. The condition of 4 cm distance and 30 psi showed the largest number of cells with the GUS expression and less variations among the three replications (Fig. 1B). At least, more than 10 blue-staining cells were observed under any combinations between the pressure range from 30 psi to 35 psi and the distance range from 4 cm to 5 cm. On the other hand, all the leaves were seriously damaged under 35 psi and 3 cm distance, resulting in less expression cells.

Efficiency of transient expression in a primary leaf of *T. durum* was tested under different combinations of the pressures and the distances. The condition of 4 cm distance and 30 psi showed the largest number of cells with the GUS expression as observed in *T. urartu*, but there were more variations among the three replications (Fig. 1C). The efficiency of transient expression under 4 cm distance was better than that under 3 cm and 5 cm distances. Specially, most of the leaves were damaged under 3 cm distance, leading to a small number of cells expressing the GUS reporter gene.

We also examined efficiency of transient expression in a primary leaf of *T. aestivum.* The condition of 5 cm distance and 25 psi showed the largest number of blue-staining cells and less variance among the three replications (Fig. 1D). All the leaves were heavily damaged under 3 cm and 4 cm distances except for 25 psi. There were a few blue-staining cells.

To test whether high DNA concentration can increase the efficiency of transient GUS expression in primary leaves of the diploid, tetraploid and hexaploid wheat species, the number of blue-staining cells was compared between 1 μg and 5 μg plasmid DNA that were used for the preparation of DNA-coated gold particles. Two pressures and one distance between a leaf and the muzzle of GDS-80 including the optimal condition of each species were examined. The high concentration did not improve the efficiency of transient expression in the diploid and tetraploid species (Figs. 2A and 2B). In *T. urartu*, higher DNA concentration generated more variance among the three replications (Fig. 2A). In *T. aestivum*, slight improvement was achieved under the combination of 30 psi and 5 cm distance (Fig. 2C). Through all the trials, under non-optimal conditions, blue-staining cells in the guard cells tended to be more frequently detected compared

Table 1. Analysis of variance (ANOVA) for the number of cells expressing GUS reporter genes with Tukey multiple comparisons

Factor	DF	Sum Sq	Mean Sq	F value	Pr(>F)	Multiple comparisons
Distance (cm)	2	4001	2000.5	8.321	0.0009***	4 cm > 3 cm, 5 cm > 3 cm
Pressure (psi)	2	3769	1884.7	7.839	0.0013**	30 psi > 25 psi, 30 psi > 35 psi
Damage	2	4810	2405.1	10.004	0.0003***	non-damaged > seriously-damaged
Ploidy level	2	1502	751.1	3.124	0.0546 NS	diploid > tetraploid
DI x PR	4	3829	957.3	3.982	0.0081**	
DI x DA	3	2747	915.6	3.808	0.0170*	
PR x DA	4	4001	2000.5	1.126	0.3574 NS	
DI x PL	4	3769	1884.7	0.871	0.4898 NS	
PR x PL	4	4810	2405.1	0.58	0.6786 NS	
DA x PL	2	1502	751.1	0.096	0.9086 NS	
DI x PR x DA	2	3829	957.3	2.07	0.1392 NS	
DI x PR x PL	6	2747	915.6	1.566	0.1815 NS	
DI x DA x PL	2	1083	270.8	2.558	0.0898 NS	
Residuals	41	837	209.3			

DI, Distance; PR, Pressure, DA, Damage, PL, Ploidy.
*P<0.05, **P<0.01, ***P<0.05.
Significantly different comparisons in Tukey's Honest Significance method were shown.

with that in the normal cells.

To examine what factors most influenced the efficiency of transient expression with GDS-80, analysis of variance (ANOVA) for the number of the GUS-expressing cells with Tukey multiple comparisons was conducted (Table 1). As expected, the distance and the pressure significantly affected the efficiency. A noteworthy finding is that the damage factor most significantly decreased the efficiency, implying that adjustment of pressure and distance without giving damage to a leaf is necessary in order to accomplish the best performance with GDS-80. Although there is a statistically significant difference between diploid and tetraploid species, the ploidy level is not main factor that is responsible for the efficiency, suggesting that GDS-80 is a robust system providing any ploidy species of *Triticum* and *Aegilops* with efficient transient expression assays.

In summary, we determined optimal conditions of gene delivery with GDS-80 for wheat species and clarified the factors influencing the efficiency of transient gene expression. The optimal distance and pressure of *T. urartu* KU-199-1, *T. durum* cv. Langdon are 4 cm and 30 psi, respectively. For *T. aestivum* cv. Norin 4, 5 cm distance and 25 psi are the best condition. These conditions minimize damage to a leaf, which was the most influential factor on the efficiency. Recently, a method for *in situ* biolistic transformation without cutting leaves has been developed by using a hand-held particle bombard system Helios Gene Gun System (Bio-Rad Laboratories, Hercules, CA, USA) (Wahara et al. 2017). Due to avoidance of wound-induced plant responses and a limited supply of nutrient and water, this method enables to analyze gene function in pathogen-infected cells for relatively long-term compared with the usage of separated leaves (Douchkov et al. 2005). Since GDS-80 is also the hand-held particle bombardment system similar to the Helios Gene Gun System, the method of Wahara et al. (2017) could be applicable. GDS-80 just needs simpler preparation for shooting, and is the highly efficient system for gene delivery, promoting elucidation of target gene function in wheat and its relatives.

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