Optimization of transient expression of GUS after DNA delivery into wheat calli and leaves

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In order to optimize the major parameters controlling DNA delivery to wheat (*Triticum aestivum* L.) scutellum and leaf tissues by particle bombardment, we studied the precipitation of DNA onto gold particles and the bombardment parameters for tissues of immature embryos or leaves. Efficiency of DNA delivery with a maize ubiquitin (*Ubi1*) promoter was assessed by scoring transient β-glucuronidase (GUS) expression in bombarded tissues. The concentrations of plasmid DNA, $CaCl_2$, and spermidine, and osmotic conditioning of tissues and bombardment parameters in leaves were analyzed for their influence on transient gene expression. The optimized transformation conditions were found to be 2.5 µg plasmid, 50 µl of 2.5 M $CaCl_2$, 20 µl of 0.1 M spermidine, and 50 µl of 50 mg/ml gold for 10 bombardments on wheat calli, with a focus tube in the microcarrier assembly. However, much higher transient transformation expression was observed in leaves without a focus tube and at an optimized target distance. A suitable bombardment procedure was developed for Alpowa wheat cultivar tissue, which allowed high-efficiency DNA delivery. This was made possible by improvements in the procedures used for studying the function of the gene and the interaction mechanism between rust and host.

Keywords: wheat; bombardment; scutellum calli; particle gun; GUS expression.

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Introduction

β-glucuronidase (GUS) is a sensitive and versatile gene fusion marker used as a reporter gene for transformation of plants. GUS is a hydrolase that catalyzes the cleavage of a wide variety of β-glucuronides, many of which are commercially available as histochemical substrates. Blue transformed cells are detected in an *in situ* enzyme assay to locate GUS activity. The transient expression of GUS in micro-projectile bombardment is a method to evaluate the efficiency of plant transformation in barley [1], maize [2], wheat [3], and rice [4].

Microprojectile bombardment technique is also known as particle bombardment, particle gun method, particle acceleration, or biolistics (biological ballistics). The Biolistic® PDS-1000 device is the only commercially available particle delivery system for gene transfer. The PDS-1000/He device is a helium-driven particle gun that accelerates a plastic cylinder called the macrocarrier, upon which millions of DNA coated with particles, have been dried. Two kinds of commonly used microprojectile particles are tungsten and gold powder. The shape and size of the particles may also influence transformation efficiency. Tungsten particles with an irregular surface are predisposed to

agglomeration, whereas gold particles with their spherical shape remain separated during the DNA coating procedure and should cause less cell damage. Southgate *et al.* used a mixture of gold particles of different sizes (ranging from 0.4 to 1.2 µm) to achieve effective transient gene expression in a wide range of plant species and explants [5). Rasco-Gaunt *et al.* observed that bombardment with 0.6 µm Bio-Rad gold particles typically resulted in finer expression than those obtained from 0.4 to 1.2 µm Heraeus and 1.0 µm Bio-Rad particles [6], indicating that the particle aggregation of smaller gold particles was less pronounced than larger particles during precipitation. Moreover, 0.6 µm particles also minimized tissue damage.

Efficient adherence of DNA onto the surface of the microprojectiles is essential for optimal DNA delivery. Presence of $CaCl_2$ and spermidine are necessary conditions for efficient precipitation of DNA, and the absence of either of them leads to loss of DNA in the washing steps. The sterile gold microprojectiles and DNA were sonicated continuously with the addition of 2.5 M $CaCl_2$ and 0.1 M spermidine and pipetted onto the surface of the macrocarrier to dry [7]. The amount of DNA used to coat the gold particles varies among the researchers. However, Rasco-Gaunt *et al.* showed that delivering DNA at more than the threshold level of 29 ng did not increase transient expression [6]. It was important to reduce the damage sustained by the target tissues by minimizing the amount of gold particles used for bombardment.

Three principle factors including vacuum, accelerating power of the device, and distance between the stopping plate and target tissue can affect microprojectile velocity and successful penetration of microprojectiles into target tissues. Low acceleration pressures (650–1,100 psi) result in larger areas being covered by particles and a more uniform distribution, which reduces tissue injury as compared to higher acceleration pressures (1,300 and 1,550 psi). The chamber vacuum pressure used routinely for deliveries with the PDS-1000/He gun is 27–28 inches Hg. Indra *et al.* recommended the use of a Petri dish containing the cultured embryos to be placed on the sample holder at level 4 (12 cm) from the top [8]. However, Rasco-Gaunt *et al.* determined the target distance that produced optimum transient expression to be 5.5 cm [6].

Much effort has been directed towards finding a strong constitutive promoter for GUS gene expression ever since the development of bombardment technology. Different promoters have different effects on gene expression through particle bombardment. Polyubiquitin gene promoters usually show strong and constitutive expression on reporter genes in transgenic plants [9]. The maize ubiquitin promoter has been widely used to express reporter genes and has been shown to cause high levels of gene expression in monocot transgenic plants [10].

In addition to its use in the production of genetically transformed organisms, high-velocity microprojectile delivery of DNA has been employed to develop a system to further analyze the regulation of the anthocyanin biosynthetic genes [11]. Yang *et al.* applied the microprojectile bombardment method to understand gene expression [12]. Co-bombardment assays utilizing the Bio-Rad gene gun have been performed to study gene function, interaction of effector and resistance genes in the tobacco system, and protein ubiquitination pathways in the interaction of barley with the powdery mildew fungus [13–15]. DNA encoding an inducer of programed cell death (PCD) is co-transformed with the GUS gene; PCD causes most of the cells expressing GUS to be eliminated [14]. Conversely, the gene suppression of PCD can be determined by restoring GUS expression in cells [14]. In the present study, we aimed to analyze the bombardment parameters to determine an optimum method for transient expression of GUS on wheat Alpowa cultivar scutellum calli and leaves in order to study the gene function in PCD and characterize the candidate effectors in the interaction of the host with the fungal

pathogen. We found that the optimized bombardment parameters for GUS expression in wheat leaves were 2.5 µg plasmid, 50 µl of 2.5 M $CaCl_2$, 20 µl of 0.1 M spermidine, and 50 µl of 50 mg/ml gold for 10 shots with the optimal target distance being that from the third slot from the bottom. These optimized conditions for DNA delivery into wheat leaves can be used to rapidly characterize candidate effectors and gene functions.

Materials and methods

Production of immature wheat embryos for tissue culture and wheat leaves for bombardment

Spring wheat line Alpowa (PI 566596) was obtained from the United States Department of Agriculture National Plant Germplasm System. Wheat was planted at the Montana State University greenhouse under the conditions of 22°C/14°C day/night temperatures and a 16-h photoperiod. The first true leaves (apical 5 - 7 cm segments) were selected for bombardment 9 - 14 days after planting.

The most mature first heads that seemed to have seeds with embryos 1 - 1.5 mm long and could be cut appeared after several days. Five plants in each of the 16 pots provided at least 1,000 embryos of the correct size range for tissue culture. The seed coats were completely peeled-off and the seeds were stored in 500 ml screw cap bottles at 4°C. 100 ml of 70% ethanol was added to the bottle and rotated by hand so that the seeds kept moving for 2 min. Sterile water (100 ml) was added and the bottle swirled gently for 10 s to rinse the seeds and the inside of the bottle in the laminar flow hood. Diluted Clorox solution (100 ml; 1 part Clorox to 3 parts distilled H_2O containing ~6 drops of Tween) was added and the bottle was placed on a rotating shaker for 17 min with vigorous shaking to keep the seeds moving. The Clorox solution was drained off and washed 5 times with sterile water, gently turning the bottle upside down to rinse the walls as well. The surface sterilized seeds were stored in tightly capped bottles at 4°C until they were ready for dissection of embryos.

Dissection of immature embryos and callus culture

Dissection of immature embryos was performed under a stereo dissecting microscope and placed with the scutellum exposed in S1 medium. Phytagel (2 g) and distilled H_2O (300 ml) were added to a 1-liter capped bottle, shaken until the phytagel dispersed in water in a 65°C water bath, and then autoclaved. Another bottle contained media ingredients including 4.42 g MS salts (Murashige and Skoog 1962) supplemented with 150 mg asparagine, 40 mg thiamine, 60 g sucrose, 1 ml of 1 mg/ml 2,4-D stock, 0.5 ml of 5 mM $CuSO_4 \cdot 5H_2O$, and distilled H_2O to a total volume of 700 ml with pH adjusted to 5.8 with 0.2 N KOH. This media (700 ml) was transferred to a 1-liter flask containing 300 ml of the autoclaved phytagel solution and stirred to mix well. When all the solution was filtered through, the bottle was placed in a 65°C water bath until it was ready to be poured. S1 was poured into standard petri plates at a count of 25 plates per liter of media. The embryos were lifted with a pair of sharp-ended forceps and sliced open with the pointed end (the end previously attached to the stalk of the mother plant) through several tissue layers. They were plated at a density of approximately 100 embryos per standard sized petri plate of S1. Callus tissue was formed adequately for bombardment after 5 - 7 days on the callus induction medium S1. The calli or leaves were incubated at 24 - 27°C during callus formation for approximately 4 days before bombardment.

Bio-Rad PDS-1000 (gene gun) bombardment

Approximately 25 mg of gold microcarriers (Bio-Rad 1652263) were weighed in a 2 ml tube and 500 µl of 70% ethanol was added to it. After ultrasonication for 15 s, particles were soaked in 70% ethanol (without vortexing) for an additional 15 min. The gold particles were spun for 1 min in a microfuge to pellet them. One milliliter (volume to be adjusted for actual amount of gold) of sterile distilled H_2O was used

to wash the particles with vigorous vortexing for 1 min. The particles were spun for another 1 min and this step was repeated thrice. After the third wash, 0.5 ml (volume to be adjusted for actual amount of gold) of sterile distilled H_2O was added to a final concentration of 50 mg/ml and aliquoted to 4 tubes and stored at 4°C.

Approximately 4 h before bombardment, calli or leaves were transferred from S1 plates to S2 plates (same as S1 with additional 72.9 g sorbitol) at 50 - 80 calli or 3 leaf segments per S2 plate. The volumes for the components in the precipitation of DNA on gold were calculated. One tube of gold was thawed for the bombardment of 8 - 10 plates and subjected to ultrasonication for 15 s. In the flow bench, GUS vector, $CaCl_2$, and spermidine were added while the tube was still being vortexed. After 30 s, the tube was closed and finger-vortexed well. The tube was removed from the vortex and the gold allowed to settle for several minutes. The supernatant was removed after centrifugation for 15 s. Ethanol (100%) was added, and the tube was finger-vortexed well. Further, 10 µl of the suspension was added onto the center of each sterilized microcarrier. PSD-1000/He device was used to deliver the gold particles to the target tissue. The bombarded S2 plates were parafilmed and incubated overnight in the dark at 25°C.

Plasmid vector

The GUS vector was supplied by Dr. Rongda Qu, Department of Crop Science, North Carolina State University, Raleigh, NC, USA. pRESQ101 vector was constructed from the original GUS gene under the control of the maize Ubi1 promoter and Sma I site originating from vector pAHC25 [16]. Plasmid DNA was isolated from bacterial cultures using the QIAGEN Plasmid Mega kit (Qiagen, Valencia, CA).

GUS staining and scoring

Histochemical GUS assays were conducted 2 days after bombardment. GUS expression was examined by immersing calli or leaves in the X-Gluc buffer containing 2 mM X-Gluc, 50 mM sodium phosphate buffer pH 7.2, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, and 0.2% Triton X-100. Blue staining was assessed after incubation overnight at 37°C. Scoring of GUS expression blue spots was done according to the average number of blue spots per embryo. The significant differences between the treatments were analyzed with a paired sample t-test using SPSS software.

Results and discussion

The gene gun bombardment parameters include size of the gold powder particles, target distance, and acceleration pressure. We recommend using the previously reported conditions to transform a similar biological system as a starting point for the optimization of biolistic transformation. Starting conditions for transformation were chosen as 0.6 µm diameter gold and 27 inches Hg as chamber vacuum pressure. For wheat, we used 1,000 psi, a 2.5 cm gap distance (distance between the rupture disk and the macrocarrier), and a 0.8 cm macrocarrier flight distance (distance between the macrocarrier and stopping screen). These conditions were standardized for the biolistic experiments (Table 1).

In this study, we evaluated the effect of changing the amounts of the components of precipitation mixture, osmotic preconditioning, and bombardment parameters.

Table 1. Parameters used for particle bombardment of wheat calli.

Gap distance	2.5 cm
Macrocarrier flight distance	0.8 cm
Gas pressure	1000 psi
Partial vacuum	27 inch Hg
Particles gold size	0.6 µm
Focus tube	Yes

Table 2. Manipulation of different precipitation parameters for optimized DNA delivery.

Item	Amount of plasmid (µg)	Amount of 2.5 M $CaCl_2$ (µl)	Amount of 0.1 M spermidine (µl)	Volume of 50 mg/ml gold (µl)	Exposure of scutellum
1	1	50	20	50	NO
2	2.5	50	20	50	NO
3	2.5	50	20	50	YES
4	2.5	25	10	50	NO
5	5.0	50	20	100	NO
6	5.0	50	20	100	YES

Optimization of DNA precipitation parameters in calli

The precipitation of DNA onto gold particles allows the determination of the potential amount of DNA to be delivered. To assess whether plasmid DNA, $CaCl_2$, and spermidine influence GUS expression, their concentrations had to be optimized. We used 1 µg, 2.5 µg, and 5 µg plasmid DNA, 25 µl and 50 µl of 2.5 M $CaCl_2$, and 10 µl and 20 µl of 0.1 M spermidine to precipitate onto gold particles and to monitor the influence on transient expression of GUS. Employing the parameters shown in Table 1, an average of 10 bombardment events were conducted at a time. A focus tube, which is a hollow steel tube, was positioned under the microcarrier holder at the microcarrier launch assembly.

Control samples were bombarded by particles without DNA and did not present any blue spots. A range of precipitation conditions were tested independently while maintaining all other conditions as standard (Table 1 and Table 2). There was a significant change in the levels of GUS expression between precipitating 1 µg and 2.5 µg DNA onto 2.5 mg gold particles for 10 bombardments (Table 2: Item 1 and Item 2; Figure 1A: column1 and column 2) at the same conditions as other precipitation complexes. The average number of GUS spots per embryo was 10 ± 2.0 when 1 µg DNA (100 ng DNA per bombardment) was precipitated (Table 2: Item 1; Figure 1A: column 1). However, a significant number of blue spots with increased GUS expression was observed at 83.6 ± 7.6 with 2.5 µg DNA used for precipitation (Table 2: Item 2; Figure 1A: column 2). The transient expression data suggested that doubling the amount of plasmid used for precipitation onto equally doubled amount of gold particles did not increase the GUS expression compared between Figure 1A column 2 and Figure 1A column 5 because increasing the number of gold particles for precipitation might cause physical damage to the target cells. Similar GUS expression was observed when 2.5 and 5 µg DNA was precipitated onto 2.5 mg and 5 mg gold particles separately, although different amounts of $CaCl_2$ and spermidine were used (Table 2: Item 4 and Item 5; Figure 1A: column 4 and column 5; and Figure 1B: picture 4 and picture 5). Therefore, 2.5 µg DNA and 50 µl gold particles (50 mg/ml) were selected to be the optimum amounts for increasing GUS transient expression.

DNA was precipitated onto microcarriers along with the addition of $CaCl_2$ and spermidine. The concentrations of $CaCl_2$ and spermidine affected the efficiency of precipitation and subsequent loss of DNA through washes. Klein *et al*. used a range of $CaCl_2$ concentrations in maize, beginning from 0.2 to 1.9 M, and found them to be optimal for transient expression of the *uidA* gene [2]. Kikkert showed that 2.5 M sterile $CaC1_2$ and 0.1 M sterile spermidine was optimum for bombardment [7]. Taylor *et al*. found that 0.1 and 0.5 M spermidine was effective for GUS expression in pearl millet embryos [17]. Different laboratories often employ slightly different

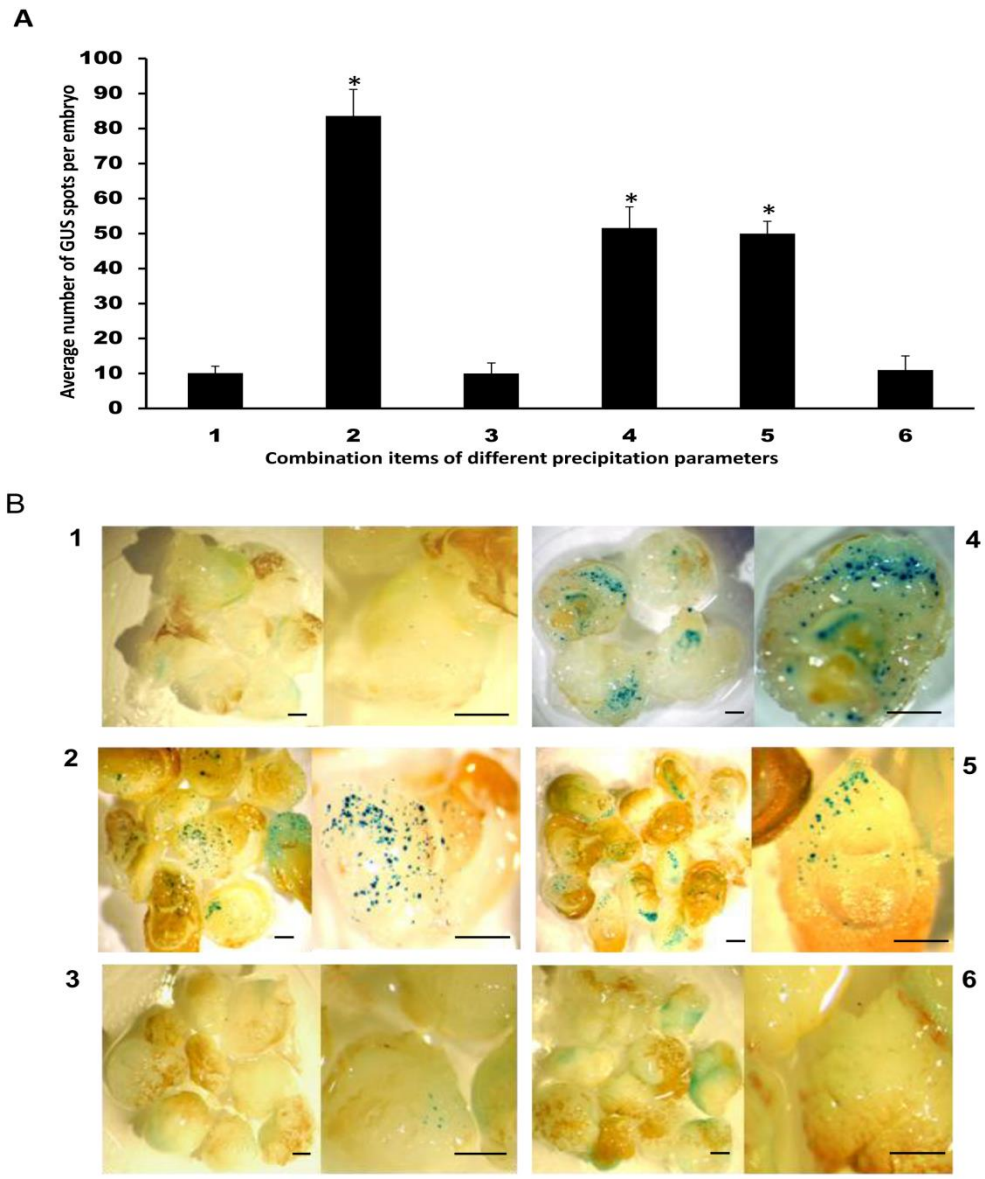

Figure 1. Transient β-glucuronidase (GUS) expression in wheat calli bombarded under different DNA precipitation conditions. **A.** Average GUS blue spots scored with different precipitation parameters. Statistical analysis of results from 10 shots was conducted for each assay. **B.** Result of GUS staining from one shot shown under different DNA precipitation conditions. The amounts of plasmid DNA, $CaCl_2$ and spermidine were optimized. (Error bars represent the variation between three independent replicates. Asterisks indicate a significant difference ($p < 0.05$) using a paired sample t-test. Bars = 0.5 mm.)

procedures for DNA precipitation. Our protocol involved the use of 2.5 M $CaC1_2$ and 0.1 M spermidine for bombardment. The GUS expression level in 50 µl $CaC1_2$ (2.5 M) and 20 µl spermidine (0.1 M) was 37.5% higher than that with half the amount of $CaC1_2$ and spermidine at the same 2.5 µg DNA precipitated onto 2.5 mg gold particles (Table 2: Item 2 and Item 4; Figure 1A: column 2 and column 4; and Figure 1B: picture 2 and picture 4).

Testing the effect of scutellum callus osmotic preconditioning

Immature embryos are the most common starting material for the establishment of embryogenic callus of cereals and grasses [18].

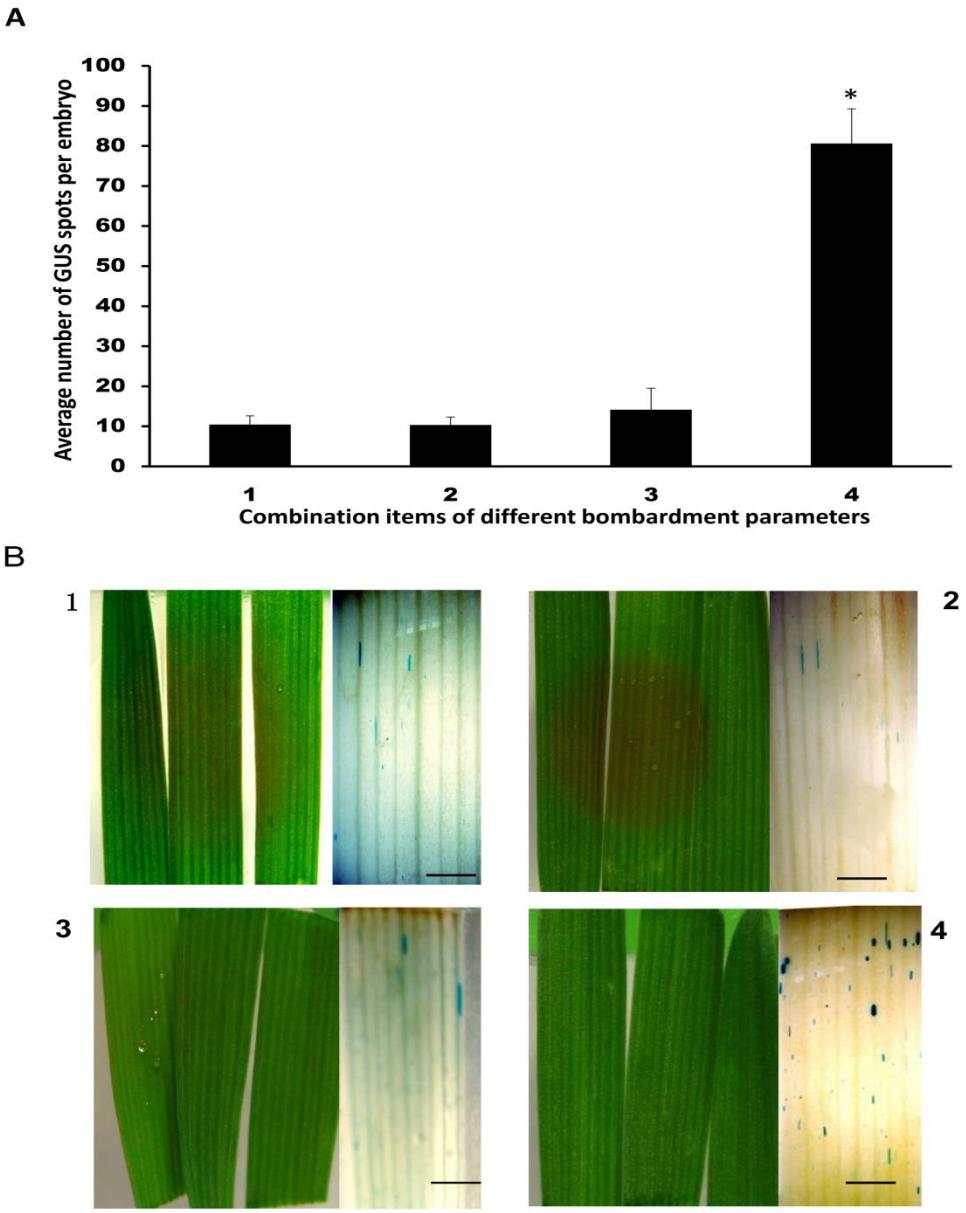

Figure 2. Transient β-glucuronidase (GUS) expression in wheat leaves bombarded under different bombardment parameters. **A.** Gus blue spots scored with different bombardment parameters. Statistical analysis of results from 10 shots was conducted for each assay. **B.** Comparison of transient GUS expression in wheat leaf tissues under different bombardment parameters. (Error bars represent the variation be tween three independent replicates. Asterisks indicate a significant difference ($p < 0.05$) using a paired sample t-test. Bars=0.5 mm.)

Wheat (*Triticum aestivum* L.) embryogenic callus is formed from two different organs, scutellum [18] and epiblast [19]. The embryos were excised and placed in medium either with the axial side up (scutellum in contact with the medium) or with the axial side down (scutellum exposed). Rasco-Gaunt *et al*. reported that embryos placed with the embryo-axial side in contact with the medium thereby exposing the scutellum, when bombarded, result in high GUS expression [6]. Scutellum calli is easy to form in Alpowa wheat cultivar irrespective of the position of the axial side. Osmotic pre-conditioning of the cells significantly influences the rate of stable transformation because plasmolysis reduces cell damage by preventing leakage of the

protoplasm of bombarded cells [20]. Our results showed only 10 GUS blue spots after the bombardment of the scutellum calli incubated far from the medium, irrespective of whether 2.5 or 5 µg DNA was used (Table 2: Item 3 and Item 6; Figure 1A: column 3 and column 6; Figure 1B: picture 3 and picture 6). The reason for the reduction in GUS expression is due to the failing osmotic pre-conditioning of the cells.

In conclusion, when the scutellum was in contact with the medium, the optimized DNA precipitation parameters were 2.5 µg plasmid, 50 µl of 2.5 M $CaCl_2$, 20 µl of 0.1 M spermidine, and 50 µl of 50 mg/ml gold particles. These parameters were used for optimization of bombardment on wheat leaves.

Optimization of the particle bombardment parameters in leaves

Several bombardment parameters influence the delivery of particles using the PDS-1000/He gun, including three adjustable distances, the gap distance (distance between the rupture disk and the macrocarrier), macrocarrier travel distance (distance between the macrocarrier and the stopping screen), and the target distance (distance between the stopping screen and the target plate). Among these distances, gap distance and macrocarrier travel distance are of lesser importance. The standard macrocarrier travel distance was 0.8 cm and the optimum gap distance was 2.5 cm [6]. To optimize the target distance for increased transformation frequency, we positioned the target plate at the second and third slot from the chamber bottom. The first true leaves were cut into 2 cm segments and incubated in the S2 plates for 4 h before bombardment. The parameters shown in Table 1 were used with or without the focus tube, and an average of 10 bombardment events were conducted at the same time. These optimized precipitation parameters (2.5 µg plasmid, 50 µl of 2.5 M $CaCl_2$, 20 µl of 0.1 M spermidine, and 50 µl of 50 mg/ml gold) were used for the optimization of bombardment parameters on leaves.

Table 3. Different bombardment parameters for optimizing β-glucuronidase (GUS) expression on wheat leaves.

Item	Focus tube	Slot from the bottom
1	Yes	2
2	Yes	3
3	No	2
4	No	3

The bombardment distance affects the velocity of particles as they enter the plant tissue and also the distribution of particles in the tissue. The control samples were bombarded with partides without DNA, and no blue spots were observed. Detailed information for the different bombardment parameters used for optimizing the GUS expression on wheat leaves was shown in Table 3. With a focus tube, the average GUS expression level was represented by 10 blue spots on one leaf segment when the leaf target plate was positioned at the second slot from the bottom (Table 3: Item 1; Figure 2A: column 1, and Figure 2B: picture 1). A similar result was obtained when the leaf target plate was positioned at the third slot from the bottom, as shown in Table 3: Item 2; Figure 2A: column 2 and Figure 2B: picture 2. A round dark range was observed when a focus tube was placed in the macrocarrier assembly (Figure 2B: picture 1 and picture 2). Without the focus tube, no round dark range was observed with the naked eye. Our results showed that the average number of blue spots was slightly higher in the absence of a focus tube compared to bombardment with a focus tube whatever the target plate was located at the second or third slot from the bottom (Figure 2A: Item 3 and Figure 2B: picture 3). A significant difference in GUS expression at 80 blue spots was observed with the target plate being placed at the third slot from the bottom (Figure 2A: Item 4 and Figure 2B: picture 4). Compared to bombardment without the focus tube, the center of the target area was exposed to a more powerful blast leading to higher microprojectile velocities that might result in significant cell damage when the focus tube was used. This cell damage resulted in the reduction

of GUS expression. In conclusion, the second slot from the bottom is the optimum target distance for the bombardment of leaves.

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