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Full Length Research Paper

High and low pressure gene gun devices give similar transformation efficiencies in maize calluses

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Maize (*Zea mays*) is a major food crop of the world. Biotechnology plays an important role in plant genetic improvement, particularly for the introduction of novel traits in order to improve agronomic performance, medical and industrial applications and food quality. Particle bombardment is a rapid and simple method that enables the generation of events and affords genes expression studies. Nonetheless, an appropriate reporter gene is necessary to visualize gene expression in the transformed cells. In this study, conditions for transient expression in maize calluses for high and low gene gun pressure devices were optimized and their efficiencies were compared. Performance of *gus* and *gfp* reporter genes, either bombarded alone or together, was measured and compared. In addition, the advantages and disadvantages of utilizing these reporter genes in maize calluses and immature wheat embryos are discussed. Finally, we report that for transient transgene expression studies, the particle inflow gun (or LPGG) caters the need while a commercial gene gun (HPGG) device is not of much need.

Key words: Biolistic, transient expression, reporter genes, plant genetic improvement.

INTRODUCTION

Maize (*Zea mays*) is a major crop worldwide and an important monocot plant model in genetics, genomics and molecular biology studies (Vega et al., 2008). Plant biotechnology aims at improving agronomic, medical and industrial applications of crops. Plant transformation techniques are essential for the introduction of novel useful traits occurring across kingdoms or available in sexually incompatible genotypes. These techniques need proper optimization in order to achieve high quality and quantity transformation events. However, transformation techniques need to be properly optimized in order to obtain high-quality and high-frequency transformation systems. Biolistic gun offers rapid delivery of candidate genes or DNA into plant cells (Rasco-Gaunt et al., 1999). Particle acceleration can be achieved through high (HPGG) or low (LPGG) helium pressure gene guns. Both biolistic devices have been previously used in cereal transformation studies (Li et al., 2003; Fadeev et al., 2005). Most of transgenic research groups use HPGG device in their assays, despite of the high input costs and patent use restrictions. Contrary to this, the LPGG does not have these restrictions.

The transformation efficiency of these devices is influenced by various parameters (Zhang et al., 2007). In order to improve efficiency, it is necessary to determine appropriate bombardment conditions. Although, physical conditions have been separately analyzed in earlier studies (Frame et al., 2000; Zhang et al., 2007). Till date there is no report on comparison between these two delivery systems.

In the plant transformation system, reporter genes were used to analyze promoter activity, evaluate selection efficiency and inheritance of foreign genes in subsequent generations. The ß-Glucuronidase (*gus*) gene has been extensively used as a gene expression reporter in plants (Jefferson et al., 1987; Finer et al., 1992; Weeks et al., 1993; Ishimaru et al., 1999). The ß-Glucuronidase activity

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Abbreviations: HPGG, High pressure gene gun; **LPGG,** low pressure gene gun; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **N6,** Chu's medium (Chu et al., 1975).

can be detected in transformed tissues following GUS histochemical analysis. This technique is a destructive and is not suitable for in *vivo* transient or stable gene expression studies. On the other hand, the visualization of Green Fluorescent Protein (*gfp*), isolated from the jellyfish *Aequorea Victoria* (Haseloff et al., 1997), implies a non-destructive test.

The aim of this work was to optimize bombardment conditions for both - high and low pressure- DNA delivery systems and identify the best conditions for transient gene expression in maize calluses. We also evaluated the interaction between two of the reporter genes most used in biotechnology: ß-Glucuronidase (*gus*) and Green Fluorescent Protein (*gfp*).

MATERIALS AND METHODS

Plasmids

Two vectors were used for the bombardment assays: pGUSCeUBI (10.150 kb) and pGFPCeUBI (8904 kb).

The pGUSCeUBI vector contains the *gus* gene, regulated by the *actin* promoter (Actin gene from rice) and the *nos* terminator (Nopaline Synthetase gene from *Agrobacterium tumefaciens*).

The PGFPCeUBI vector contains the *gfp* gene, regulated by the *actin* promoter and the *3´ocs* terminator (Octopine Synthetase gene from *A. tumefaciens*).

Both vectors contain a selection cassette with the *bar* gene, regulated by the *ubiquitin* promoter (*ubiquitin1* gene from maize) (Gordon-Kamn et al., 2002) and the *nos* gene terminator. The bar gene codes for the enzyme PAT, which inactivates the herbicide phosphinotricin or ammonium glufosinate by acetylation process (Murakami et al., 1986). Phosphinotricin is a glutamate analog that inhibits glutamine synthetase. The inhibition results in the accumulation of NH4+ which is toxic for the plant cell. Transgenic plants expressing a chimeric bar gene are resistant to high doses of phosphinotricin (De Block et al., 1987; 1988).

To obtain pGUSCeUBI, the pAct1D vector (provided by CAMBIA) was digested with *Not*I. The pUBI-BAR-NOS vector (kindly provided by Dr. del Vas, Biotechnology Institute, INTA, Argentina) was digested with *Not*I and inserted into the *Not*I site of pAct1D.

The plasmid pGFPCeUBI was constructed as follows: The *gfp* cDNA was amplified from the pBINm-gfp5ER vector (kindly provided by Dr J. Hasseloff, MRC Laboratory of Molecular Biology, Cambridge, England) using the primers *xgfpfor* (5'GCGCCTCGAGAAGGAGATATAACATGAAGAC 3') and *hgfprev* (5'GCCAAGCTTTTAAAGCTCATCATGTTTGTA 3'). The PCR product was digested with *Xho*I and *Hin*dIII, and inserted into the *Xho*I/*Hin*dIII site of pH8 (provided by Dr. del Vas, Biotechnology Institute, INTA, Argentina). The pH8 vector contains the *Actin* promoter and the 3´*ocs* terminator. This construct, called pGFPCe was digested with *Not*I. The pUBI-BAR-NOS vector was digested with *Not*I and inserted into the *Not*I site of pGFPCe.

Plant material

Maize

Maize (*Z. mays L.*) embryogenic type II calluses (Valdez et al., 2004) were initiated from immature embryos of the Hi-II genotype (Gordon-Kamm et al., 2002). Immature embryos were aseptically isolated from Hi-II seeds, 10 - 12 days post pollination. Embryos were cultured in the dark at 28°C on 2,4-D N6 culture medium (Chu et al., 1975). Highly embryogenic calluses were sub-cultured to fresh N6 medium every two weeks.

Wheat

Plants of genotype ProINTA Federal were grown in a growth chamber at 18/15°C thermoperiod and 16/8h (day/night) photoperiod to be used as scutella donor plants. Scutella, of approximately 1 mm in size, were dissected in aseptically conditions from immature embryos and were used as target for gene transfer following the biolistic procedure described in Pellegrineschi et al. (1998, 1999).

Bombardment of maize cells

Ten days subculture calluses were used for transformation assays. Four hours before bombardment assays, calluses were placed in osmotic medium (N6 medium containing manitol 0.4 M and sorbitol 0.4 M) (Aulinger et al., 2003). The calluses remained in the osmotic medium for 16 h post-bombardment and were then placed in N6 medium for 10 days. Embryogenic Hi II calluses were bombarded using two devices, the particle inflow gun (P.I.G.) and the PDS 1000 He from Bio Rad. The Particle Inflow Gun (PIG) was hand-made constructed using equipment and supplies that were readily available from equipment supply companies and following Finer (1992) instructions. The P.I.G helium pressure range commonly used is between 5 and 9 bar (72.5 and 130.5 psi) (Finer, 1992). On the other hand, the PDS 1000 He helium pressure range commonly used is between 300 and 2000 psi (user guide Bio Rad Laboratories).

The particle suspension was obtained out by the sequential addition of 10 µl of 50 mg/ml gold particles, 10 µl of 1µg/µl plasmid DNA, 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1M spermidine.

Particle inflow gun (LPGG) bombardment conditions

For each bombardment, 10 µl of the particle suspension was placed in the center of the screen in a disassembled syringe filter unit. Embryogenic calluses were placed on the shelves, either 3, 6 or 9 cm away from the syringe filter unit. Tissues were protected with a 250-µm nylon screen baffle. A vacuum of -0.9 bar was applied inside the chamber and the particles were discharged when helium gas was released by activation of the solenoid. Three different helium pressures 6 bar (87 PSI), 7 bar (101.5 PSI) and 8 bar (116 PSI) were studied.

PDS 1000 (HPGG) bombardment conditions

For each bombardment, 10 µl of the particle suspension was placed in the center of the macrocarrier membrane. Retention screens and rupture membranes for the helium pressure of 650 PSI, 900 PSI or 1100 PSI were used in each case of transformation assay. Embryogenic calluses were placed 3, 6 and 9 cm away from the macrocarrier position. A vacuum of -0.9 bar was applied and the particles were discharged when the helium broke the rupture membrane. Three different helium pressures (650 PSI, 900 PSI and 1100 PSI) were used.

Amounts of DNA used

For LPGG, the amounts of DNA delivered in each experiment were 0.5; 1.0 or 2.0 µg, whereas, for HPGG, DNA variation was 0.25; 0.5; 1.0; 1.5 and 2.0 µg.

Table 1. Bombardment conditions that gave best results.

	HPGG (PDS-1000/He)	LPGG (P.I.G.)
Pressure	1100 Psi	101.5 Psi
Size particle	1µm	1µm
Distance	6 cm	9 cm (*gus*)/ 3 cm (*gfp*)
Average number of blue dots (GUS)	1266	1864
Average number of fluorescent areas (GFP)	11.07	11.3

Visualization of *gus* gene expression

The activity of the β-glucuronidase enzyme was visualized by histochemical reaction with 2 mM X-Gluc substrate (5-Br-4-Cl-3-indolyl-b-D-glucuronic acid). The visualization of *gus* expression was carried out under binocular microscope (10x and 30x). The photographs were taken with a digital camera (Leica MZ16F).

Visualization of *gfp* gene expression

Bombarded calluses were cultured in 2,4-D+N6 medium at 26°C for 5 days after which *gfp* transient expression was visualized under binocular microscope (Leica DFC model FX 300,10x and 30x) and observed with a Leica 106Z lamp for blue light, with a 390 -460 nm excitation range and a 480 - 520 nm emission spectrum range.

Comparison of *gus* and *gfp* transient gene co-expression

Transient reporter gene expression for plasmids pGUS-CE-UBI and pGFP-CE-UBI vectors were used in co-bombardment. The total DNA amount used in each bombardment was 1 µg, containing equimolar amounts of both vectors and als containing the same copy quantity of each plasmid in the mix. Assays were carried out on maize calluses as well as on wheat immature embryos. In both cases the bombardment conditions were as follows: 1100 Psi helium pressure, 6 cm distance, 0.9 bar pressure vacuum and 1.0 µm gold particle diameter. In co-bombarded maize calluses, *gfp* gene transient expression was observed 5 days after bombardment followed by β-glucuronidase visualization. On the other hand, in wheat embryos, *gfp* gene and GUS transient expression were observed one day after bombardment.

Statistical analysis

For all statistical analysis, the Sigma Stat program was used. The normality of the data was analyzed by Kolmogorov-Smirnov test. It was necessary to convert the data according to the data^{-1} formula in order to achieve its normal distribution. This modification is allowed within the ANOVA analysis. A two-way ANOVA was carried out. The differences ($p < 0.001$) found after conducting a two-way ANOVA test, were analyzed with Tukey's test ($\alpha = 0.05$).

RESULTS

The transformation efficiency was measured as the number of gene insertions per treatment (blue spots for *gus* gene or green fluorescent area for *gfp* gene). The observations shown as follows are only an indirect assay or presence or absence of the transgene involved in the experiments and not a quantity of gene expression.

The best conditions and results obtained with HPGG and LPGG are shown in Table 1. No significant differences ($p > 0.001$) were found between both devices in terms of transformation efficiency. However, the HPGG-bombarded calluses showed more reproducible transformation efficiency between treatments than LPGG-bombarded calluses, in terms of standard deviation.

Gus gene transient expression analysis

High pressure gene gun

The highest *gus* gene insertion per treatment were observed at 1100 Psi when the bombardment distance was 6 cm using 1 µm particles. Under these conditions, average values of 1266 blue dots were observed. In contrast, the lowest number insertion per treatment was observed at 650 psi, 9 cm and 0.6 µm particles. In both cases, the blue GUS spots were observed in the peripheral area.

Significant differences ($p < 0.001$) were found when 1.0 µm particles and 6 cm of distance were used (Figure 1).

Low pressure gene gun

The highest *gus* gene insertion per treatment was observed at 101.5 Psi, 9 cm and 1.0 µm particles (Figure 2). At these conditions, an average value of 1864 blue dots was obtained. In contrast, the lowest level of insertion per treatment was observed at 87 Psi pressure for all distance and particle size conditions. Although low reproducibility were observed between the assays (Figure 2), significant differences ($p < 0.001$) were found with 101.5 Psi pressure, 1.0 µm particles and 6 cm of distance. Even though the 116 Psi caused the displacement of the calluses, the *gus* gene visualization was more homogeneous on the calluses that did not displace.

Gfp gene transient expression analysis

High pressure gene gun

In maize calluses, the *gfp* gene was observed as defined

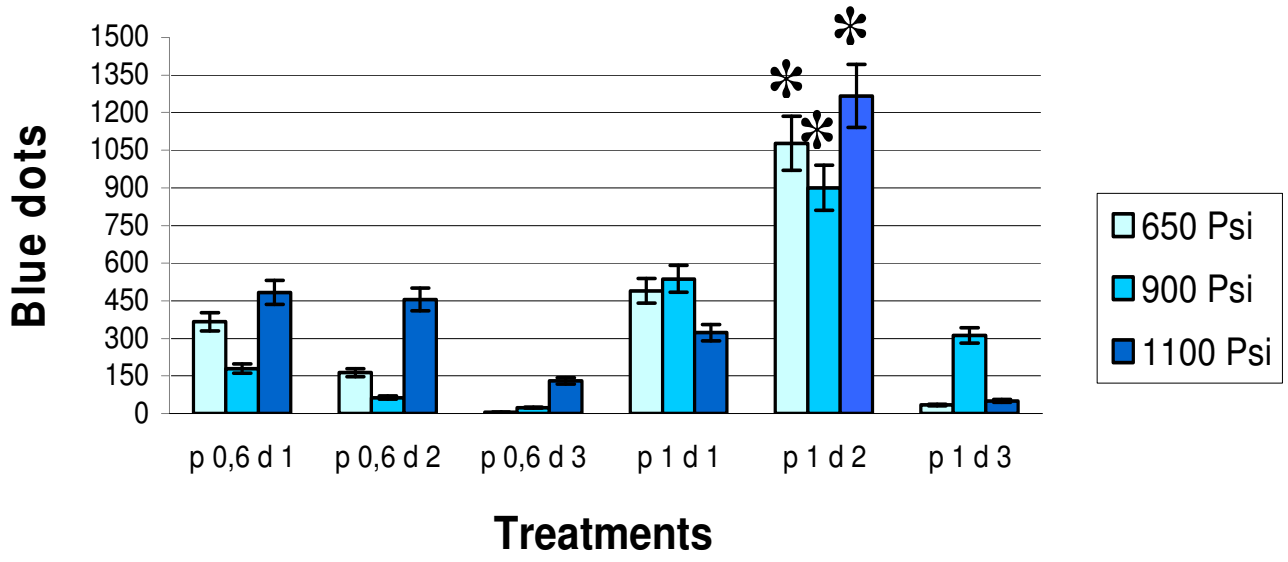

Figure 1. Average of bombardment assays. References: **p** is particle size (0.6 and 1 µm); **d** is distance (**d1**: 3 cm; **d2**: 6 cm; **d3**: 9 cm).
* indicates treatments with significant differences ($p < 0.001$).

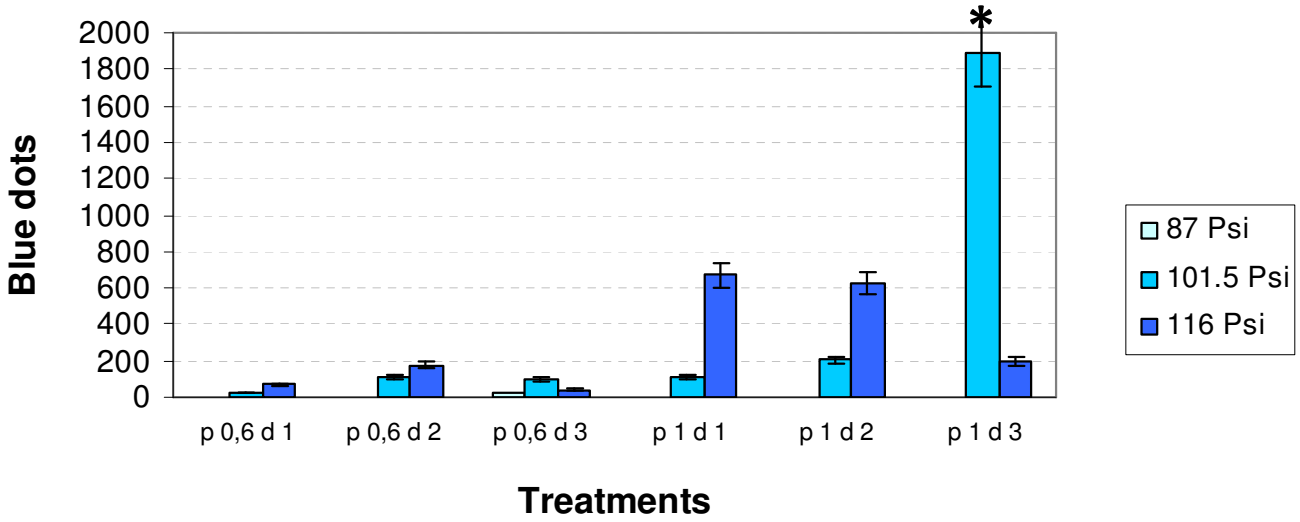

Figure 2. Average of bombardment assays. References: **p** is particle size (0.6 and 1 µm); **d** is distance (**d1**: 3 cm; **d2**: 6 cm; **d3**: 9 cm)
*indicates treatments with significant differences ($p < 0.001$).

areas instead of the blue dots observed with the *gus* gene. We therefore changed the scoring criterion to "green fluorescent areas per callus". The highest *gfp* gene insertion per treatment was observed at 1100 Psi, 6 cm and 1.0 µm particles (Figure 3). Under these conditions, an average value of 11.07 fluorescent areas per callus was obtained. In contrast, the lowest gene insertion per treatment was observed at 650 Psi, 6 cm and 0.6 µm particles. Significant differences ($p < 0.001$) were found when 1100 Psi pressure, 1 µm particles and 3 / 6 cm of distance were used.

Low pressure gene gun

Statistically significant differences ($p < 0.001$) were found between treatments. The highest gene insertion was observed at 101.5 psi pressure, 3 cm distance and 1.0 µm particles (Figure 4). Under these conditions, an average

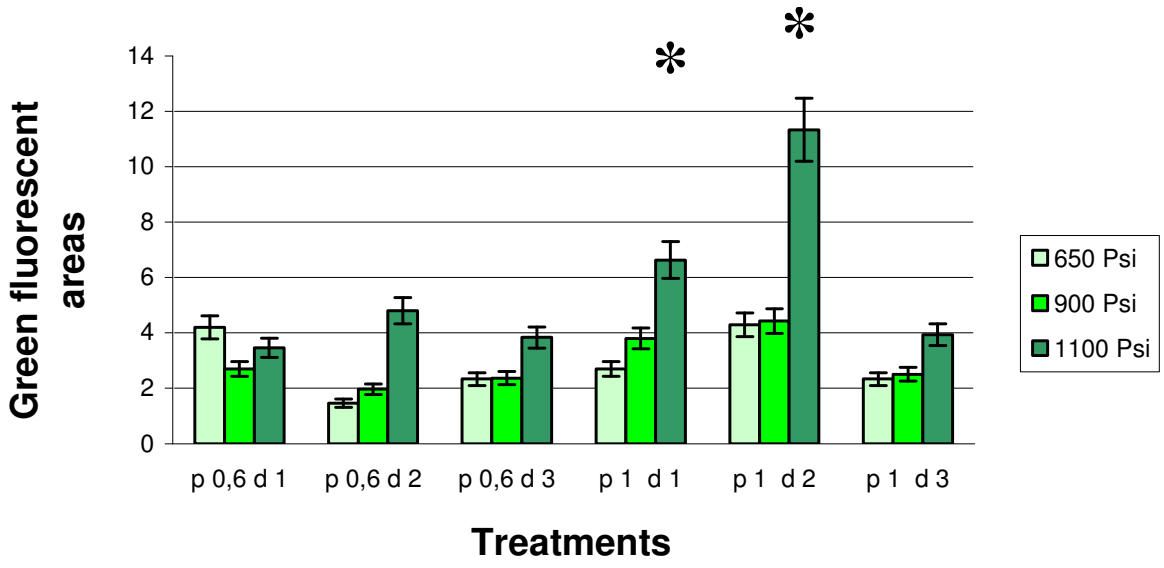

Figure 3. Average of bombardment assays. References: p is particle size (0.6 and 1 µm); d is distance (d1: 3 cm; d2: 6 cm; d3: 9 cm).
*indicates treatments with significant differences (p <0.001).

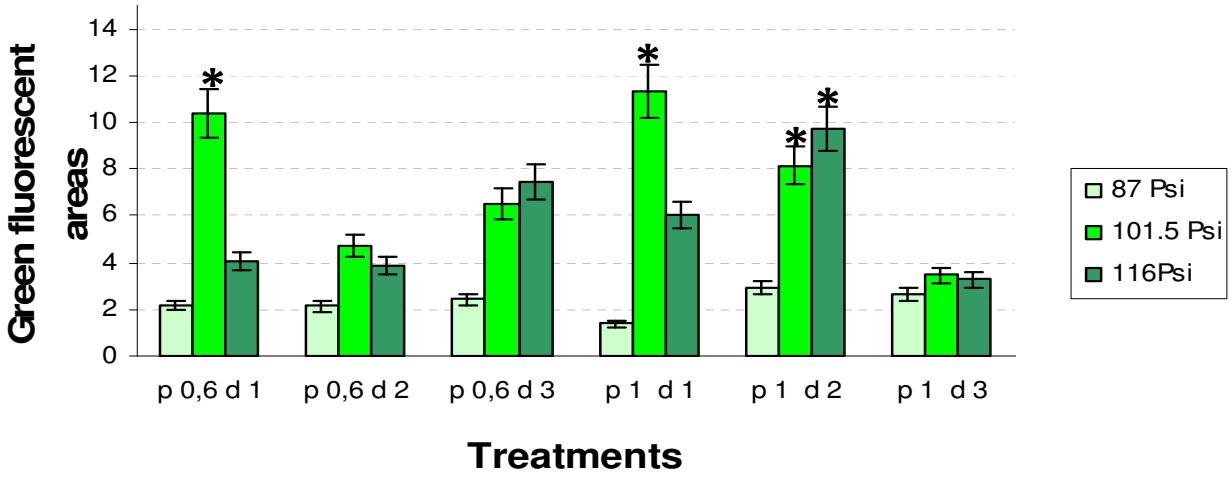

Figure 4. Average of bombardment assays. References: **p** is particle size (0.6 and 1 µm); **d** is distance (**d1**: 3 cm; **d2**: 6 cm; **d3**: 9 cm).
* indicates treatments with significant differences (p <0.001).

value of 11.3 fluorescent areas was obtained. In contrast, the lowest expression level was observed at 87 Psi, 3 cm and 1.0 µm particles.

Effect of DNA amount variation on transformation efficiencies

High pressure gene gun

We tested how varying the amount of DNA bombarded impacted on the transformation efficiency, under the best conditions identified for HPGG. In treatments with 1.0 µg, 1.5 µg, 2.0 µg DNA per shoot, transient expression levels were higher than 0.25 and 0.5 µg DNA per shoot (Figure 5) Statistically significant differences ($p < 0.001$) were found between treatments. Thus, *gus* insertion increased with DNA amount per shoot, up to 1.0 µg, and then remained relatively constant. In fact, transient *gus* gene insertion were similar when 1.0, 1.5 or 2.0 µg DNA per shoot were used.

The *gus* gene insertion ratio between 1.0 and 2.0 µg was

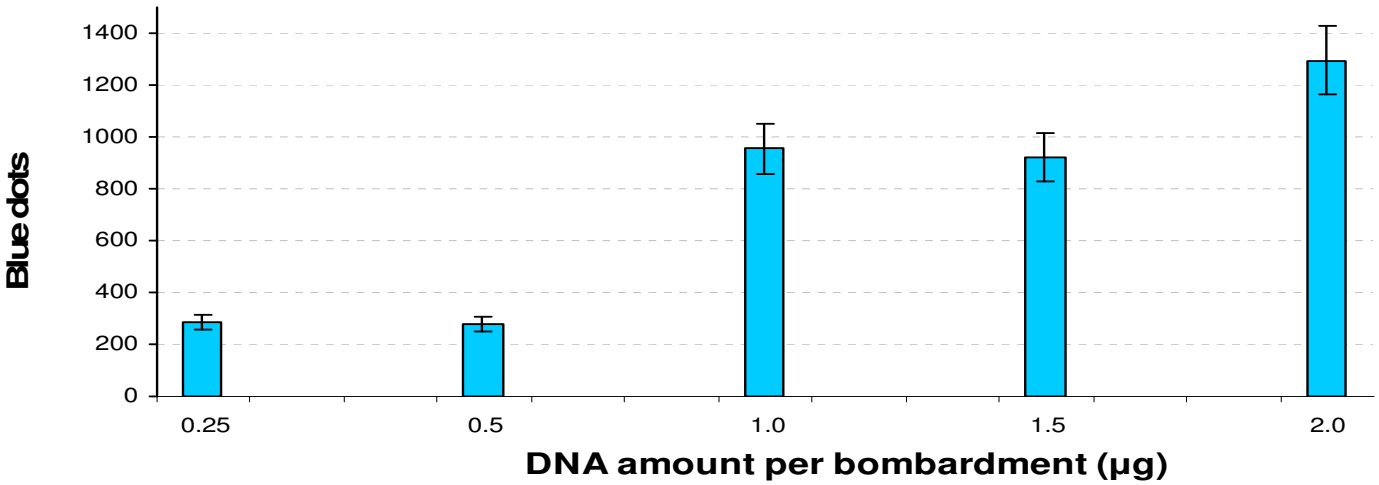

Figure 5. Relationship between the number of blue spots and the amount of DNA used. Bars indicate standard deviation corresponding to three repeats per treatment.

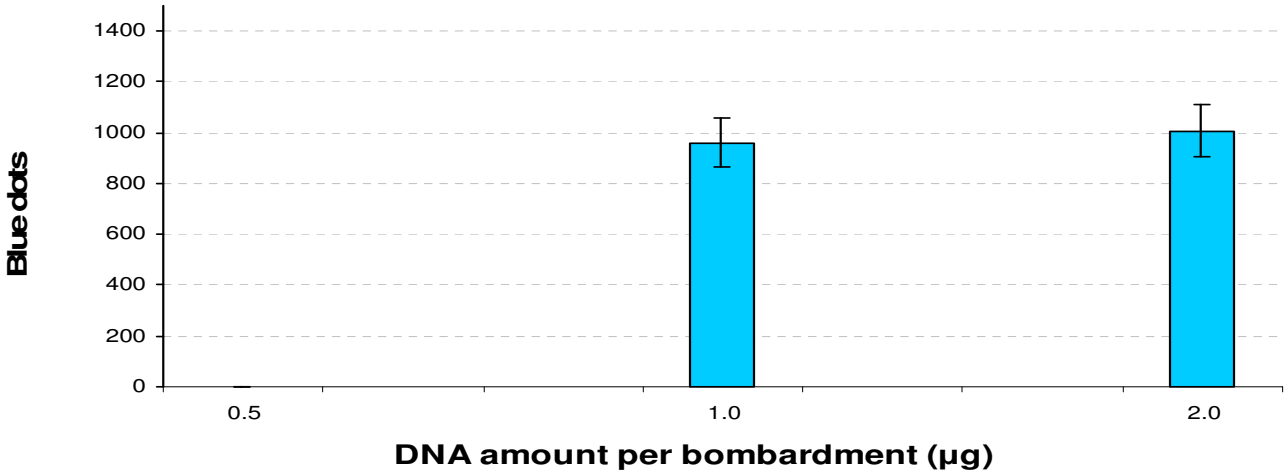

Figure 6. Relationship between the number of blue spots and the amount of DNA used. Bars indicate standard deviation corresponding to three repeats per treatment.

0.89. At these conditions, doubling DNA amount per shoot from 1.0 - 2.0 µg represented an improvement of only 11% in *gus* gene insertion.

Low pressure gun

Statistically significant differences ($p < 0.001$) were found between 0.5, 1.0 and 2.0 µg DNA per shoot. Using 0.5 µg DNA per shoot, the *gus* gene insertion was zero. The number of insertions was similar between 1.0 and 2.0 µg DNA per shoot.

These results are consistent in their tendency and absolute values with the results obtained with HPGG (Figure 6).

Comparison between the *gus* and *gfp genes*

In maize, both reporter genes were visualized by different assays: The presence of the *gus* gene was detected as dots, whereas, the *gfp* gene was detected as defined areas (Figures 7a and b). However, in immature wheat embryos, both reporter genes were observed as dots (Figures 7c and d). In maize calluses, due to the interference by GUS staining with GFP detection, we first visualized

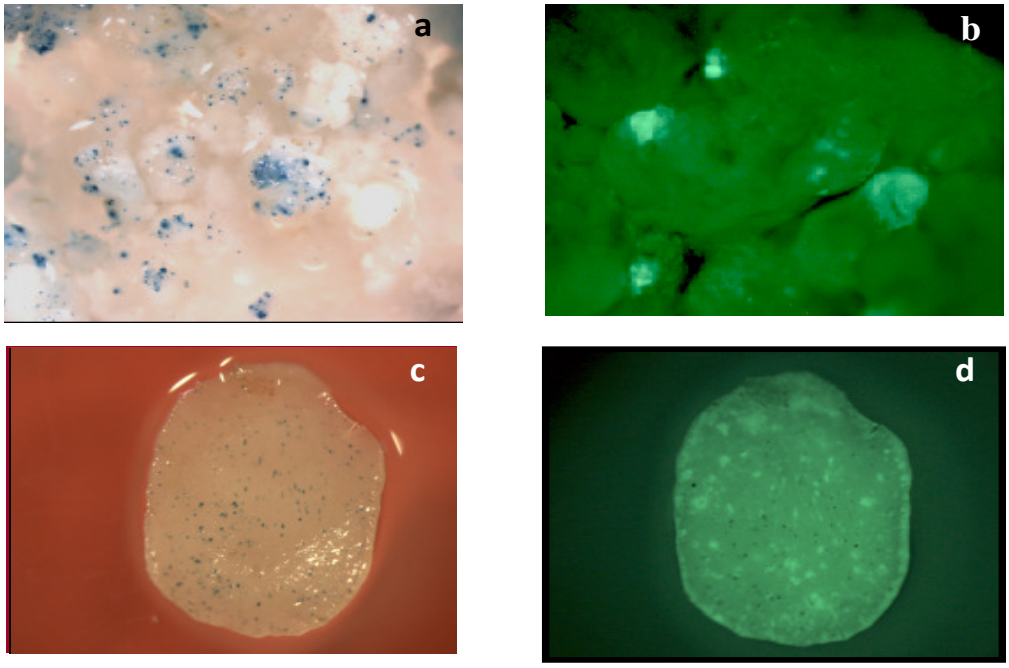

Figure 7. a- *gus* gene transient expression in a maize callus .b- *gfp* gene transient expression in the same maize callus. c- *gus* gene transient expression in a wheat embryo. d- *gfp* gene transient expression in the same wheat embryo.

Table 2. Averages of blues dots (*gus*) and green fluorescent areas (*gfp*) observed in co-bombardment and single reporter gene bombardment in maize calluses.

Co- bombardment		Single reporter gene bombardment	
gus	***gfp***	***gus***	***gfp***
1279.36	10.46	1266	11.07

GFP protein activity under fluorescent microscope, and then assayed for *gus* gene expression. In contrast, in immature wheat embryos it was possible to observe GFP activity even after applying X-Gluc substrate.

No differences were found in the number of gene insertions in co-bombardment values when comparing with single-gene bombardment results in maize calluses (Table 2).

DISCUSSION

The distance between the particle source from target tissue and helium gas pressure are known to influence the transformation frequency (Rasco-Gaunt et al., 1999; Fadeev et al., 2005). The findings in the present study with maize type II callus are in agreement with these findings. In HPGG, the lowest expression level was found at 9 cm distance with all pressures. We suppose that as the distance increases, particles dispersion increase and spread across larger area by diluting the point of bombardment. In contrast, LPGG showed a different behavior across the distances with no homogenous behavior. For the pGFPCeUBI vector, the best distance was 3 cm, whereas, for the pGUSCeUBI vector, it was found to be 9 cm. These differences might be due to the low reproducibility of the LPGG rather than to the intrinsic properties of the vectors. In LPGG, the lower acceleration might cause a lower particle tissue insertion.

The transient transformation efficiency varies with the particle size. In both gene guns, best results were obtained with 1.0 µm particles. This might be because 1.0 µm particles have a greater penetration force into cells, resulting in greater number of insertions.

Lower gene insertions per treatments were found when 0.6 µm particles were used. Under these conditions, the transformation is better with 1.0 µm particles but, for stable transformation and reducing gold particle size from 1.0 - 0.6 µm has a favorable effect on stable clone recovery from bombarded calluses (Frame et al., 2000). One reason for this might be that sub-cellular damage decreases when 0.6 µm particles are used, which would

represent an advantage for cell recovery and plant regeneration in stable expression assays.

Transient transformation efficiencies increase with higher pressures. In both devices, best conditions were found when higher pressures were used. Although 116 Psi was the best LPGG pressure, the calluses were propelled on the gene gun sidewalls. This condition was not suitable because it was impossible to recover the bombarded calluses without a risk of contamination or a possible loss of callus viability. This problem was observed only with LPGG and described previously by Finer in 1992. In LPGG, the helium column reaches the cell plate with very high force because it is retained only by a filter mesh. In contrast, in HPGG, helium is retained first by the rupture disk and then by the screen mesh, resulting in a lower direct column force on the calluses. In fact, HPGG did not dislodge the calluses, even when using high pressures. This might account for the more homogeneous results obtained with HPGG.

The increase in DNA amount per shoot from 0.25 µg up to 1.0 µg enhanced transient transformation levels. These values remained relatively constant when more than 1.0 µg DNA were used. The averages of gene insertion showed that both gene gun devices have a similar performance, at least at transient level. Thus, the DNA amount parameter was independent of the gene gun device.

The visualization time for both reporter genes was important to record the data. The *gus* gene visualization was carried out one day after bombardment. However, *gfp* visualization was carried out five days after bombardment. Five days might allow more time for cell recovery and proliferation.

Blue dots were distributed in the peripheral area of bombarded calluses. This distribution was described by Finer in 1992. This distribution might be due to a great stress of the bombarded cells caused by the high pressure and particle penetration. In contrast, this kind of distribution was not observed with the *gfp* gene. However, we cannot explain why the blue dots were still observed only in the peripheral area five days after the bombardment.

In maize calluses, the co-bombardment did not show an increase or decrease in the number of gene insertions as compared with the one-vector bombardment, suggesting that there were no interferences in the gene insertion efficiency between both genes.

In immature wheat embryos, GFP protein was expressed as fluorescent dots and in maize as fluorescent areas. This difference might be due to the single time-point of gene visualization.

In maize calluses, X-Gluc substrate interferes with *gfp* visualization. To solve this problem, the *gfp* visualization was carried out first, and the *gus* visualization with X-Gluc later. This visualization problem was not encountered in immature wheat embryos. This might be due to the different kind of tissues used: the maize calluses are not organized tissues, whereas, immature wheat embryos are more compact and organized.

Conclusion

The best conditions to evaluate the transient transformation efficiency for HPGG and LPGG were established. To date, this is the first work that compares efficiency and effect of bombardment parameters of two gene gun devices along with two different reporter genes. Several works have described conditions for stable transformation for each devices. Both gene guns can be used for transient experiments for both reporter genes but the choice of one of them for stable transformation depends on the kind of target tissue.

The advantages and disadvantages of working with both reporter genes were established in maize and wheat. The highest *gus* gene insertion number were observed at 1100 Psi when the bombardment distance was 6 cm using 1 µm particles. The optimal amount of DNA to be used in bombardment was 1.0 µg DNA per shoot for both HPGG and LPGG. Our results show that there are no interferences between *gus* and *gfp* transient expression in maize calluses and immature wheat embryos. To date, no reports about two reporter genes like *gus* and *gfp* interaction in plant tissues had been published. Indeed, this report gives data for the *gfp* reporter gene driven by a monocot promoter in maize calluses. These results may be useful to shed light on co-transformation experiments where different promoters and candidate genes of interest are used to ascertain the transformation and transgene expression patterns. Additionally, even tough LPGG may not be the greatest device in terms of reproducibility, is a suitable approach in transient gene expression experiments, and this allows to conclude that both devices may be used in transient expression genes studies in maize calluses and wheat embryos, taking into account appropriate setting conditions for each one.

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