# Optimization of Gene Delivery into Arabidopsis, Tobacco and Birch Using the Helios™ Gene Gun System

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## Summary

Particle bombardment is a physical method of cell transformation in which high density, sub-cellular sized particles are accelerated to high velocity to carry DNA/RNA into living cells. It is a versatile technique that can be used both for transient expression studies (*e.g.* promoter analysis) and creating stable transformants. The Helios Gene Gun particle delivery product uses a technique based on DNA-coated gold particles, precipitated on the inner wall of a plastic tube and accelerated by pressurized helium. The aim of this study was to optimize parameters for transient expression of gene constructs into plant material with the Helios Gene Gun system. As target plants, we used thale cress (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*), both generally used as model organisms in plant molecular biology and genetics, and silver birch (*Betula pendula*) as a representative of woody plants. To investigate the transient gene delivery, we used constructs containing the constitutively active promoter of the Cauliflower Mosaic Virus (CaMV) 35S transcript fused with reporter genes encoding luciferase (LUC) and β-glucuronidase (GUS). The optimization was performed in a step by step manner. The most critical parameters were helium pressure, the optimum of which varied in different plant species, and amount of gold. Gold particles with a diameter of 0.6 µm were optimal for all plant species studied. The optimization increased the expression levels five- to ten-fold. However, the variation in and between the experiments remained high.

## Introduction

Particle bombardment is a physical method of cell transformation in which high density, sub-cellular sized particles are accelerated to high velocity in order to carry DNA/RNA into living cells. The technique was first described as a method of gene transfer into plants (Klein *et al.*, 1987). Because it is a physical method, particle bombardment is readily applicable to a variety of biological systems and it also effectively overcomes physical barriers to gene transfer, such as the cell wall of plants. It is a versatile technique that can be used both for transient expression studies (*e.g.* promoter analysis) and for creating stable transformants (Christou, 1994). Particle bombardment has also been used for wounding plants in order to promote Agrobacterium transformation (Bidney *et al.,* 1992).

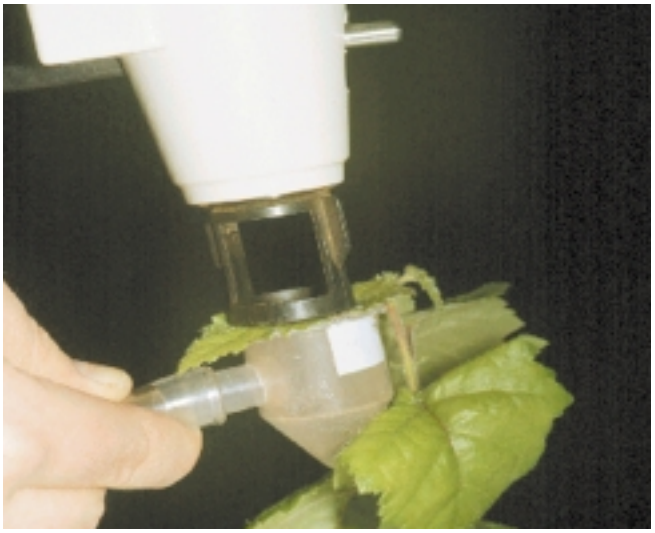


*Fig. 1. Bombarding birch with the Helios Gene Gun system.*

The Helios Gene Gun particle delivery product line uses a technique based on DNA-coated gold particles, precipitated on the inner wall of a plastic tube and accelerated by a flow of pressurized helium (Helios Gene Gun System Instruction Manual, 1996). The most significant difference between it and Bio-Rad's chambered Biolistic® device the PDS-1000/He is that the Helios Gene Gun system requires no vacuum, removing limitations to the target and its size. Moreover, the cartridges can be stored for several months and the bombardment procedure is much faster in comparison with the PDS-1000/He instrument. In practice these two particle delivery products complement each other, the vacuum chamber method providing a more controlled bombardment environment, and the Helios Gene Gun system providing a much wider selection of target material.

The aim of this study was to optimize parameters for transient expression of gene constructs into plant material with the Helios Gene Gun system. As target plants we used thale cress (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*), both generally used as model organisms in plant molecular biology and genetics, and silver birch (*Betula pendula*) as a representative of woody plants. In order to investigate the transient gene delivery, we used constructs containing the constitutively active promoter of the CaMV 35S transcript fused with reporter genes encoding luciferase (LUC) and β-glucuronidase (GUS).

## Methods

### PLANT MATERIAL

*Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg *erecta* plants were grown in peat at 20 °C with an 8 hour photoperiod. The 4–5 week old plants were bombarded before they started to bolt. *Nicotiana tabacum* (L.) cv. Petit Havana SR1 plants were grown at 23 °C with a 16 hour photoperiod in vermiculite and fertilized with a commercial fertilizer (Substral®, Thompson Siegel, Germany). The birch plants used were young greenhouse *Betula pendula* (Roth.) JR 1/4 clone plantlets grown in forest peat-vermiculite (2:1 ratio) mixture (Finnpeat, Kekkilä, Finland) with a 16 hour photoperiod.

### PLASMID CONSTRUCTS

The reporter gene constructs used were pANU21 (5.2 kb) containing the *uidA* gene encoding β-glucuronidase (GUS) from *Escherichia coli* and pHTT308 (6.6 kb) containing the *luc* gene from firefly encoding luciferase (LUC). Both plasmid constructs contain the reporter gene under the control of the constitutive promoter CaMV 35S (regular promoter for *uidA* and 4x35S for *luc*). In both plasmids the promoter is followed by the TMV leader Ω that functions as a translational enhancer (Gallie *et al.*, 1987). Particles used for control bombardments were coated with pUC19 (Yanisch-Perron *et al.*, 1985), the vector used for the reporter constructs.

### CARTRIDGE PREPARATION

All procedures of cartridge preparation were according to the Helios Gene Gun System Instruction Manual (1996) and are briefly summarized below.

Precipitation of DNA onto microcarriers. Gold particles were coated each time with a molar 1:1 mixture of plasmids pANU21 and pHTT308. The needed amount of gold microcarriers, 50 mM spermidine and the calculated amount of DNA in Tris-EDTA were combined together. One M $CaCl_2$ was added dropwise to associate the DNA with the gold particles. The gold suspension was pelleted and washed three times with dry ethanol prior to resuspension into a polyvinylpyrrolidone (PVP) solution in ethanol.

Cartridge preparation with the Tubing Prep Station. The Gold-Coat tubing was first dried by purging with nitrogen for 15 minutes. The suspension was drawn into the Gold-Coat tubing and placed into the Tubing Prep Station. The microcarriers were allowed to settle for a few minutes, after which ethanol was removed slowly. The Gold-Coat tubing was rotated and the particles were spread onto the inner surface of the tubing and subsequently dried with a flow of nitrogen. Any unevenly coated sections were discarded before the remaining tubing was cut into 0.5” pieces with the Tubing Cutter.

### EXPERIMENTAL DESIGN

Two separate coatings were made for each optimization step and kept separate during the whole experiment. In each step, the results were obtained from at least two independent experiments with 3–5 parallel shots.

### BOMBARDMENT CONDITIONS AND TRANSIENT EXPRESSION

Intact leaves of greenhouse grown arabidopsis, tobacco and birch plants were used as target material. The leaves were held in place during the bombardment by flattening them against a fine mesh with the help of a suction pipe attached to a vacuum cleaner (Figure 1). In some experiments a diffusion screen was used on the base of the Helios Gene Gun barrel to reduce tissue damage in the center of the shot. After bombardment, the plants were placed in the greenhouse for 24 hours before assaying for the enzyme activities. Each bombarded area was cut into two equal halves, one for the histochemical GUS assay and one for the quantitative LUC assay.

### ENZYMATIC ASSAYS

Histochemical GUS assay. One half of the leaf disk was submerged in 1 mM 5-bromo-4-chloro-indolyl-β-D-glucuronide (X-Gluc) in buffered solution [100 mM Na-phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 1.0 mM X-Gluc, 0.1% Triton® X-100] and incubated in the dark for 16 hours at 37 °C (Stomp, 1992). After staining, chlorophyll was removed with absolute ethanol in order to better visualize the blue spots, which were counted under a stereo microscope.

Luciferase assay. The other half of the bombarded area was homogenized in 200 µl ice-cold modified lux-buffer [50 mM Na-phosphate (pH 7.0), 4% soluble PVP (MW 360,000), 2 mM EDTA, 20 mM DTT] (Herrera-Estrella *et al.*, 1994). Cell debris was removed by a 10 minute centrifugation at maximal speed and the supernatant was assayed for luciferase activity. Twenty µl of plant extract was mixed with 100 µl of Luciferase Assay Reagent (Promega, USA) at room temperature and the emitted light was measured for a period of 10 seconds in a luminometer (Model 1254–001, Bio-Orbit, Finland). For tobacco, 10 µl of extract and 50 µl of Luciferase Assay Reagent were used after the first step of optimization.

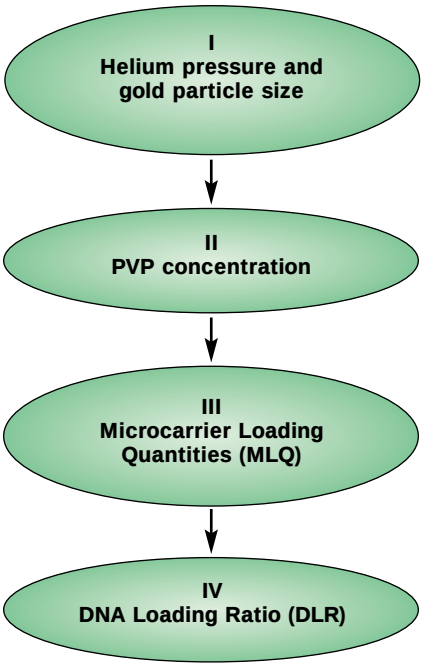
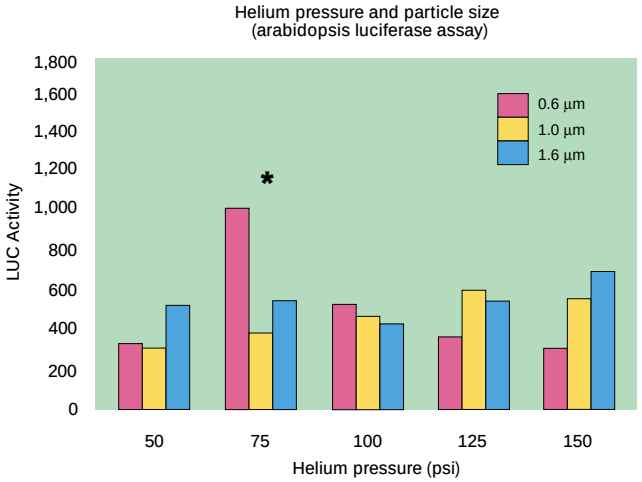
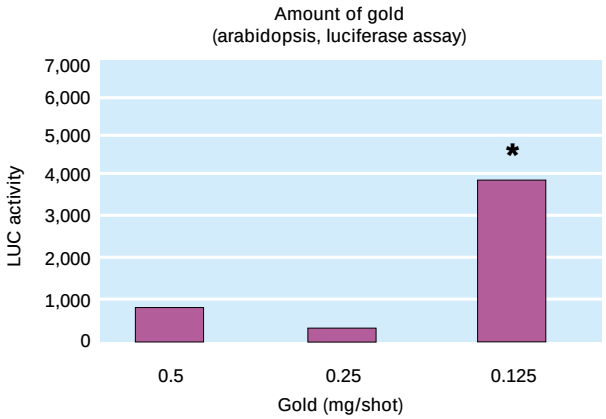
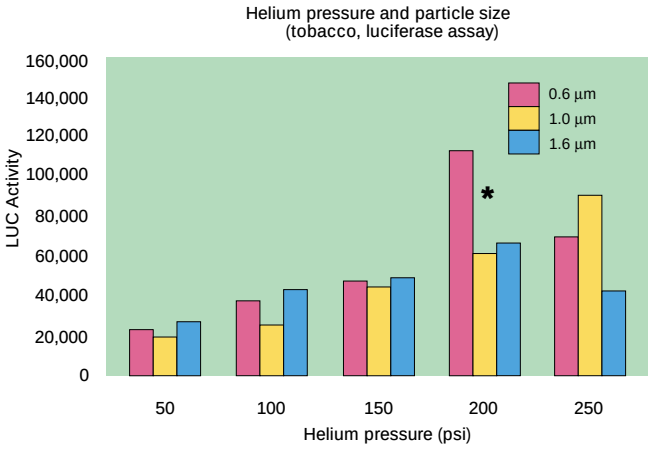
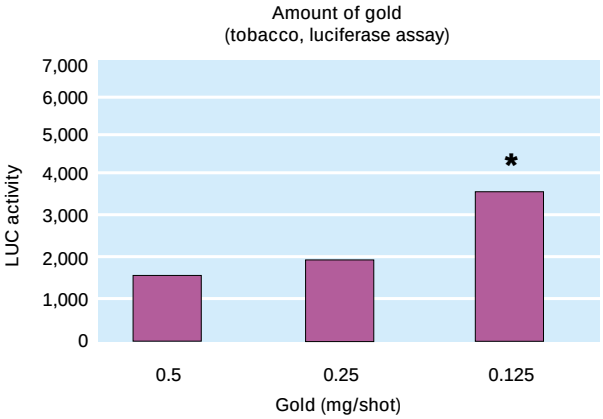


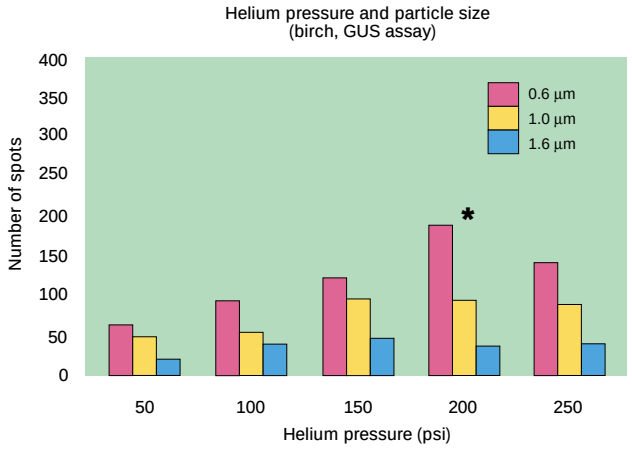

*Fig. 2. Outline of the optimization procedure.*



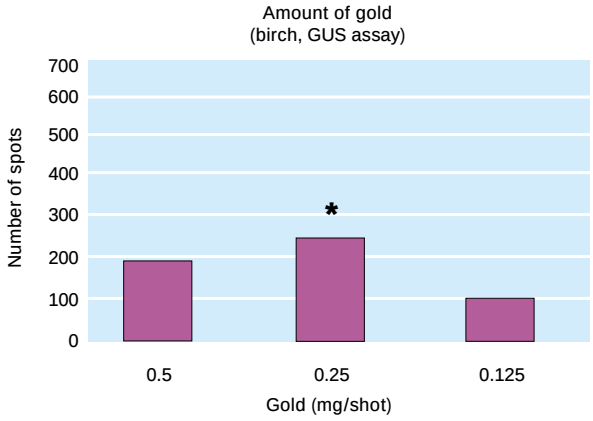




*Fig. 3. The effect of helium pressure and gold particle size. Asterisk shows the chosen combination for further optimization.*

*Fig. 4. The effect of the amount of gold. Asterisk shows the chosen combination for further optimization.*

**Table 1. Optimization Parameters and their Optimum Values for Arabidopsis, Tobacco and Birch**

| *Parameter* | *Tested options* | *Optimum* |
|---|---|---|
| **ARABIDOPSIS** | | |
| Pressure of helium | 50, 75, 100, 125, and 150 psi | 75 psi |
| Size of gold particles | 0.6, 1.0 and 1.6 µm | 0.6 µm |
| Concentration of PVP | 0, 0.05, and 0.1 mg/ml | no significant difference, 0.05 mg/ml chosen |
| Amount of gold per shot | 0.5, 0.25 and 0.125 mg | 0.125 mg |
| Amount of DNA per shot | 5, 1, 0.2 and 0.04 µg | 1 µg |
| **TOBACCO** | | |
| Pressure of helium | 50, 100, 150, 200 and 250 psi | 200 psi |
| Size of gold particles | 0.6, 1.0 and 1.6 µm | 0.6 µm |
| Concentration of PVP | 0, 0.05, and 0.1 mg/ml | no significant difference, 0.05 mg/ml chosen |
| Amount of gold per shot | 0.5, 0.25 and 0.125 mg | 0.125 mg |
| Amount of DNA per shot | 5, 1, 0.2 and 0.04 µg | 1 µg |
| **BIRCH** | | |
| Pressure of helium | 50, 100, 150, 200 and 250 psi | 200 psi |
| Size of gold particles | 0.6, 1.0 and 1.6 µm | 0.6 µm |
| Concentration of PVP | 0, 0.05, and 0.1 mg/ml | no significant difference, 0.05 mg/ml chosen |
| Amount of gold per shot | 0.5, 0.25 and 0.125 mg | 0.25 mg |
| Amount of DNA per shot | 5, 1, 0.2 and 0.04 µg | 1 µg |

## Results

The optimization was performed in a step by step manner, as suggested in the Helios Gene Gun system instruction manual and outlined in Figure 2. The principle of this procedure is that one parameter is varied while the others are kept constant. However, in the first optimization step, the helium pressure and the size of microcarriers were optimized together, and different pressures were used for each gold particle size. In the second step, polyvinylpyrrolidone (PVP) concentration was varied. PVP serves as an adhesive during the cartridge preparation process. In the final steps, the amounts of gold and DNA were optimized. The tested options for different parameters and the results of the optimization are listed in Table 1. The most critical parameters were helium pressure, the optimum of which varied in different plant species (Figure 3) and amount of gold (Figure 4). Reducing the amount of particles raised significantly transient expression levels particularly in arabidopsis and tobacco. Gold particles with a diameter of 0.6 µm were optimal for all plant species studied (Figure 3).

For arabidopsis and tobacco, the parallel assays for luciferase activity and GUS histochemistry (number of blue spots) correlated well and led to identical optimal parameters in most steps of optimization. The number of blue spots per shot was similar for all species studied. However, in tobacco the amount of luciferase in the extracts was 10–100 times higher than in arabidopsis. In birch samples we could not recover any reliable amounts of active luciferase. This problem was probably caused by inhibitory compounds that are co-extracted (Loponen 1998), as mixing of birch extract with tobacco extract led to dramatic loss of activity (not shown). The inhibition was specific to luciferase, as quantitative GUS assay (using 4-methylumbelliferryl-β-glucuronide, Jefferson, 1987) of birch extracts worked well (not shown).

We were also interested in investigating whether there are differences between batches of cartridges (*i.e.* coatings) and between stages of the plant material. No significant difference was detected between different coatings (data not shown). However, there were significant differences between leaves of different age within single tobacco (Figure 5) and birch plants.

As a final step of optimization, arabidopsis, tobacco and birch leaves were bombarded with three different pressures with and without a diffusion screen. Even though visible tissue damage was reduced by using the diffusion screen, no significantly higher expression of the reporter genes was observed. The results of the experiment with arabidopsis are shown in Figure 6.

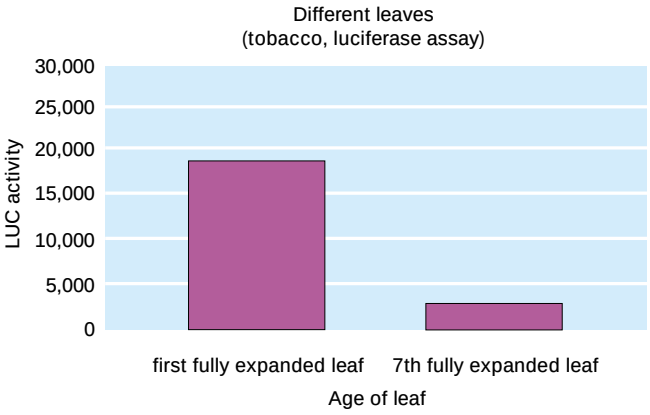



*Fig. 5. The effect of the age of the leaf in tobacco. The conditions used: helium pressure 200 psi, amount of gold 0.125 mg and amount of DNA 1 µg per shot.*

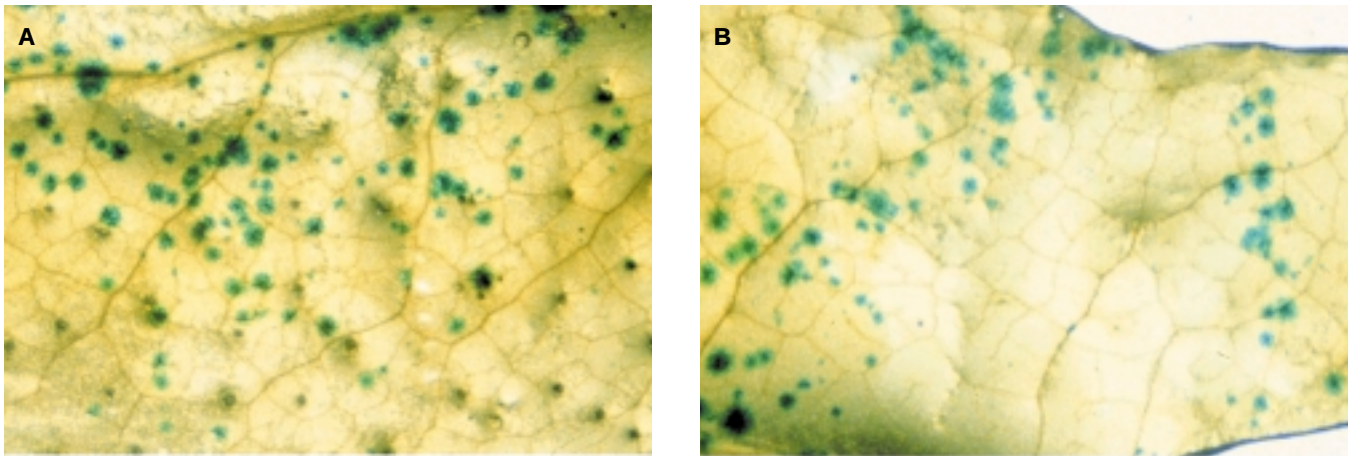



*Fig. 6. Arabidopsis bombarded with (A) and without (B) diffusion screen. Conditions used: helium pressure: 75 psi, amount of gold: 0.125 mg/shot and amount of DNA 1 µg/shot.*

## Discussion

We have shown that the Helios Gene Gun system can be successfully used for transient gene expression studies in arabidopsis, tobacco and birch. The Helios system is fast and easy to use. Nevertheless, it was necessary to optimize key parameters for each target. During the optimization we were able to increase the expression levels five- to ten-fold.

In general, helium pressure and the amount of gold were shown to be the most critical parameters (Figures 3 and 4). Especially in arabidopsis and tobacco, the most significant improvement of transient expression levels came with the reduction of the amount of gold. However, in birch the amount of gold did not have an equally significant influence. The particle size also had some effect in all plant material tested, the best particle size being 0.6 µm for all targets. The optimal amount of DNA was 1 µg in all plant materials, but significant transient expression was also observed when using only 0.04 µg of DNA per shot. It should also be noted that the amount of DNA consisted of two plasmid constructs mixed in molar 1:1 ratio.

In this study the transient expression levels of two different reporter genes (coding for LUC and GUS) were measured from the same shot. Luciferase was measured quantitatively from plant extracts, while for GUS we counted the number of transformation loci after histochemical staining. In general, the results correlated with each other well. In case of discrepancy between the different assays, we followed the luciferase results. For birch, we could apply only the histochemical method (discussed above).

According to our experience, the biggest problem with the Helios Gene Gun system is the high degree of variation in and between the experiments. This, however, is characteristic for all the particle bombardment systems. According to Christou (1992), the variation can be due to physical, environmental and biological parameters. Little is known *e.g.* about biological interactions between physical parameters and target tissue, or about the fate of DNA from the time the particles enter cells. Environmental and biological variation are especially hard to control. This is also reflected in our results showing a significant difference in the transient expression levels between leaves of different developmental stage in the same plant (Figure 5). Therefore, statistical comparison of the results is usually quite difficult (Ritala, 1995). In this study, only physical parameters were optimized.

The diffusion screen became available for the Helios Gene Gun system during the optimization process and it was tested in a separate experiment. The diffusion screen did not increase expression significantly, but reduced the damage in the center of the shot and made the transformation loci more evenly distributed (Figure 6). It was also possible to use higher pressures when using the screen, which may be important as pressures lower than 50 psi are hard to control with the instrument. The screen could possibly be useful when aiming at stable transformation, as optimal conditions for that tend to be gentler than for transient expression. While the highest transient expression is generally obtained with rather violent treatments giving better particle penetration, these conditions may impair cell division or growth (Sanford *et al.*, 1993).

In conclusion, to achieve optimum results, we recommend the following measures:

- Use plants grown under defined growth conditions to get as homogenous plant material as possible.
- Use leaves that are as near the same age and condition with each other as possible.
- Optimize helium pressure and amount of gold for each new target.
- Have at least five parallel shots per construct in order to control the variation in results.
- Use an internal control in every shot while doing e.g. promoter deletion analysis.
- Support leaves during bombardment. Using suction to keep the leaves in place and to flatten them evenly is especially recommended with arabidopsis leaves.

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