# Transient gene expression assays in rose tissues using a Bio-Rad Helios® hand-held gene gun

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**Abstract**

Rose tissues of different varieties were transformed using a Bio-Rad Helios® hand-held biolistic gun. Parameters for optimum transient expression were optimized and included rose variety, flower age, tissue, gold particle size, DNA loading ratio. Smooth flowers without thick waxy layers and young unopened actively growing flowers were found to be better suited for the transient expression assays. The DNA amounts, gold particle amounts and size etc were not found to influence the efficiency of the transient transformation in these tissues. These studies indicate that biolistic transformation using hand-held guns can be used for successful transient expression assays in rose flower tissues. This is especially useful for a quick and easy analysis of genes and their expression before attempting stable transformation.

**Keywords**

Biolistic transformation, Gene gun, Helios, Rosa hybrida, Petals, Transient Assay, GFP

## 1. Introduction

Transient expression assays are used for quick analysis of genes and regulatory elements in plants. Stable transformation of many plants especially plants of woody nature like roses takes long durations (Durzan., 1990). In the era of genomics when many genes and regulatory sequences are isolated in large numbers, their analysis by stable transformation can be very time and resource consuming (Wroblewski et al., 2005). Even model systems like *Arabidopsis,* where a quick stable transformation method is available, may take weeks before gene analysis could be carried out (Clough & Bent., 1998). Some plants especially cereals, legumes and woody plants are considered very recalcitrant to transformation and tissue culture and may take a long time before the effect of the transferred gene can be observed (Birch., 1997). Moreover, stable transgene insertions do not often provide reliable assays as they are influenced by the genomic location of their integration through position effects and may also lead to transcriptional or post transcriptional gene silencing (Vaucheret et al., 1998). A transient assay offers advantages over stable transformation including relatively quick analysis of introduced genes and is not generally influenced by the plant genetic constitution. (Kapila et al., 1997).

The development of a quick and reliable method for assaying genes in plant tissues will be helpful for obtaining proof of function for many genes. Many transformation methods are available for plants and among them biolistic bombardment and *Agrobacterium* mediated transformation are commonly used (Birch., 1997). The particle bombardment system has been used for transformation of a wide variety of organisms and organelles (Klein et al., 1992). Many variants of the system had been used previously but lacked portability and mostly required tissues be placed in a vacuum chamber often limiting the types of target tissues that could be used for transformation. Application of vacuum can also lead to damage of the tissue being used (Finer et al., 1999). The Helios® gene gun from Bio-Rad is a very easy but powerful tool that could be employed to deliver DNA into tissues for obtaining transient expression. The Helios® gun is a portable hand-held device and therefore provides many advantages over the previous biolistic systems which limited their usage to specific tissues and conditions. The system also provides flexibility in amounts of DNA and gold used per delivery by controlling and thus a large number of samples could be tested with many different parameters over a short period of time. The hand-held gun can be used on

intact live organisms as well as direct the DNA coated particles to specific tissues and locations on a live organism thus making *in situ* targeted gene delivery a reality. This has led to widespread use in gene therapy experiments in animal models (Lin et al., 2000) and tissue specific transformation in plants (Helenius et al., 2000).

This study describes the use of the Bio-Rad Helios® handheld gun for transient gene expression of GFP in rose petals. We optimized the parameters for the handheld gun including the DNA loading ratio (DLR) and the pressure used for bombardment of rose leaves and tissues. Our results indicate that many factors influence successful transient transformation of rose tissues and may include type of tissue, age of tissue and plant variety. Other parameters like gold particle size and DNA concentration did not have a major influence on the outcome of this study.

## 2. Materials and methods

### *2.1. Plant material*

The plant materials used for particle bombardment were tissues of white flowered rose (*Rosa hybrida)* varieties Tineke and Denise attached to the flower. Prior to bombardment, fresh rose flowers cut from a local rose farm (Ludwig's Roses, Pretoria, South Africa) were maintained in a home-made preservative solution composed of 1 liter of purified water, 1 tsp of sugar, 1 tsp of household bleach and 2 tsp of lemon juice (Broocker., 2003). Approximately 5cm of flower stem was cut under this solution at 45˚ angle. This was to remove any air bubbles blocking the xylem tissue when the stems were cut from the plant. The flowers were then stored in a 25˚C growth chamber. The rose flowers remained healthier for longer periods and displayed slight growth under these conditions.

In addition to the rose tissues of Tineke and Denise, a few flowers from greenhouse grown rose plants from the UP greenhouse of the varieties Delilah and Memoir were used in order to compare the response of the flowers grown under the different conditions to transformation.

### *2.2. Reporter cassette*

The reporter gene used in this study was a mutated green fluorescent protein (mGFP) from the vector pBIN19-mGFP5-ER and was used for this experiment (Haseloff., 1998).

The construct had a GFP reporter gene driven by a CaMV35S promoter and a Nos terminator.

Bacterial colonies with the construct from plates were inoculated in 100 ml of LB broth containing kanamycin. The cultures were incubated in a 37˚C shaker for 16 hours. Plasmid DNA was purified from bacterial cells following alkaline lysis plasmid DNA purification protocol (Sambrook & Russell., 2001). The DNA was checked on a 1% agarose gel to ascertain the purity and quantified using a Nanodrop 2000 (Thermo scientific). This DNA was then used for preparation of the cartridges for bombardment.

*2.3. Preparation of gold particles*

The Bio-Rad Helios® gene gun optimization kit was used for optimizing all the conditions. All laboratory glassware and plastic ware used were sterilized and dried thoroughly. Cartridges were prepared according to the Helios® gene gun system instruction manual (Bio-Rad Laboratories, 1996). The same instructions had been previously used in studies by Helenius et al., (2000) and Carsono & Yoshida (2008). For the cartridge preparation, the concentration of spermidine was 0.05 M and that of Calcium chloride was 1M. 100 µl of each of these solutions was added to the suspension. It was always noted that the total volume of DNA added was never greater than that of spermidine and Calcium chloride. Aseptic conditions were maintained throughout the process.

The weighing out of gold particles and precipitation of gold particles was performed at room temperature on a laboratory bench with a clean spatula. Cartridge preparation with a Bio-Rad Tubing Prep Station was performed under a laminar flow hood cleaned with 70% ethanol. The tubing material was cleansed and dried by purging with nitrogen gas through it for 3-5 minutes. The tube was then fixed on the tubing prep station and the suspended gold particles were slowly injected to the tube using a syringe. The tubes were then rotated for 5 minutes to evenly coat the inner surface uniformly. The gold micro-carrier coated tubes were then cut into pieces and inserted into the cartridge holder. Different amounts of gold and DNA were chosen for the optimization of the parameters as shown in supplementary table1. The bombardments were carried out with and without the diffusion screen.

*2.4. Observation of GFP expression*

The GFP was visualized at the Microscopy and microanalysis facility at the University of Pretoria using a Zeiss fluorescent microscope using an excitation wavelength of 450-490nm and using an emission wavelength filter in the range of 515-565nm. The photographs were taken using an Axiocam (Zeiss) attached to the microscope with exposure at 1.10s.The green fluorescent protein was visualized in the tissues showing intense green fluorescent spots clearly visible over the background.

## 3. Results and Discussion

*3.1. Optimization of gene gun Parameters*

*3.1.1. Helium gas pressure used in bombardment*

The pressure of helium gas to be used in the experiment is very important. High pressure can cause damage while low pressures can lead to insufficient penetration of the target tissues by the gold micro-carrier. As different tissues in the plants differ in their thickness and surface characteristics, it is important to optimize the conditions needed for each tissue type individually (Helenius et al., 2000).

We used empty cartridges and leftover cartridges of a previous experiment to optimize the pressure of helium gas. The bombardments were carried out on leaf and flower tissues at various pressures. We tried a range of pressures *viz.* 100Psi, 150Psi, 180Psi, 200Psi, 220Psi, 250Psi, and 300Psi. At pressures below 200Psi there was no effect on the tissue that was bombarded. However, at pressures above 220Psi, the flower petals were damaged extensively and some where even shattered. At pressures above 250Psi, the even the leaf tissues were damaged. Therefore we decided to use the pressure at 220Psi for leaves and flowers for the experiment. A diffusion screen was used in half of the experiments to scatter the gold particles instead of concentrating them to one region on the tissue.

Although no damage was externally visible on tissues at 220Psi, there was extensive damage to tissues in thinner petals when observed under microscope (Supplementary material Fig.1). We therefore reduced the pressure range in the subsequent experiments. A pressure range between 180 and 220Psi and use of a diffusion screen was found to be the best

method as per this experiment to prevent damage to the petals. For leaf tissues however, a pressure of 220Psi could be used without much damage to the tissues.

*3.1.2. Size and amount of gold particles*

The initial experiments were carried out with gold particle sizes of 1µ. Since this was damaging the tissues particles of 0.6 µ size were used in all the subsequent experiments. This led to less damage when compared to the larger particles. The use of a smaller gold particle size at the right pressure ensured that more particles were delivered into the tissue per shot with lesser damage to the tissue. This also meant that more DNA molecules were actually delivered into the tissues per shot and since there is a marked reduction in damage leading to better expression of gene.

We tried a range of amounts for the gold particles (0.125 to 1.0 mg) used per shot in the experiment. However, any direct correlation between the amounts of gold used per shot was not observed in the experiment and therefore all further experiments were carried out using the same concentrations (0.5 mg and 0.125 mg). The use of a diffusion screen also did not improve transformation efficiency although it did reduce damage caused on tissues due to direct bombardment. Previous experiments have also shown a wide range of variability between gold particle size, ratio and the gene expression (Helenius et al., 2000).

*3.1.3. Optimisation of DNA concentrations*

The DNA concentrations used in the experiment also did not show direct correlation with gene expression in the tissues (Fig. 1). There was expression of the GFP reporter gene in some tissues whereas in others nothing was visualized. The concentration of DNA and its effects in biolistic bombardment experiments were shown to be highly inconsistent in previous studies and were attributed to the various external and biological factors within an experiment, ranging from the plant growth conditions to anatomy of the tissues (Helenius et al., 2000).

*3.2. Transformation efficiency of leaf vs. petals*

Leaf tissues were more efficiently transformed than the petals under the conditions we tested. As evident from the Fig 2 A-H, the leaf tissues showed more spots of transient

GFP expression than the petals in Fig 2 I-Q. This may be due to the characteristics of the harder leaf tissue being able to withstand the effects of bombardment than the delicate petals. This could also be due to the browning caused by high amount of phenolics in the tissues during damage caused by bombardment of the petals. Presence of high phenolic content in tissues has been earlier shown to be inhibitory to transformation and is a critical problem in some plants like coffee (Da Silva & Menéndez-Yuffá., 2003). The rose leaves were more effective in withstanding damage to tissue during the bombardment experiments. To confirm that DNA was transferred to the leaves, PCR was randomly carried out on few bombarded tissues with primers specific to the bombarded vector. (Data not shown)

*3.3. Age of the tissue*

The petals of the cut flowers used are almost entering the senescence state when used and not growing any more. The efficiency of transformation was very less in these petals probably due to senescent nature of the tissue. To confirm this we also carried out bombardments on a limited number of young flowers from the greenhouse plants which were in their active growing stages. There was remarkable difference in the response to the bombardment in these tissues including less damage and browning. The actively growing tissues responded better than the cut flowers in the experiment and we were able to obtain the large spot of green fluorescence than any other tissue used (Fig 2 R). This indicates that the transformed cells divided as the tissue grew and thus gave rise to a larger region of fluorescence. Fig. 2 G&H also indicates the same as leaves of Delilah variety used in these experiments were small leaves near the flower buds in growing stages. They showed a larger area of fluorescence probably due to the actively dividing cells in the region.

*3.4. Rose Varieties*

Petals of Denise variety showed more number of GFP expression spots per tissue but this could not be attributed to the rose variety as this pattern was not consistent across all experiments. However, an interesting observation here was the texture of petals of Tineke and Denise when observed under the microscope (Compare Fig 2 I and J with Fig. 2 N). The surface texture of Tineke was different from that of Denise by having scaly protection on the surface probably of wax. This may have prevented the successful penetration of the petals by the projectiles and may have resulted in very few GFP spots in Tineke petals. However, the

numbers of petals in Denise showing GFP expression were not significant enough to confirm this. There could have been differences in growth of the plant as each of the flowers came from different plants. This could have also influenced the outcome of the experiments to some extend. This suggested the importance of using flowers at the same stage from plants grown under uniform conditions to give consistent results in transient expression studies of flowers. Therefore, further experiments have to be carried out under more uniform conditions to confirm whether Denise is indeed more amenable for the transient expression studies than Tineke.

## 4. Conclusion

These were preliminary studies in order to develop an easy and efficient method for transient expression of genes in rose flowers. These studies indicate that a variety of factors have to be taken into account while developing a consistent method. The age of the flower is one of the most important criteria to consider as transformations of older flowers were less efficient than that of young flowers. Rose varieties also influence the efficiency of transformation to some extend. As observed in the microscopic studies, the texture of the petals differs across varieties and this may be factor in determining the transformation efficiency. In all experiments, the leaf tissues showed more number of GFP spots than petals and could be attributed to the presence of fewer amounts of phenolics in the leaf when compared to petals. Other factors like size and amount of gold particle and DNA loading ratio did not influence the results in our studies and is consistent with previous studies. Previous studies also mention inconsistency in results especially due to environmental and biological variation that are very hard to optimise (Helenius et al., 2000). Our results indicate that biolistic bombardment can be used for transient expression studies for providing a reliable assay of candidate genes. However, differences in varieties and tissues can affect efficiency of transformation and conditions may have to be optimized separately for each variety.

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**Figure Legends**

Fig. 1 – Summary of bombardment experiment of petals and leaves of Tineke (T) and Denise(D) with Diffusion Screen (D) or without Diffusion screen (S). 1,2,3,4 indicate different DNA loading ratios (Details in supplementary Table 1)

Fig. 2. A,B- Unbombarded controls of Tineke; C,D- bombarded Tineke leaves; E,F- Denise leaves; G,H- Delilah leaves; I,J – Unbombarded control petals of Denise; K,L,M- Bombarded petals of Denise; N- Unbombarded control petals of Tineke; O,P,Q – Bombarded petals of Tineke; R- Bombarded young flower of Memoir showing intensely fluorescent growing region.

Supplementary material Fig 1. A) A normal rose petal which is not bombarded and B) A Bombarded Petal showing cell death due to tissue damage under white light

**Table legend**

Supplementary material Table 1. Amount of Gold and DNA and DNA loading ratio (DLR) used for preparation of the cartridges in a typical experiment

**Figure 1**
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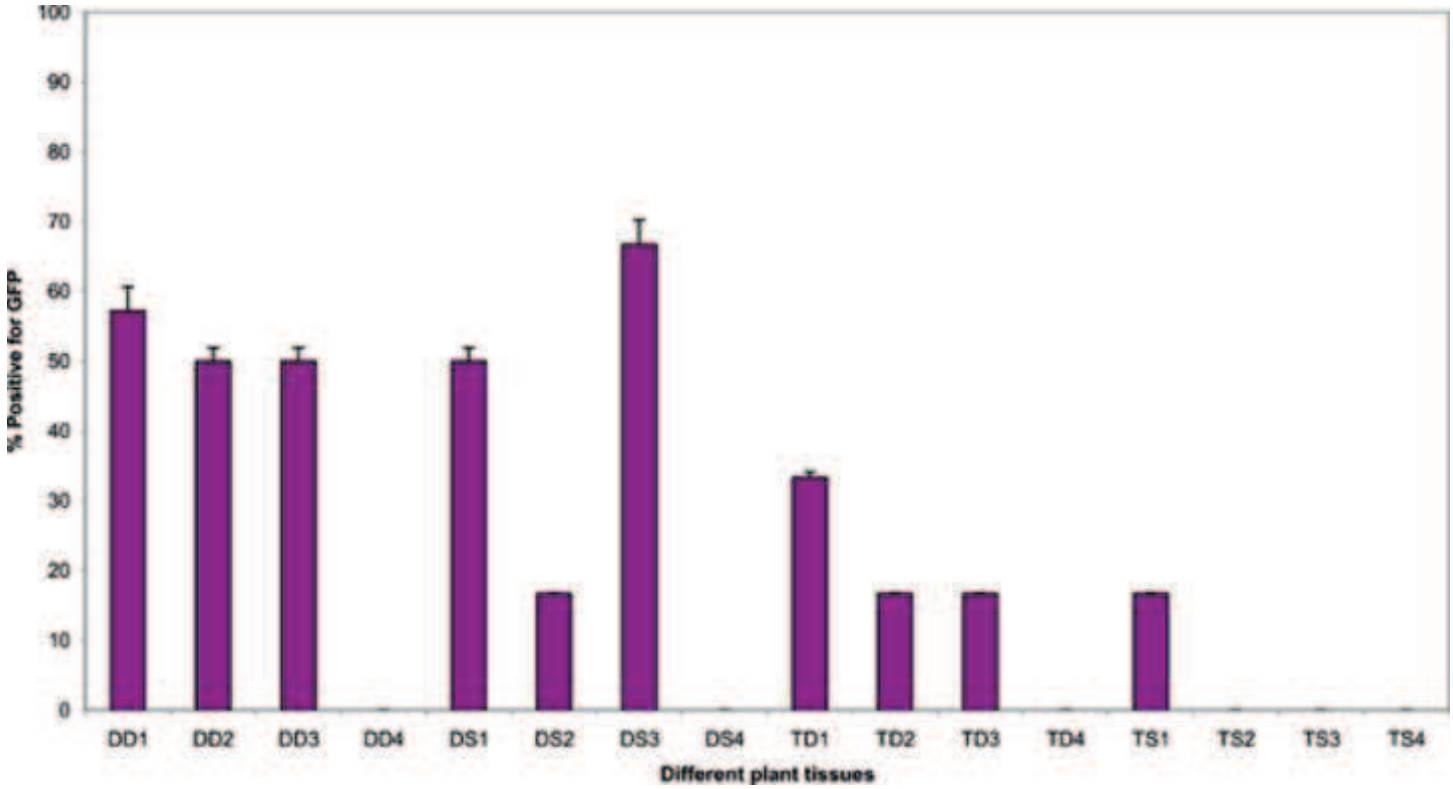

**Figure 2**

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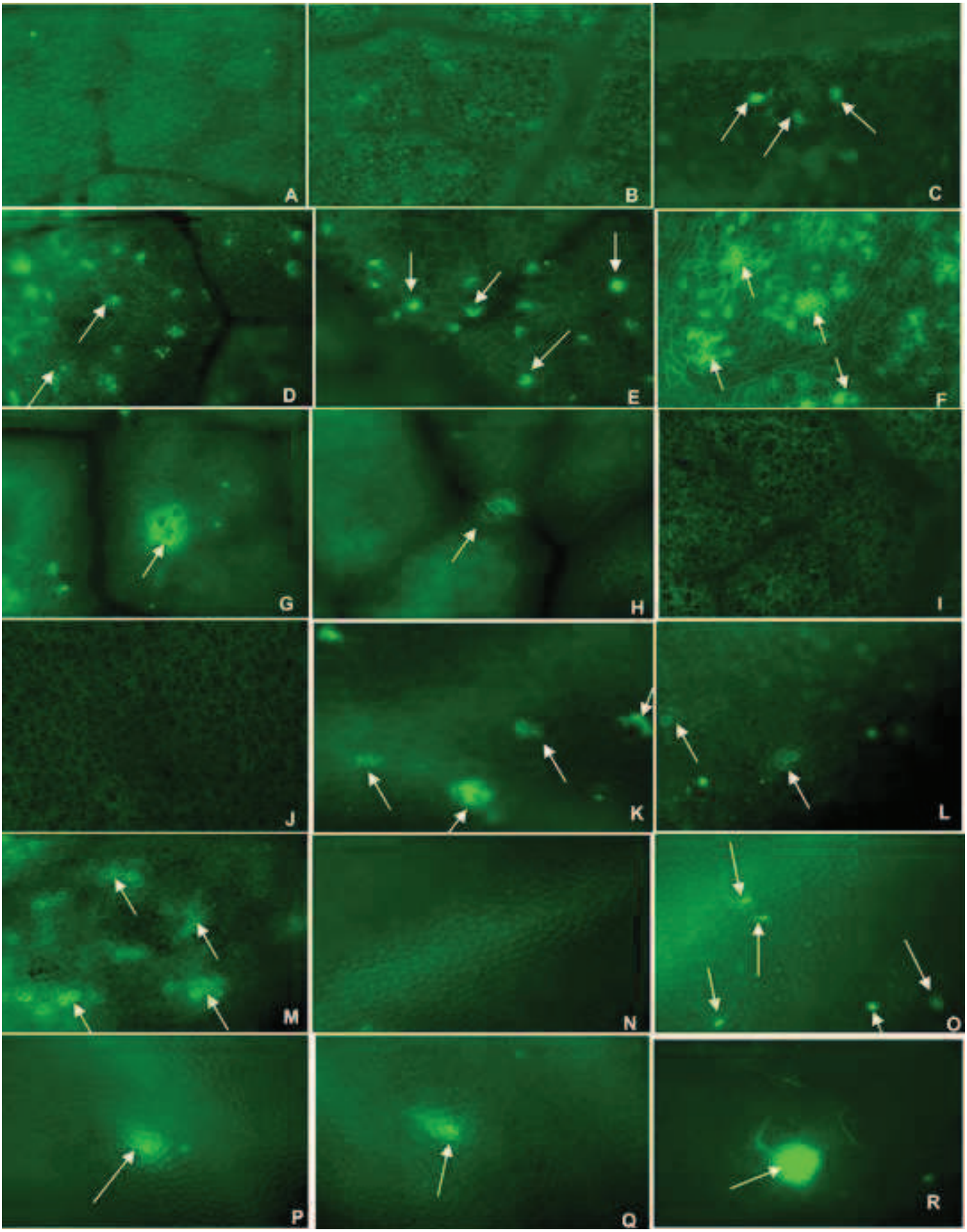

**Supplementary Figure 1**
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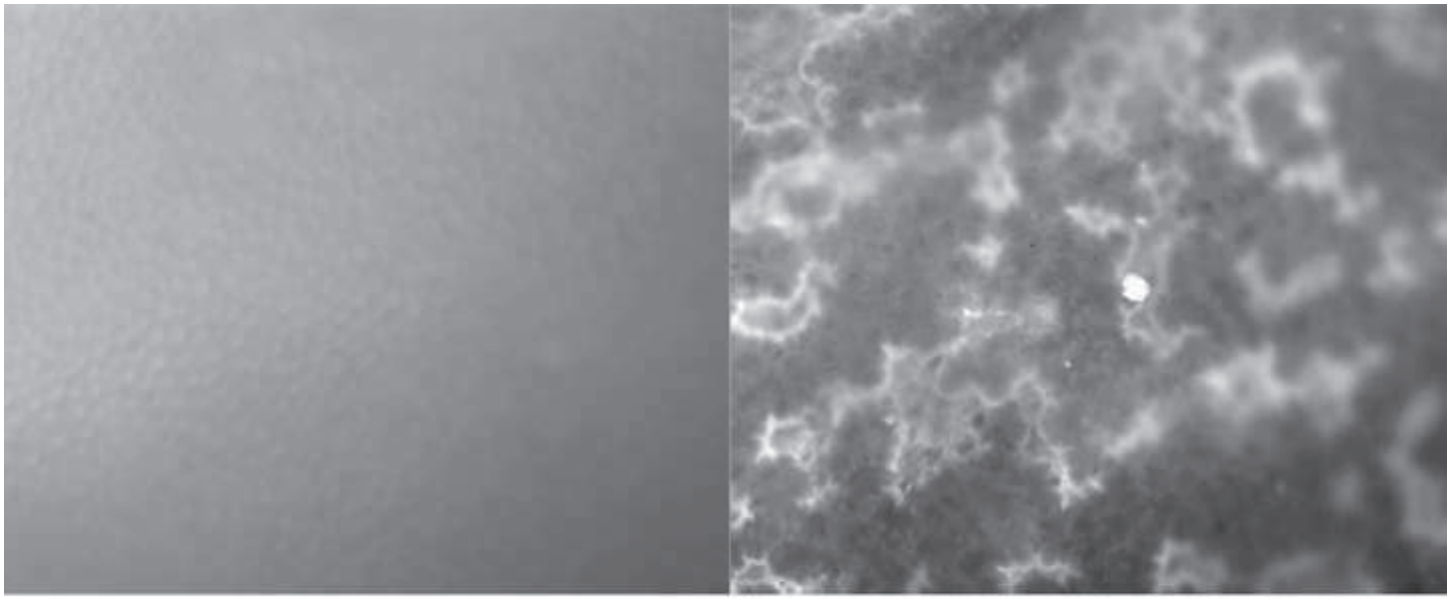

A B

**Supplementary Table**

| Exp | Gold (mg) | DNA (µg) | Gold (mg) | DNA (µg) | DLR | With Screen | Without Screen | Total | Shots/ flower | Shots/ variety | # leaves |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **GFP1** | 0.5 | 0.5 | 25 | 25 | 1 | 3 | 3 | 6 | 3 | 18 | 2 |
| **GFP2** | 0.125 | 0.25 | 6.25 | 12.5 | 2 | 3 | 3 | 6 | 3 | 18 | 2 |
| **GFP3** | 0.5 | 1 | 25 | 50 | 2 | 3 | 3 | 6 | 3 | 18 | 2 |
| **GFP4** | 0.125 | 0.125 | 6.25 | 6.25 | 1 | 3 | 3 | 6 | 3 | 18 | 2 |

Supplementary material Table 1. Amount of Gold and DNA and DNA loading ratio (DLR) used for preparation of the cartridges in a typical experiment