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## Efficient transient expression of the $\beta$ -glucuronidase reporter gene in garlic (*Allium sativum* L.)

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**Abstract** – A biolistic particle delivery system was used to introduce DNA containing a  $\beta$ -glucuronidase (*gus*) reporter gene under the control of the CaMV35S promoter in three different garlic (*Allium sativum* L.) tissues: embryogenic calli, leaves and basal plate discs. Expression of the reporter gene was assayed histochemically and fluorimetrically when the tissues were bombarded with 1  $\mu$ m diameter gold particles coated with DNA, at a distance of 3 cm from the stopping plate and using 1100 psi rupture discs. Following bombardment, high levels of  $\beta$ -glucuronidase (GUS) were found without the need for treatment to block previously reported putative endogenous nuclease activity.

*Allium sativum* L. / transient expression / GUS / garlic

**Résumé** – Expression transitoire du gène rapporteur de la  $\beta$ -glucuronidase chez l'ail (*Allium sativum* L.). Le système biolistique a été utilisé pour introduire l'ADN du gène rapporteur de la  $\beta$ -glucuronidase (*gus*) sous le contrôle du promoteur CaMV35S, dans trois tissus différents d'ail (*Allium sativum* L.) : cals embryogéniques, feuilles et lames de disques basaux. L'expression du gène rapporteur a été testée par histochimie et fluorimétrie après que les tissus aient été bombardés à l'aide de particules d'or de 1  $\mu$ m de diamètre enrobées d'ADN. Après le bombardement, on a trouvé de hauts niveaux du  $\beta$ -glucuronidase (GUS) et ce sans qu'un traitement pour bloquer l'activité nucléase endogène soit nécessaire.

*Allium sativum* L. / expression transitoire / GUS / ail

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## 1. Introduction

The transfer of foreign genes into plants is used to improve agronomic traits and to investigate fundamental aspects of genetics and development. It is of great importance for use with species such as garlic (*Allium sativum* L.) which is considered sterile for practical purposes. Although several fertile clones from the Caucasus have been described [6] garlic cultivars are reproduced as clones because they do not flower in the growing conditions of the countries where they are cultivated. The breeding practices used with this species have been limited to clonal selection and production of virus-free clones by meristem culture.

Particle bombardment to deliver DNA into plant cells has been used successfully for transient and stable transformation studies in a large number of plant species [8], including onion [5, 10] and garlic [1]. However, in this last species, a strong endogenous nuclease activity has been reported in different kinds of tissue. This prevents the detection of transient expression of the  $\beta$ -glucuronidase (*gus*) reporter gene unless nucleases are previously blocked by nuclease inhibitors that have been vacuum-infiltrated into the tissues. This cumbersome procedure is a serious handicap since large numbers of target tissues have to be bombarded to ensure efficient production of transgenic plants.

This paper shows the transient expression of the *gus* gene driven by the CaMV35S promoter after transferring DNA into garlic tissues, without treatment to inhibit nuclease activity.

## 2. Materials and methods

### 2.1. Plant material and tissue culture

The garlic clone used in this study was the cultivar "Moraluz". This was selected from eight originally tested since it more successfully develops regenerant plants both from embryogenic calli and basal plate discs. Three different types of tissue were subjected to bombardment with DNA-coated

microparticles: young leaves, basal plates and two month old calli. Calli were obtained from basal plates on Murashige and Skoog medium (MS) [13] supplemented with  $1 \text{ mg}\cdot\text{l}^{-1}$  kinetin and  $1 \text{ mg}\cdot\text{l}^{-1}$  2,4-dichlorophenoxyacetic acid, 3% sucrose, pH 5.8, and solidified with 3% Phytigel<sup>TM</sup>. Cultures were maintained in the dark at 27 °C and subcultured on the same medium every four weeks. Thin basal plate discs were cultured in that medium and subjected to the above conditions during four days. Small pieces of leaves and thin discs of basal plates without previous treatment were also used. Four hours prior to bombardment, callus or explant pieces (about 3 mm diameter) were placed inside a 2 cm circle at the centre of a plate containing the above medium supplemented with 400 mM sorbitol and 400 mM mannitol.

### 2.2. Plasmids

The plasmid 35SPro-GUS/UBI-BAR was used for bombardment experiments. It is a pUC-derived plasmid carrying the *gus* coding sequences under the control of the CaMV35S promoter and the *bar* gene encoding the enzyme phosphinothricin acetyltransferase driven by the maize ubiquitin promoter, first exon and first intron. Plasmid DNA was isolated from *Escherichia coli* using the Quiagen large-scale plasmid purification system.

### 2.3. Bombardment

DNA-coated microparticles were prepared using a  $\text{CaCl}_2$ /spermidine method. The preparation contained 50  $\mu\text{l}$  of gold suspension (3 mg of 1  $\mu\text{m}$  gold), 5  $\mu\text{l}$  of plasmid DNA at a concentration of  $1 \text{ }\mu\text{g}\cdot\mu\text{l}^{-1}$ , 50  $\mu\text{l}$  of 2.5 M  $\text{CaCl}_2$  and 20  $\mu\text{l}$  of 100 mM spermidine. The solution was pipette-mixed during preparation followed by vortexing for 3 min and centrifugation for 10 s. The supernatant was discarded. DNA-coated gold particles were rinsed in 250  $\mu\text{l}$  absolute ethanol, centrifuged and then resuspended in 56  $\mu\text{l}$  of ethanol. Bombardments were made with 7  $\mu\text{l}$  of this suspension using a BioRad biolistic PDS-1000 He.

#### 2.4. Histochemical and fluorimetric GUS assays

After bombardment the plates were kept in darkness at 27 °C for 16 h. The tissue of each bombarded sample was divided into two halves. One was subjected to histochemical staining and the other to fluorimetric determination of GUS activity. For the former, the tissue was flooded with a staining solution containing 500  $\mu$ l of GUS reaction buffer (50 mM phosphate buffer pH 7, 10 mM EDTA, 500  $\mu$ M potassium ferricyanide, 500  $\mu$ M potassium ferrocyanide, 0.1% Triton X-100, 2% methanol and 250  $\mu$ M 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-gluc)) and incubated overnight at 37 °C. Quantitative fluorimetric GUS assays were performed using 4-methylumbelliferyl  $\beta$ -glucuronide (MUG) as a substrate following the method of Jefferson [9]. Protein content was determined using the Bio-Rad kit with bovine serum albumin as a standard. Endogenous fluorescence values from control explants bombarded with gold particles without DNA were subtracted from the GUS enzymatic activity fluorescence values. Data were expressed in pmol 4-methylumbelliferone (4-MU)·min<sup>-1</sup> per milligram of extracted protein.

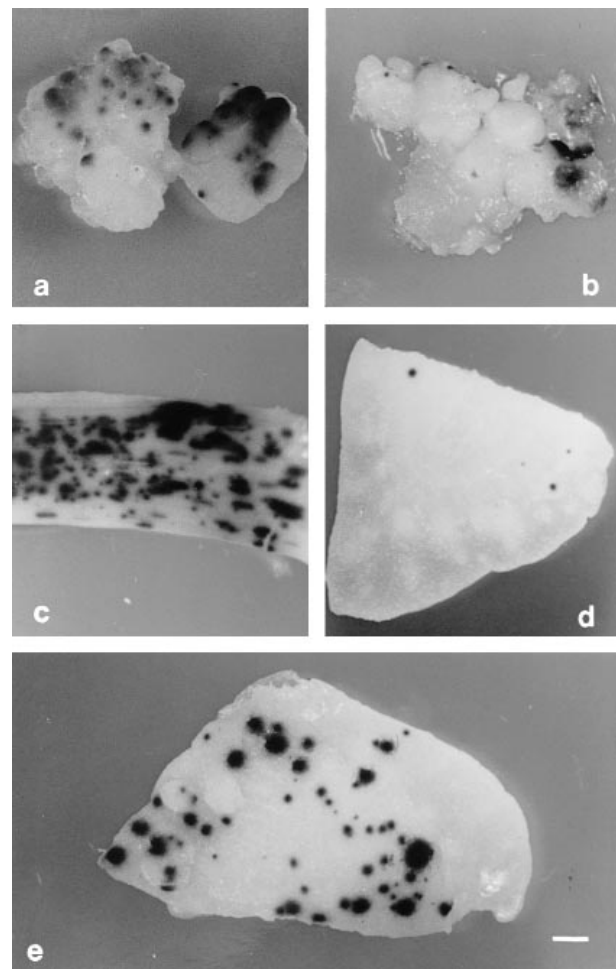
### 3. Results and discussion

The choice of plasmid used in this investigation was based on the results of previous bombardment experiments (Ferrer et al. unpublished) [1] which showed the CaMV35S promoter to be superior to others, such as maize ubiquitin or rice actin genes, in driving the expression of reporter genes in garlic tissues. This behaviour has also been observed in onion, a species related to garlic [5].

Preliminary work indicated that the most important physical factors influencing transient GUS activity in embryogenic calli were bombardment pressure and target distance. The experiments were conducted using 1  $\mu$ m diameter gold particles coated with DNA under 25 in Hg vacuum, at two bombardment pressures, 1300 psi and 1100 psi, and at two target distances, 3 cm and 6 cm from the stopping plate. Ten samples were bombarded for each

set of conditions. As a negative control, calli were bombarded with 1  $\mu$ m diameter gold particles without DNA.

A value of 3 (strong transient *gus* expression) was given to those calli showing large numbers of blue spots over their surfaces after histochemical staining with X-gluc. (Fig. 1a). A value of 1 was given for weak expression, i.e. where only a few blue spots were seen (Fig. 1b). Calli bombarded



**Figure 1.** Histochemical GUS assay of garlic tissues. (a) Callus expressing high level of GUS activity. (b) Callus expressing low level of GUS activity. (c) Leaf expressing high level of GUS activity. (d) Basal plate disc expressing low level of GUS activity. (e) Four days cultured basal plate disc expressing high level of GUS activity. Bar = 1 mm.

**Table I.** GUS activity of four different tissues measured by MUG assay (pmol MU·mg<sup>-1</sup> protein × min) (mean ± standard error of 10 experiments).

Tissue	MUG assay
Callus	2143.1 ± 1527.8
Leaf	1416.9 ± 219.1
Cultured basal plate	2418.5 ± 1534.4
Basal plate	162.3 ± 40.9

using 1100 psi rupture discs and at 3 cm from the stopping plate obtained values of 2 or 3. Bombardment at 1300 psi and 6 cm produced the worst results with several calli showing no blue spots at all. No GUS activity was observed in negative controls.

To confirm these preliminary results, and to see whether different tissues responded differently to bombardment, three different kinds of tissue were chosen: embryogenic calli, young leaves and basal plate discs. This last tissue offered the possibility of directly regenerating plants via micropropagation in a previously tested culture medium consisting of Gambor's B5 medium supplemented with 0.05 mg·l<sup>-1</sup>  $\alpha$ -naphthalenetic acid and 2 mg·l<sup>-1</sup> N-benzyl-9-(2-tetrahydropyranil)adenine. Ten samples of each explant were bombarded at 1100 psi from 3 cm. To better estimate GUS activity a fluorimetric assay was conducted parallel to the histochemical assay. Table I shows quantitative GUS activities and Figures 1a, 1c and 1d show samples of the three tissues after staining with X-gluc. Variation in *gus* expression among different samples of the same tissue was noted, reflecting inter-bombardment-experiment differences. Similar variation has been widely observed in other studies [5, 7, 8, 11, 15] which points to an important limitation of this microprojectile-mediated gene transfer technique – the need for many bombardments to assure plant transformation.

Different levels of transient *gus* expression were also found depending on the target tissue used (Tab. I). High levels of GUS activity were found both in embryogenic calli and leaves. These levels were similar to those reported by other authors in

different species and tissues [4, 11, 12, 21]. In contrast, basal plate discs showed the lowest level with very few blue spots on the samples treated with X-gluc. Because this could have been due to the low mitotic activity of these cells, a new assay was conducted with basal plate discs after inducing cell division. This was achieved by culturing basal plate discs in a similar way to those employed with immature cereal plant embryos [20]. In this experiment, high levels of GUS activity were found (Fig. 1e, Tab. I).

These results contrast sharply with those obtained by Barandiaran et al. [1] who found high nuclease activities in three different garlic tissues, something which prevented their detecting any GUS activity. Positive results were obtained only after treatment to inhibit the nuclease activity. Even then, a 100% response, as measured by the number of bombarded explants which stained blue, was never seen. In the present investigation, all the calli, cultured basal plates and leaves bombarded showed *gus* expression. An important difference between these two investigations is the cultivar used. Differences in GUS activity dependent on the genotype bombarded have been observed in onion [5] but not so strongly as in garlic. The influence of genotype in obtaining transformed plants is generally recognised [3]. However, this influence has been more attributed to variation in the performance of different genotypes during tissue culturing aimed at producing regenerant plants, than to the process of gene transfer by particle bombardment. Due to the physical nature of this method there appears to be no biological constraint to the actual delivery of DNA. It is considered therefore genotype-independent [15]. Variation in GUS activity has also been noted in plant tissues of different transgenic plants [14]. Inhibition of GUS due to several secondary compounds is amongst the reasons offered to explain non-uniformity in GUS activity [16, 19]. Nuclease inhibition of the expression of foreign DNA has also been reported in transformation experiments of maize pollen [2] and *Brassica napus* microspores [8]. It may be that different garlic clones show different levels of nuclease activity – or of other inhibitor compounds – which might account for the differences observed

between the present results and those of Barandiaran et al. [1].

It is also worth mentioning that the bombarded tissues in the present investigation were previously subjected to osmotic treatment. Improved efficiencies of transient expression and stable transformations have been reported following osmotic treatment [18]. It is believed that cell plasmolysis prior to bombardment enhances the cell chances to survive the shock of bombardment.

Other differences between these sets of experiments in garlic are seen in the responses of embryogenic calli and leaves. Barandiaran et al. [1] reported a greater activity in leaves than calli, but the results of the present investigation reveal no significant differences in transient *gus* activity. This might be explained by the age of the calli at the time of bombardment, a factor which has a strong influence on transient gene expression [3, 5, 12, 17]. It is encouraging that two month-old embryogenic calli and, more important, basal plate discs with a high regenerant ability of the garlic cultivar "Moraluz", show high levels of transient *gus* expression with this standard procedure. It is a good starting point from which to obtain transformed plants of this asexually reproduced and agronomically important crop.

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