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Optimization of parameters for particle bombardment of embryogenic suspension cultures of cassava (*Manihot esculenta* Crantz) using computer image analysis

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Abstract Tissue derived from embryogenic suspension cultures of cassava was bombarded with microparticles coated with a plasmid containing the *uidA* gene, which codes for β-glucuronidase (GUS). After 3 days, the effect of different bombardment parameters was evaluated by comparing the numbers of blue spots that resulted from histological GUS assays. Counting of blue spots was performed using a system comprised of a black and white video camera, a stereoscope and a personal computer. A reproducible counting method was established by optimizing GUS assay conditions, preparation of tissue samples and acquisition of video images in view of attaining the highest possible contrast between the blue spots and the surrounding tissue. The effects of bombardment pressure, microparticle size, number of bombardments, and osmotic pretreatment on GUS expression were investigated. Optimal transient expression of the *uidA* gene was observed after bombardment at 1100 psi, with a particle size of 1 μm, an osmotic pretreatment and two bombardments per sample. The highest number of blue spots observed was 2400 per square centimeter of bombarded tissue.

Key words Embryogenic suspension cultures · β-Glucuronidase · Microparticle bombardment · Image analysis · Ferricyanide, ferrocyanide

Introduction

Cassava is a tuber crop of major importance in tropical countries, especially in Africa (Cock 1985). Although efforts to genetically transform cassava were initiated about a decade ago (Calderón 1988), only recently was the regeneration of transgenic plants achieved (Schöpke et al. 1996). In this study the genetic transformation of cassava was accomplished through particle bombardment of embryogenic suspension cultures. In the present article we report the optimization of bombardment parameters for embryogenic suspensions of cassava using a plasmid containing the *uidA* gene. The expression of this gene is revealed through a histological assay (Jefferson 1987) that results in the development of a blue stain. Because of its simplicity, this assay has been used for the optimization of microparticle bombardment protocols in a number of species (Vain et al. 1993; Ritala et al. 1993; Bommineni et al. 1994) and for studies comparing the effect of different promoters on transient gene expression (Franche et al. 1991; Charest et al. 1993). The effect of different parameters on transient GUS expression can be analyzed by comparing the numbers of GUS-positive, blue spots. This is usually done by visually counting spots with the help of a stereoscope. When we began to establish parameters for the microbombardment of embryogenic suspension cultures of cassava, we found that this method is extremely time-consuming and has a high error rate, which was particularly evident when the treatments resulted in high densities of blue spots. An alternative system for the quantification of blue spots, based on image analysis, is presented here. This system eliminates counting errors caused by the experimenter and greatly reduces the time required for the evaluation of multiple samples.

Areas in plant tissue culture where image analysis has been used include somatic embryogenesis (Plata et al. 1991; Dutta et al. 1991; Chi et al. 1996), measurement of growth (Coles et al. 1991; Motooka et al. 1991; Olofsdotter et al. 1994; Anthony et al. 1994) and automated handling of in vitro plant material (Tillet 1990). One report describes image analysis with regard to GUS-positive areas in tobacco leaf explants transformed with *Agrobacterium tumefaciens* (Owens De Novoa and Coles 1994). The aim of the latter study was to compare the transformation efficiency of different bacterial strains by comparing areas of leaves that

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contain GUS. However, this system is different from ours in that it evaluates images at a macroscopic rather than microscopic level, where problems of contrast are far less critical than in the plant transformation system that we are studying.

Materials and methods

Plant material

Embryogenic suspensions were initiated from friable embryogenic callus of cassava cultivar 'TMS 60444' (Taylor et al. 1996). They were cultured in 250-ml flasks containing 50 ml liquid SH-medium (Schenck and Hildebrandt 1972) supplemented with MS vitamins (Murashige and Skoog 1962), 50 μM picloram and 60 g/l sucrose. The suspensions were incubated on a shaker (150 rpm) at 25°C in a photoperiod of 16 h at 20–25 $\mu mol\ s^{-1}\ m^{-2}$ PAR provided by fluorescent lamps (Sylvania Cool White). The culture medium was replaced with fresh medium every 2 days. The cultures used for particle bombardment were between 8 and 12 months old.

Preparation of tissue for bombardment and culture conditions

Twelve-to-fourteen-day-old suspensions were drawn in and out of a 10-ml syringe several times in order to dissociate clumps of embryogenic tissue into smaller units. The resulting suspension was sieved, and the fraction composed of units 100–500 µm in diameter was resuspended in SH-medium. Settled cell volume (SCV) was determined by leaving suspensions undisturbed for 30 min in a 15-ml graduated centrifuge tube. Aliquots of 300 µl SCV were pipetted onto filter paper disks (Whatman, grade 3 MM, size 23 mm) to obtain a monolayer of embryogenic units. Filters with tissue were placed on solidified GD-medium (Gresshoff and Doy 1974) containing 50 µm picloram, 20 g/l sucrose and 2 g/l Phytagel (Sigma). Immediately prior to bombardment the tissue-covered filters were transferred onto a dry filter paper in an empty petri dish. After bombardment, the filters were transferred to the GD-medium described above and cultured for 3 days. In one experiment the filters were cultured 4 h before and 16 h after the bombardment on GD medium with equimolar mixtures of sorbitol and mannitol (combined molarity 0.2, 0.4, and 0.6 *M*) according to Vain et al. (1993).

Microparticle bombardment

Gold particles with diameters of 1.0 µm, 1.6 µm (BioRad, USA), and 1.8–2.3 µm (Cat. # 00767, Alfa Aesar Research Chemicals, USA) were coated with the plasmid pILTAB28 (=pMON505 containing the construct 35S-GUS-7S; Schöpke et al. 1993). The coating of particles with DNA was performed according to Sivamani et al. (1996). The Particle Delivery System PDS1000/He (BioRad) was used for the bombardment of coated particles. The following conditions were chosen: distance rupture disc assembly – macrocarrier cover (gap): 1/4 inch (6.35 mm); distance macrocarrier – stopping screen: 6 mm (upper position of screen); distance rupture disk retaining cap to the tissue to be bombarded: 9 cm. Samples of DNA particle suspension (50-µl aliquots) were prepared and used for up to 3 h after their preparation. Bombardments were performed in a partial vacuum (9.1 kPa abs. pressure). Five microliters of DNA particle suspension containing 1.0 µg DNA and 0.3 mg particles were used per bombardment. Each treatment consisted of the bombardment of seven filter disks covered with suspension-derived embryogenic tissue. Each experiment was repeated three times. The effect of helium pressure (400, 650, 900, 1100 psi), microprojectile size (see above), the number of bombardments per sample (1 or 2) and an osmotic pretreatment was investigated.

Histological GUS assays

Three days after bombardment, GUS assays modified from that of Jefferson (1987) were performed with the bombarded tissue on they filter disks. The assay buffer included 0.08 *M* sodium phosphate at pH 7.0, 7.7 m*M* X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexyl-ammonium salt), 20% (v/v) methanol and 0.16% Triton X 100. In one experiment potassium ferricyanide and potassium ferrocyanide were added in concentrations of 1.6, 3.2, 6.4, 12.8 and 25.6 m*M* each. For the experiment in which the numbers of blue spots resulting from different bombardment parameters were compared, a concentration of 6.4 m*M* was chosen. After 2 h in the assay buffer at 37°C the filters were washed several times with water without disrupting the original position of the tissue. These were then transferred to a mixture of 160 g chloral hydrate +50 ml glycerol +50 ml water, pH 7.0. After several days in this clearing solution, the filters with tissue were placed on dry filter paper to absorb excess liquid, mounted on a microscope slide and embedded in phenolic glycerol gelatin (Sigma, St. Louis, USA).

Quantification of blue spots

A black and white video camera (Sony XC-77) in conjunction with a stereoscope (Zeiss SV8) and a computer (MacIntosh IIvx) were used to obtain images from the cleared and embedded tissues. Transmitted light was provided by a fiber optic illuminator. The stereoscope was used with its standard lens, the zoom was set to 1.0 and the aperture to 5–6. The computer was equipped with a LG-3 Scientific Frame Grabber and TV-3 RS-170 (CCIR) display board (both from Scion Corp, Md., USA). Image analysis was performed using the public domain software NIH Image (by W. Rasband, NIH; available through the Internet at http://rsb.info.nih.gov/nih-image/ or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, USA; part number PB93-504868). Images were captured and displayed through NIH Image on a monitor (Apple Performa Plus) with a 256-grayscale. The option 'Threshold' was used to highlight those areas with gray values ≥90, which coincided with most of the blue spots. Spots composed of 2–100 pixels were then counted with the 'Analyze Particles' command. An area of 1 cm^2 per bombarded filter (4.25 cm^2) was evaluated.

Results and discussion

Optimization of bombardment parameters

For any plant tissue that is used for particle bombardment for the first time, optimal parameters for transient or stable gene expression must be established. Because transient expression of introduced genes can be studied during the first days after bombardment (as opposed to weeks or months for gene expression in stably transformed cells), it is very useful for optimizing variables affecting the efficiency of DNA transfer through microparticles. We investigated several of the factors that have the highest impact on the efficiency of transient expression (Sanford et al. 1993). The results of our studies are summarized in Fig. 1. There is a clear tendency for an increase in the number of blue spots in microbombarded embryogenic suspensions of cassava with an increase in bombardment pressure. This increase is roughly linear between pressures of 400 psi and 900 psi and levels off at 1100 psi. Since only particles of a certain size have the ability to penetrate a cell wall without damaging or killing cells of specific cell type, it is not surpris-

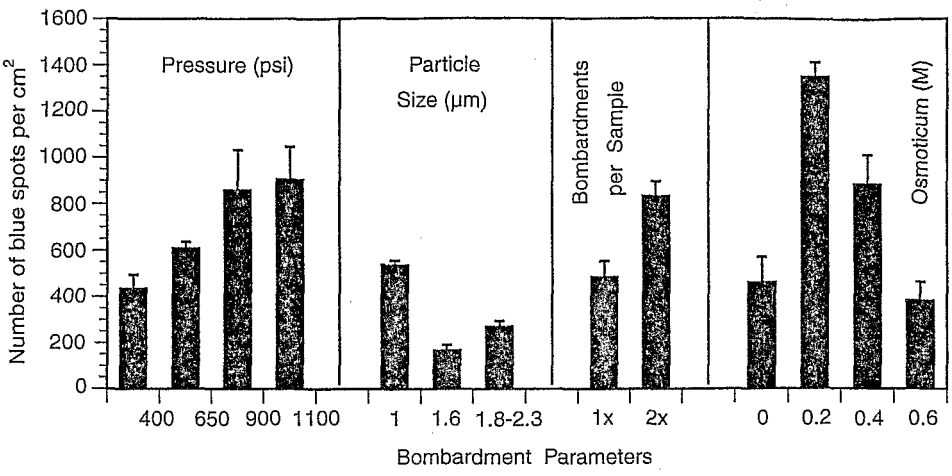

Fig. 1 Effect of bombardment pressure, particle size, number of bombardments per sample and osmotic treatment on the expression of GUS in embryogenic tissue of cassava. Tissue from embryogenic suspensions was distributed onto filter disks and bombarded with a plasmid containing the *uidA* gene. Three days after bombardment histological GUS assays were performed. The number of blue spots per square centimeter was counted using an image processing system. For each treatment seven disks were bombarded, and each experiment was repeated three times. *Bars* indicate the average number (+ standard error) of blue spots from 21 disks from one treatment. Except for the factor that was tested in each experiment, the following parameters were kept constant: bombardment pressure 1100 psi, particle size 1.0 µm, number of bombardments per sample 1 and no osmotic treatment

ing that of the three particle sizes tested one is clearly more efficient than the others (1.0 µm). Bombarding one sample twice doubles the number of blue spots. When the optimal bombardment pressure (1100 psi) and the optimal particle size (1.0 µm) were used, treatment with a 0.2 *M* mixture of sorbitol and mannitol in equimolar amounts had a dramatic effect on the number of blue spots per square centimeter: compared to the control without osmotic treatment, the number of spots increased from about 450 to 1400. This effect was less pronounced at 0.4 *M*, and the number of blue spots resulting from a treatment with 0.6 *M* osmoticum was comparable to the control. The highest number of blue spots (2400/cm^2) was obtained in a preliminary experiment (data not shown) where an osmotic treatment was combined with double bombardment at a helium pressure of 650 psi. It remains to be seen whether a further increase is possible by using higher pressure.

Each of the experiments summarized in Fig. 2 includes the following treatment: bombardment pressure 1100 psi, particle size 1.0 µm, bombarded once, no osmoticum. In the experiment in which the effect of different pressures was investigated, the value for this treatment is about 900 blue spots per square centimeter, while in the three other experiments this value is around 500. The reason for this difference is not clear. One possible explanation is that the embryogenic suspension at the time it was used for the pressure experiment was for some unknown reason in a different physiological state. This interpretation is supported by the observation that in spite of 'controlled' conditions, i.e. constant subculture intervals and controlled temperature and light, we observed slight variations in the color of the suspensions (different tints of yellow) and in the proportion of large versus small cell aggregates.

Quantification of blue spots

A quantification procedure for 'blue spots', i.e. dark areas on a bright background, that is based on image analysis must fulfill several requirements. The spots should be well-defined, i.e. their boundaries should be as sharp as possible. The contrast between the spots and the background should be very high, and the spots should be arranged in a single plane of focus. The light source used to illuminate the samples to be evaluated should provide a homogenous field of illumination. In addition, the bombarded tissue should be in its original position for the counting procedure. This is important if the material is only loosely attached to its support, as is the case with embryogenic suspensions.

In studies to optimize the parameters for particle bombardment, transient expression of the introduced *uidA* gene is usually analyzed 1 to several days after bombardment. At this time, transient GUS expression should result mainly in single GUS-positive cells, or, in a system where very rapid cell division might occur, two-cell stages. However, the blue spots obtained with GUS assays often are not confined to single cells. Instead, the blue stain is detected in groups of many cells (in the range of 5–50), the size of the spots can vary considerably, and the margin of the spots is diffuse. Therefore, authors refer to 'blue foci' (e.g., Vain et al. 1993), 'GUS foci' (Bommineni et al. 1994), 'expression units' (Charest et al. 1993) or simply 'blue spots'. At low densities of spots per bombarded surface area this does not pose a problem for counting, but at higher densities the probability that large spots overlap increases and makes comparisons difficult. The apparent diffusion of the blue stain is caused by the diffusion of the product of the action of β-glucuronidase, 5-bromo-4-chloroindoxyl, whose oxidative dimerization leads to the forma-

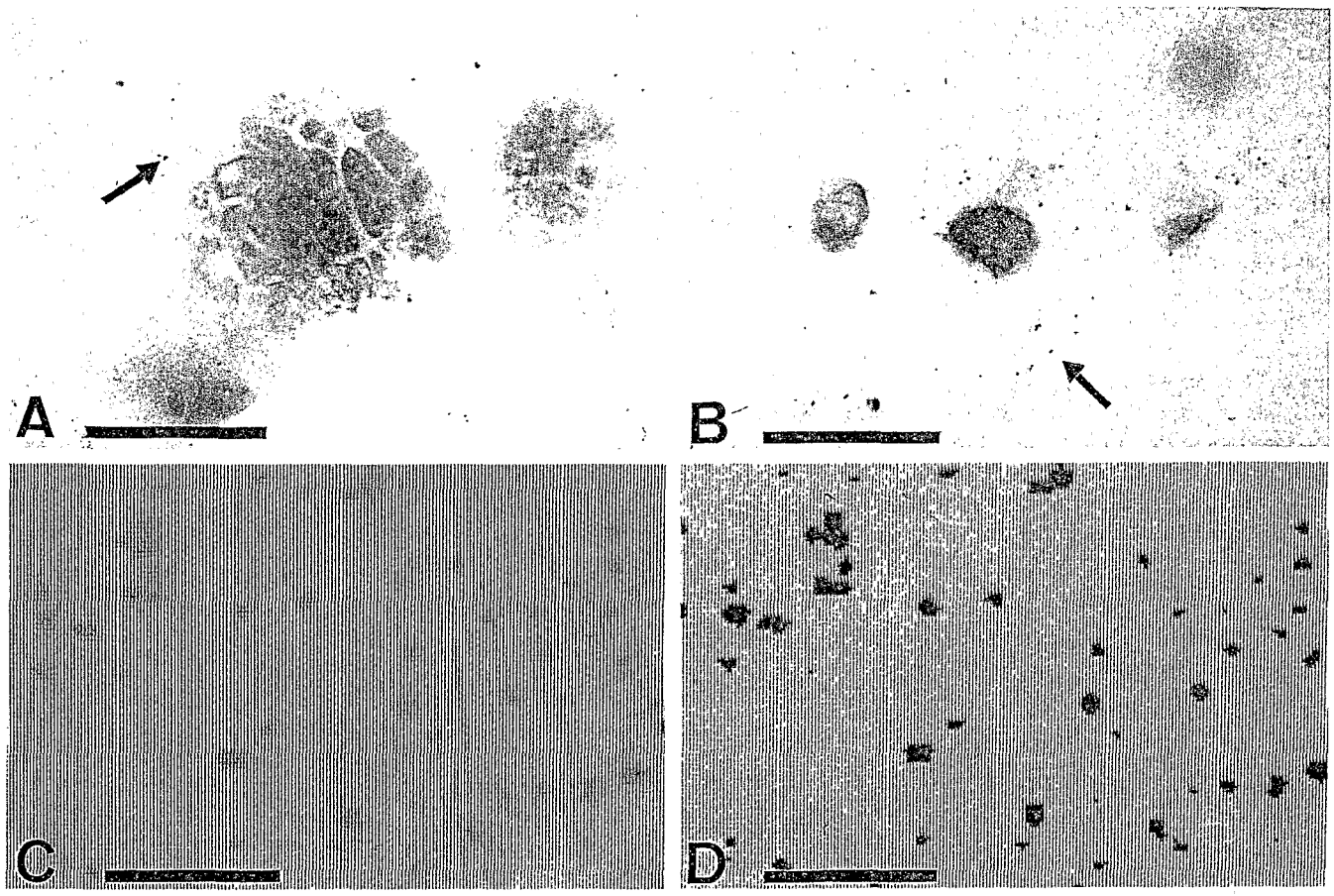

Fig. 2A–D GUS expression in microbombarded embryogenic tissue of cassava 3 days after bombardment. **A** Assay without ferri/ferrocyanide, **B** assay with 6.4 m*M* ferri/ferrocyanide, **C** close-up of an image taken from a computer monitor (for settings see Materials and methods); **D** as **C** but the *gray* values are thresholded. *Gray* values above 90 are shown *black*, those below are *white*. *Bars* in **A** and **B**: 60 μm, in **C** and **D** 300 μm. The *small dots* (*arrows*) in **A** and **B** are gold particles

tion of the insoluble indigo dye. This dimerization can be enhanced by oxidation catalysts such as potassium ferri- and ferrocyanide (Holt and Withers 1958; Lojda 1970).

We found that, depending on the concentration of potassium ferri- and ferrocyanide, the size of blue spots in microbombarded embryogenic suspension cultures of cassava varies dramatically. In tissue treated with buffer without the catalysts, there are areas with varying intensities of blue, and no sharply defined spots confined to single cells (Fig. 2A). With increasing concentrations of catalysts (1.6 m*M*, 3.2 m*M*, 6.4 m*M* each), the size of the spots is reduced. At 6.4 m*M*, the majority of spots are comprised of one dark blue cell surrounded by one layer of light to very light blue cells (Fig. 2B). At higher concentrations (12.8 and 25.6 m*M*), the number of blue spots is reduced, probably due to inhibition of the β-glucuronidase. The concentration of 6.4 m*M* of each catalyst was therefore chosen for further experiments.

A high contrast between the blue spots and the surrounding tissue was achieved by clearing tissues after the GUS assays with a chloralhydrate/glycerol solution. This left the tissue nearly transparent, while the blue stain was not affected. Cleared tissue was embedded in phenolic glycerol gelatin on microscope slides, which made the handling of samples much easier. Embedded samples were stored for more than 1 year without noticeable change in stain intensity. Due to the way the tissue samples were prepared for bombardment (a layer of embryogenic tissue about 0.5 mm thick on a filter paper circle), most of the blue spots were in the same plane. While the filter paper reduced contrast somewhat, it was necessary in order to support the tissue throughout the different manipulations.

The contrast of an image captured by a video camera and displayed on a monitor depends on the object, the settings of the optical system used (lens, magnification, aperture), the light intensity, the settings of the video camera and the software used to display it. Once an image is captured, it can be enhanced by image processing, for example through sharpening, contrast enhancement or elimination of 'noise'.

The number of 'countable' spots is influenced by varying any of the above-mentioned parameters and by image enhancement. The settings we chose (see Materials and methods) represent a compromise. Our primary goal was to develop a quick method to compare numbers of blue

spots and not to obtain highly accurate absolute numbers. Therefore, except for thresholding no image processing was included in the procedure. The number of blue spots that can be counted with these settings corresponds roughly to two-thirds of the spots that are counted visually. Weakly blue cells are not counted because the gray values by which they are represented on the computer image overlap with the background. At very high densities, blue spots that lie close to each other may be counted as one, i.e. the actual number is underestimated.

In order to count areas or spots with the 'Analyze Particle' command in NIH Image, the different gray values have to be segmented, i.e. grouped according to their gray values. With the command 'Threshold' a gray level can be chosen that renders all pixels with a value above it (=darker) as black, and the remainder as white. The number of black areas then can be counted automatically. Figure 2C and D show the same image, taken with the settings as described in Materials and methods, before (2C) and after thresholding (2D). The density of 'blue' spots measured in this specific sample was 2257 per square centimeter.

There are several advantages of this counting procedure over visual evaluation. First, many samples can be evaluated in a short period. At densities above 1000 blue spots per square centimeter visual counting on average takes at least 20 min/cm^2, while counting using the image analysis system is achieved in less than 5 min. Second, the results are stored in a form that can be used for statistical analysis. Third, the reproducibility of the results is much higher. Variation due to counting errors caused by fatigue or by differences in the eyesight of different experimenters are excluded.

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