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Transient gene expression in strawberry (*Fragaria* × *ananassa Duch.*) protoplasts and the recovery of transgenic plants

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Summary. A transient ß-glucuronidase (GUS)-assay was performed to evaluate electroporation parameters and optimize DNA delivery conditions into strawberry protoplasts. Optimal GUS-activity was obtained when protoplasts were subjected to 400 V/cm for 20 ms. GUS-activity could be further increased by the addition of carrier DNA to the electroporation mixture. Callus selected on 10 µg/ml hygromycin produced shoots which exhibited GUS-activity. The transformed nature of the shoots obtained after selection was confirmed by DNA-analysis.

Abbreviations: CaMV, cauliflower mosaic virus; dCTP, deoxycytidine-triphosphate; EtBr, ethidium bromide; GUS, ß-glucuronidase; MES, 2(N-morpholino) -ethanesulfonic acid; X-gluc, 5-bromo-4-chloro-3-indolyl glucuronide

Introduction

Strawberry (*Fragaria x ananassa)* is a vegetatively propagated octoploid (2n=8x=56) species with strong heterozygosity. The high ploidy level and heterozygosity are characters that impose difficulties when this species is bred according to conventional strategies. This makes strawberry an especially suitable target for manipulation on a gene level. One avenue to genetic improvment is the use of direct gene transfer mediated by electroporation (Rhodes et al. 1988; Yu-Wen et al. 1989). To evaluate electroporation conditions into plant protoplasts, transient gene expression assays have been performed (Fromm et al. 1985; Hauptman et al. 1987; Shaun et al. 1990). The ß-glucuronidase (GUS) gene has been shown to be a useful and versatile reporter gene for this purpose (Jefferson et al. 1987) and it has also been used extensively to identify transformed tissue. Recently, successful Agrobacterium mediated transformation of strawberry has been reported using a modification of the leaf-disc system developed by Horsch et al. (1985)(James et al. 1990; Nehra et al. 1990). Although Agrobacterium provides an efficient vehicle for stable transfer of foreign genes into plant cells, a direct gene transfer method has the advantage of requiring no particular vector construction. DNA-mediated transformation of protoplasts also allows the integration of a large number of foreign genes into the host genome and to a certain extent influence the number of copies integrated (Paszkowski et al. 1989). Furthermore an efficient electroporation protocol can be used to compare different gene constructs in transient assays. Finally, the possibility of isolating viable protoplasts with the capacity to regenerate into cytologically normal plants motivated us to evaluate a direct gene transfer method such as electroporation. The aim of this work was to characterize factors influencing the expression of the GUS gene which had been introduced by electroporation into strawberry protoplasts and to obtain transgenic plants.

Material and methods

Plasmid. The vector, pRT88HPT, was constructed by insertion of the hygromycin phosphotransferase gene from pPCV707, as an *Eco RI - Hind III* fragment, into the polylinker of pRT88. The 5.2 kb vector pRT88 also contains the coding region of ß-glucuronidase, which as well as the hygromycin gene are under the control of the CaMV 35S promotor and the nopaline synthase 3′ terminator sequence. Plasmid DNA was purified in large scale essentially after Birnboim and Doly (1979) followed by CsCl/EtBr gradient centrifugation.

Plant material and protoplast isolation. Leaves and petioles from 3-4 week-old shoots of the octoploid breeding line, 77101 were cut into pieces in a preplasmolysis solution containing 0.3 M sorbitol and 0.05 M $CaCl_2$ x H_2O. After one hour the solution was replaced by an enzyme solution consisting of 1.0 % Cellulysin (Calbiochem-Boehring) and 0.1 % Mazerozyme (Calbiochem-Boehring) dissolved in medium K3 (Menzel et al. 1981) with 0.4 M sucrose.

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Incubation took place overnight (15-18 h) in darkness at 25°C. Protoplasts were purified according to Nyman and Wallin (1988), after which they were suspended in an electroporation buffer consisting of 10 mM MES 0.5 M glucose and 1.0 mM $CaCl_2$ (Hoefer Scientific Instruments, Technical Bulletin 118). Protoplast density was 1 x 10^6 protoplasts/ml if not otherwise stated.

Electroporation. The electroporator used in this study was a Progenetor 101(Hoefer Scientific Instruments) delivering rectangular pulses. The protoplast suspension (400µl) was pipetted into each well of a multipetri dish, after which the DNA was added. Different parameters were tested to optimize the DNA delivery; Protoplast density ($5x10^5$ - $5x10^6$ protoplasts/ml), DNA concentration (25-300 µg/ml), field strength (100-700 V/cm), pulse length (5 - 20 ms), and electroporation with or without carrier DNA (50-200 µg/ml sonicated calf thymus). In order to determine the effect of temperature , protoplasts were incubated on ice 10 min before and after electroporation. Protoplasts were also subjected to heat-shock (5 min at 45°C before electroporation and the addition of DNA), in some experiments followed by incubation on ice for 10 sec. DNA concentration was 100 µglml if not otherwise stated. Figures represent the mean +/- standard deviation from three independent experiments.

Spectrophotometric GUS-assay. Transient GUS activity was determined using the spectrophotometric assay of Jefferson et al. (1987). Protoplasts were harvested 30 h after electroporation by transferring the cells to centrifuge tubes and pelleting at 500 rpm for 5 min. Protoplasts were resuspended in 900 µl lysis buffer and transferred to Eppendorf tubes. 100 µl of 10 mM p-nitrophenyl beta-D-glucuronide in lysis buffer was added and the reaction was allowed to proceed at 37°C for 15 h. The reaction was terminated by the addition of 0.4 ml 1.0 M 2-amino-2-methyl propanediol, followed by measurement of the absorbance at 415 nm.

Viability test. Protoplast viability was determined 24 hours after electroporation. Since protoplasts subjected to electroporation tend to burst, it is difficult to get reliable figures on protoplast survival by conventional staining methods. We therefore decided to combine one of these methods, trypan blue exclusion, with the determination of protoplast density in a counting chamber .

Selection of stable transformants. Protoplasts were cultured mainly according to Nyman and Wallin (1988) except that the protoplasts were embedded in agarose with liquid medium surrounding the beads. Selection of transformants was initiated two weeks after electroporation by adding hygromycin B to the liquid medium to a final concentration of 10 µg/ml. The stable transformation experiments was performed using following electroporation conditions; $1x10^6$ protoplasts/ml, 100 µg DNA/ml, 400 V/cm, 20 ms.

Histochemical GUS-assay. Small pieces of tissue from callus grown under selection pressure were histochemically stained using 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as a substrate (Jefferson et al. 1987). Callus exhibiting GUS activity was transferred to regeneration medium, a MS-medium (Murashige and Skoog 1962) with 5.0 mg/l Thidiazuron, 0.2 mg/l NAA and 3.0 % sucrose. Regenerated shoots were histochemically stained as described above.

DNA-analysis. DNA was isolated from both transformed and untransformed control shoots as described by Dellaporta et al. (1983). DNA was digested with *Hind III*, run on 0.8 % agarose gel and blotted onto supported nitrocellulose membranes (Hybond-C, Amersham) according to the manufacturer's instructions. The filter was hybridized with the plasmid pRT88HPT and labelled with ^{32}P-dCTP using a multiprime labelling kit (Amersham).

Results and discussion

Nonspecific staining. It has been reported that several species exhibit intrinsic GUS-like activities (Ching-yeh et al. 1990). We could not detect such activities in strawberry by using the methods described above.

Effect of DNA concentration and the presence of carrier DNA Between 25-200 µg DNA/ml there was an approximately doubling of enzyme activity when the DNA concentration was doubled. However, after 200 µg the increase was rather low indicating that 200 µg was a suitable concentration under these conditions (10^6 protoplasts/ml) and that in this case the amount of protoplasts is the limiting factor (Fig. 1). The most efficient transformation procedures make use of carrier DNA in the transformation mixture (Shillito et al. 1985; Negrutiu et al. 1987). In conformity with these results our experiments shows that the GUS activity could be further increased by the addition of carrier DNA. Even at the optimum DNA concentration, 200 µg/ml, activity could be increased threefold by adding the same amount of carrier. The absence of carrier DNA could not be compensated for by the addition of the same concentration of plasmid-DNA (Fig 2).

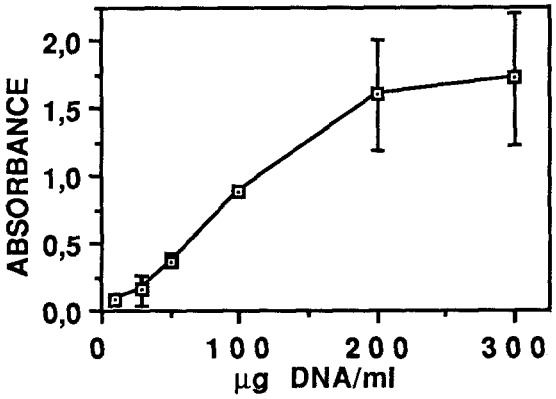

Fig.1. Effect of DNA-concentration on GUS-activity.

Protoplast density. Since protoplasts became the limiting factor around 100-200 µg DNA/ml, we decided to investigate the effect of increased protoplast density at a given DNA-concentration (100 µg DNA/ml). Under these conditions there was a nearly linear relationship between the number of protoplasts and GUS activity up to a density of about 2.5 x 10^6 protoplasts/ml; then the relationship breaks down and

GUS activity on a per protoplast basis was reduced compared with that of lower densities (Fig. 3) This indicated that plasmid availability became the limiting factor.

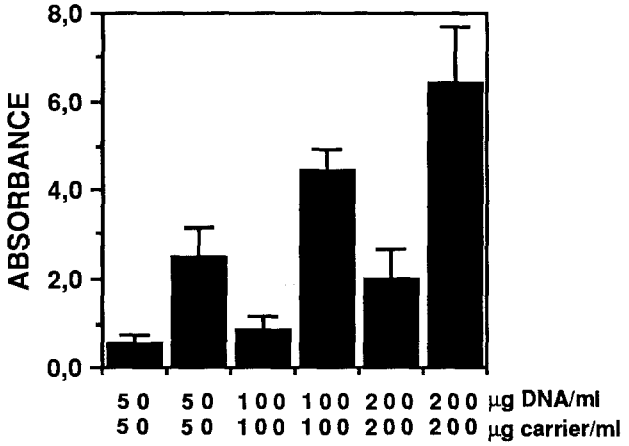

Fig.2. Effect of carrier-DNA on GUS-activity.

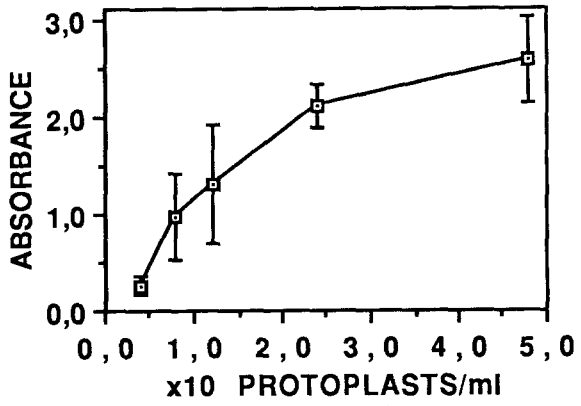

Fig.3. Effect of protoplast density on GUS-activity. DNA-concentration; 100 µg/ml.

Effect of field strength and pulse length. Field strength affected both survival and GUS activity (Fig. 4). Viability decreased from approximately 80 to 15 % over the 100 - 700 V/cm range. The optimal activity was found at 400 V/cm, where we also had a reasonable amount of surviving protoplasts. When comparing protoplasts subjected to 100 and 700 V/cm respectively, the GUS activity was almost the same. On the other hand the survival was drastically lower at the higher field strength, indicating that there was a greater uptake of plasmids during this treatment. Lower concentration of input DNA is correlated to a lower copy number of foreign DNA integrated into the genome (Paszkowski et al. 1989). Consequently by using protoplasts treated with high voltage, the number of genes introduced to each protoplast would be increased. Rhodes et al. (1988) reported that electroporation with 625 V/cm produced higher transformation frequencies than electroporation with lower voltage despite greater reductions in the percentage of viable protoplasts. The pulse length had no effect on protoplast viability while it had a pronounced effect on GUS expression (Fig. 4).

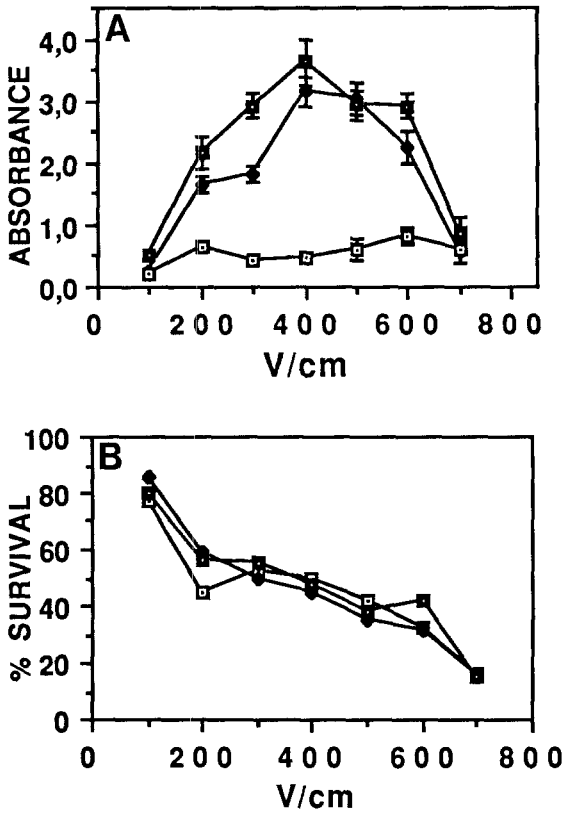

Fig.4. Effect of field strength and pulse length on; A) GUS-activity and B) Protoplast survival.

Effect of heat-shock and cold treatment. It has been reported that a heat-shock i.e. 45°C for 5 minutes, increase the transient activity and transformation frequency (Séguin and Lalonde 1988; Shillito et al. 1985). Our results are not consistent. There was a positive effect in one experiment and a negative in the other two. Although standardized growth conditions were used in all experiments, the inconsistent results might be due to differences in the material. The condition of the protoplasts could be of great importance when the material is subjected to such a harsh treatment. The same phenomenon was reported for maize protoplasts isolated from embryogenic cell suspensions (Rhodes et al. 1988). Cold treatment i.e. protoplasts on ice 10 minutes before and after electroporation, is a widely used parameter in electroporation protocols (Hauptmann et al. 1987, Bekkaoui et al. 1988, Tautorus et al. 1989). However, in our system this treatment had

a negative effect on GUS-activity. In three independent experiments cold treatment decreased the activity while viability was unaffected (data not shown).

Stable transformation. Callus grown under selection pressure exhibited the characteristic blue staining after treatment with X-gluc. Shoots produced from this callus also showed GUS-activity. Southern blot analysis confirmed the transformed nature of the selected plants (Fig. 5). The transformation frequency based on the number of selected calluses / plated protoplast was in the range of 1-5 x 10^{-4}. However, preliminary experiments where we excluded hygromycin from our culture media point to the fact that transformation frequency is higher than the number of calluses recovered indicate. Electroporation is a widely applied and effective means of introducing cloned genes into a variety of cell types. We have extended the list of plants transformed in this way with strawberry. Furthermore, different Fragaria species with various ploidy levels will be transformed for use in somatic hybridization experiments.

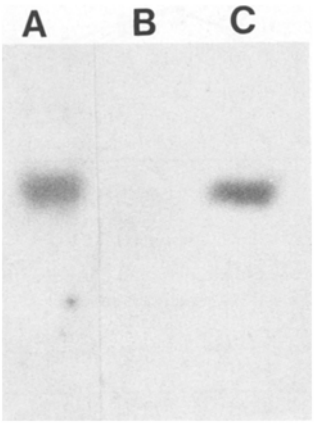

Fig. 5. Southern blot analysis of *Hind III*-digested DNA from;
A) Transformed plant grown in greenhouse
B) Untransformed control plant
C) Transformed plant grown in vitro

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