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Delivery of foreign genes to intact barley cells by high-velocity microprojectiles

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Summary. Foreign DNA was introduced through the cell walls of intact suspension culture cells of barley (*Hordeum vulgare* L.) by utilizing the particle acceleration approach. DNA-coated microscopic tungsten particles were accelerated to velocities that permitted their penetration of intact cells. Chimaeric constructs of β-glucuronidase and neomycin phosphotransferase II under the control of the dual *Agrobacterium* T_R 1′2′ promoter or the cauliflower mosaic virus 35S promoter served as reporter genes. Three days after particle delivery, high-level expression of both reporter genes was observed. That plasmid size could be critical for stabilizing DNA in the course of particle delivery will be discussed.

Key words: Gene transfer – Barley (*Hordeum vulgare* L.) – Suspension culture cells – Particle acceleration – Transient expression

Introduction

The production of whole, stably transformed plants of species of the Gramineae family has proven difficult, due to the inability to efficiently regenerate fertile plants from single cells or because of the limited host-range specificity of *Agrobacterium*. Techniques such as electroporation (Fromm et al. 1986), polyethylene glycol-stimulated direct DNA uptake (Lörz et al. 1985) and microinjection (Crossway et al. 1985) generally require the enzymatic removal of cell walls. Although great progress has been made in regenerating plants from transformed protoplasts of rice (Toriyama et al. 1988) and maize (Rhodes et al. 1988), only recently has a protoplast-to-plant regeneration system been reported for wheat (Harris et al. 1988), and no such system is as yet available for barley and rye.

Alternative techniques like the direct injection of DNA into young floral tillers of rye (De la Pena et al. 1987) and the microinjection into microspore-derived embryoids of rapeseed (Neuhaus et al. 1988) have not, until now, been reported to be successfully applicable to other species. Furthermore, *Agrobacterium tumefaciens* was shown to be capable of introducing foreign DNA into cells of maize (Grimsley et al. 1987); however, this technique has not yielded stably transformed plants yet.

In order to transform barley cells, we therefore chose the particle acceleration approach. Klein et al. (1987) have shown that small DNA-coated tungsten particles can be accelerated to velocities that permit their penetration of intact cells and tissues, thereby introducing foreign genetic material. This approach has recently been used for DNA delivery into cells of suspension cultures of maize (Klein et al. 1988a, b), rice and wheat (Wang et al. 1988), for the stable transformation of soybean (McCabe et al. 1988) and for the transformation of mitochondria (Johnston et al. 1988) and chloroplasts (Boynton et al. 1988). Here we describe the application of this novel approach for delivery of foreign DNA through the cell walls of intact suspension cells of barley, with subsequent expression of two chimaeric reporter genes [β-glucuronidase (GUS) and neomycin phosphotransferase II (NPT II)] in these cells.

Materials and methods

Plant material

Cell suspensions of *Hordeum vulgare*, winter-type cv Borwina, were cultured in MS medium (Murashige and Skoog 1962) containing 2 mg/l 2,4-dichlorophenoxyacetic acid. The cells were maintained on a rotary shaker (130 rpm) and subcultured twice a week. According to Klein et al. (1988a), exponentially grow-

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ing cells (300–400 mg) were collected on a filter paper (Filtrak MN6, 5 cm diameter), and care was taken to layer the cells evenly over the surface of the filter paper. The filter paper was then transferred to modified B5 medium (B. Müller et al., unpublished results), solidified with 1% agar.

Plasmids

The plasmids used are shown in Fig. 1. The 14.2-kb binary *Agrobacterium* vector pGSGLUC1 (J. Botterman, unpublished results) contains the NPT coding region under control of the T_R1' promoter and the 3′ polyadenylation region of octopine synthase, as well as the GUS coding region under control of the T_R2' promoter and the 3′ region of transcript 7 of *Agrobacterium tumefaciens* T-DNA. The $T_R1'2'$ promoter is a dual promoter from the T_R-DNA of an octopine Ti-plasmid (Velten et al. 1984); pGSGLUC1 was kindly provided by J. Leemans, Plant Genetic Systems, Ghent, Belgium. The 12.8-kb binary *Agrobacterium* vector pC27 (Alliotte et al. 1988) contains the NPT coding region under control of the T_R1' promoter and the 3′ polyadenylation region of octopine synthase (the plasmid was kindly provided by M. Van Montagu, Rijksuniversiteit, Ghent, Belgium). The 5.1-kb plasmid pRT103GUS (Töpfer et al. 1988) contains the GUS coding region under control of the CaMV 35S promoter and the CaMV 3′ polyadenylation region (the plasmid was kindly provided by H. H. Steinbiß, MPI für Züchtungsforschung, Köln, FRG). The 5.1 kb-plasmid pHP23 (J. Paszkowski, unpublished results) contains the NPT coding region inserted into the *Sma*I site of pDH 51 (Pietrzak et al. 1986), i.e. under control of the CaMV 35S promoter and the corresponding CaMV 3′ termination region (the plasmid was kindly provided by J. Paszkowski, ETH Zürich, Switzerland).

Particle treatment of suspension cells

The particle gun device was constructed as described by Klein et al. (1987). The tungsten particles used had an average size of 0.6 µm (size range 0.1–1.0 µm). To adsorb DNA to the microprojectiles, 5 µl of CsCl-purified supercoiled DNA (1 µg per µl of water) were added to 1 mg tungsten particles, followed by 10 µl of 0.9 *M* $CaCl_2$. After 10 min, 1 vol. of a buffer was added that consisted of 20 m*M* HEPES (pH 7.05), 137 m*M* NaCl, 0.7 m*M* Na-phosphate, 5 m*M* KCl and 5 m*M*α-D-glucose. The mixture was kept for 30 min at room temperature, followed by 5 min centrifugation (12,000 rpm in a microcentrifuge) and removal of the supernatant. The final microprojectile-DNA-sediment containing 5 µg DNA per mg of tungsten was placed on the front surface of the macroprojectile. Cells were positioned 15 cm from the end of the barrel of the acceleration device. The experiments were performed in triplicate.

Analysis of GUS and NPT expression

Following particle treatment, the cells were incubated at 25 °C in the dark on the filter paper on which they were particle-treated. After 60 h, the filter with the cells was removed from the agar support and cut into two halves, one for the GUS assay and the other for the NPT assay.

GUS assay. According to Klein et al. (1988 a), the filter with the cells was transferred to a 5.5-cm petri dish containing 100 µl of the GUS substrate mixture, spotted over the plate such that the filter paper adsorbed the substrate evenly over its area. The substrate mixture contained 0.3% (w/v) of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Genofit, Geneva) in 0.1 *M* K/Na-phosphate buffer (pH 7.0), 10 m*M* EDTA, 0,1% Triton X-100, 5 m*M* K-ferricyanide and 5 m*M* K-ferrocyanide. The petri dish was sealed, incubated for 24 h at 25 °C and thereafter microscopically inspected. The number of cells or clusters of cells that developed the blue precipitate was counted.

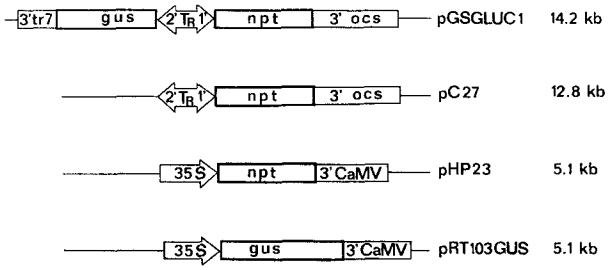

Fig. 1. Schematic drawing of the chimaeric reporter gene constructs used. For details and references, see 'Materials and methods'

NPT II assay. NPT activity was determined after electrophoresis of cell extracts in non-denaturating polyacrylamide gels by the in situ gel assay of Reiss et al. (1984) in the modification of Van den Broek et al. (1985). Equal amounts of protein were applied to each gel slot.

Results

Intact cells of *H. vulgare* suspension cultures produced detectable levels of GUS activity 2 days after treatment with particles that had been coated with plasmids containing the GUS gene under control of plant-specific promoters. GUS-expressing cells showed a dense blue precipitate throughout the interior of the cell (Fig. 2). In most cases, the blue cells occurred as single cells, but small blue cell aggregates were also observed. The control samples were treated with particles coated with plasmids containing the NPT II gene but not the GUS gene. These samples did not give any blue cell, instead they showed NPI II activity (Table 1). All three NPT II constructs used were able to produce NPT II activity in barley cells.

The GUS and NPT II genes were transferred to the barley cells by particles coated either with one plasmid type (pGSGLUC1 containing GUS and NPT II genes physically linked on one plasmid) or coated with a mixture of two plasmid types (pHP23 and pRT103GUS) containing the GUS and NPT II genes on separate plasmids (Fig. 1). However, in the former case the activities of GUS and NPT were low (only 15%) compared to the latter. The former plasmid pGSGLUC1 is 14.2 kb in size and contains GUS and NPT II under control of the dual $T_R1'2'$ promoter, whereas the latter plasmids pHP23 and pRT103GUS are smaller (5.1 kb) with GUS and NPT II under control of the 35S promoter. To rule out that the difference in activities is construct-specific, we replaced pGSGLUC1 by a plasmid of comparable size (12.8 kb) with T_R1'-NPT II. Again, the transient activities caused by this plasmid were low (8%) compared to pHP23 (Fig. 3).

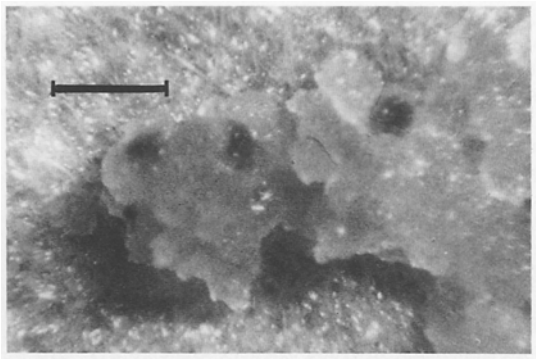

Fig. 2. GUS-expressing barley cells 3 days after particle treatment with microprojectiles coated with a mixture of plasmids pRT103GUS and pHP23. GUS activity was visualized exactly as described in 'Materials and methods'. The dark-blue stained GUS-expressing cells appear black in the black-and-white print of the original color slide (*bar* = 60 μm)

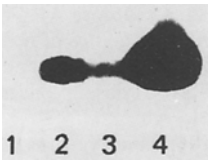

Fig. 3. NPT II activity in barley cells 3 days after particle treatment. The microprojectiles were coated with equal amounts of the following plasmids: *lane 1* – no DNA; *lane 2* – pGSGLUC1; *lane 3* – pC27; *lane 4* – pHP23 + pRT103GUS

Table 1. Activities of GUS and NPT II in barley cells 3 days after treatment with microprojectiles coated with equal amounts of the plasmids indicated

Plasmids	Reporter genes	GUS activity (number of blue cells ± SE)	NPT II-activity (cpm/100 μg protein ± SE)
pGSGLUC1	NPT/GUS	22 ± 6	8,070 ± 1,160
pHP23 + pRT103GUS	NPT + GUS	166 ± 58	55,600 ± 12,900
pC27	NPT	0	4,380 ± 980
pHP23	NPT	0	52,100 ± 14,300

Discussion

The results show that foreign DNA can be delivered through the cell walls of intact cells of *H. vulgare* by the particle-acceleration approach. Two reporter genes (GUS and NPT II) were introduced and expressed in barley suspension cells. Co-delivery using both the GUS and NPT II constructs was shown to be effective. Recently, Klein et al. (1988a) and Wang et al. (1988) bombarded suspension cells of maize, rice and wheat with a 35S-GUS construct (microprojectile size 1.2 μm, treatment under vacuum) and obtained activities of 100–500 blue cells per dish. Our results (166 blue cells per dish) with a similar 35S-GUS construct show that it is possible to obtain comparable expression signals when using a particle size of 0.6 μm and operating the acceleration device under normal atmospheric pressure (Table 1). The 35S-NPT II expression levels obtained are comparable to those produced in similar numbers of protoplasts transformed with the same vector by electroporation (not shown).

GUS activities, as well as NPT II activities, observed after particle treatment show that the large plasmids used (12.8 kb and 14.2 kb) were considerably less efficient than the smaller plasmids (5.1 kb). To some extent, this is due to the copy number of plasmids transferred (2–3 times more small plasmids than large plasmids were transferred at constant DNA amounts). The large constructs contain the marker genes under control of the $T_R1'2'$ promoter, whereas the small constructs are 35S promoter constructs. However, this does not explain the considerable difference in activity levels, since we tested exactly the same constructs at constant DNA amounts in barley protoplasts after DNA transfer by electroporation, and obtained for both the T_R1' and 35S promoter constructs similar activity levels (Mendel et al., unpublished results). Hence it could be assumed that plasmid size per se is critical for stabilizing DNA molecules during the process of particle acceleration, with small plasmids being more stable than larger ones. Further experiments are needed to investigate this problem.

Several physical factors of particle delivery (e.g. particle size and volume, particle coating procedure, DNA quantity, depth of aperture of the barrel, etc.) were investigated by Klein et al. (1988a) and Wang et al. (1988) and were found to strongly influence the efficiency of gene transfer. Also the physiological state and the morphological character of the target cells have to be taken into account. Thus, the testing of all factors influencing particle delivery to a given cell type might allow us to considerably increase the efficiency of this novel gene transfer approach for barley cells.

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