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SHORT COMMUNICATION

Transient gene expression in shoot apical meristems of sugarbeet seedlings after particle bombardment

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Abstract

Sugarbeet apices were used as targets for particle bombardment with a microtargeting device. Before examining gene expression, particle penetration experiments were carried out. Transient GUS expression was detected within the first and second cell layers of the meristem. Dividing cells with GUS activity demonstrated that cells survived the bombardment procedure.

Key words: *Beta vulgaris*, direct gene transfer, microtargeting, particle bombardment, shoot apical meristem.

Introduction

With respect to genetic engineering, sugarbeet is one of the recalcitrant species. Although sugarbeet cells are susceptible to *Agrobacterium tumefaciens*, it has always proved difficult to regenerate plantlets from transformed cells. Difficulties in sugarbeet tissue culture, i.e. strong genotype dependence, somaclonal variation and low reproducibility, are described in several reports (e.g. Mikami *et al.*, 1989; Catlin, 1990). Thus, transformation procedures for sugarbeet using *Agrobacterium*-mediated gene transfer into shoot-base tissue (Lindsey and Gallois, 1990), cotyledonary explants (Fry *et al.*, 1991) and embryogenic callus (D'Halluin *et al.*, 1992) are difficult to reproduce or are not generally usable. Therefore, an alternative transformation system to circumvent these problems would be desirable.

A plant tissue that generates whole shoots in a relatively short time and without complex tissue culture conditions is the shoot apical meristem. The rationale behind the transformation method used is to stably transform cells that contribute to the germline and to generate plants expressing the foreign gene in at least parts of their inflorescence, thus leading to transgenic offspring. Since the cells of the subepidermal layer (L2) of the meristem are considered to be the precursors of germline cells (Satina *et al.*, 1940) the probability of getting chimeras with partially transgenic flowers should be highest by transformation of those cells.

For the investigations presented in this paper, the apices of sugarbeet seedlings were used as targets for particle bombardment to study the penetration of particles into the apex, the transient expression of marker genes and the viability of cells after the bombardment. For this, the microtargeting apparatus developed by Sautter *et al.* (1991) was chosen. Using this device, the transient expression of marker genes occurs in meristems of wheat immature embryos (Iglesias *et al.*, 1994), seedlings (Bilang *et al.*, 1993) and inflorescences (Leduc *et al.*, 1994), and stable transformation occurs in tobacco (Sautter *et al.*, 1991). This technique has various advantages especially for the bombardment of small targets which are difficult to isolate.

Materials and methods

Preparation of the plant material

Monogerm sugarbeet fruits (*Beta vulgaris* L. var. *altissima*; line 9B2416) were provided by Planta Ltd., Einbeck, Germany. To facilitate handling, fruits were soaked in water for about 1 h after which the outer tissue of the fruits was softened. Seeds

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were excised from fruits and imbibed in water at 24 °C in the dark for 18 h. The age of the mature embryos/seedlings was measured from the beginning of imbibition and stated as 'hours after seed isolation (h ASI)'. Surface sterilization was performed by stirring seeds for 20 min in 15% sodium hypochlorite. Embryos were aseptically isolated from seeds using a binocular microscope (Zeiss DRC, Oberkochen, Germany). To expose the shoot apex, the outer cotyledon was broken off. The embryos were placed in Petri dishes containing either MS basal medium (MS salts and vitamins, 2% sucrose; Murashige and Skoog, 1962) or MSH medium (MS basal medium supplemented with 0.5 mg l^{-1} benzyl amino purine (BA), 0.25 mg l^{-1} indole acetic acid (IAA) and 0.1 mg l^{-1} gibberellic acid (GA_3)) and cultivated at 25 °C with a photoperiod of 16 h until use.

Particle preparation

Uniformly-sized gold particles (batches of 0.8, 1.2, 1.8, and 2.25 μm particles) made according to Sautter *et al.* (1991), and Aldrich gold (Aldrich-Chemie, Steinheim, Germany; particles 0.7–4.9 μm in diameter, average of 1.7 μm), were stored in 40% polyethylene glycol (mol. wt. 4000). Prior to bombardment, particle aliquots were rinsed in distilled water and mixed with a 5 μg μl^{-1} DNA solution. The final particle concentration was 5×10^5 μl^{-1}. For both transient expression and penetration experiments, the plasmid pFF 19G (Timmermans *et al.*, 1990; kindly provided by J. Messing) was used.

Bombardment

For bombardment experiments, the microtargeting apparatus developed by Sautter *et al.* (1991) was used. A 20 nl aliquot of a particle/DNA suspension is injected into a Pitot's tube and atomized and propelled by a pressure pulse of compressed nitrogen. The particles are further accelerated by passing through a glass capillary of 10 mm length, the 'restriction', and hit the target after a free flight of about 10 mm. Five or eight hours prior to particle bombardment, embryos were placed individually in Eppendorf tube lids containing either MS (in penetration experiments and the first series of transient-expression experiments) or MSH (in all further experiments) supplemented with 13% or 28% mannitol as osmoticum and solidified with 3% low-melting-point agarose (Sea Plaque; FMC BioProducts, Rockland, ME, USA). The lid was placed on to the sample holder of the microtargeting device with the meristem facing the restriction aperture (140 μm in diameter, unless stated otherwise). For penetration experiments, the particle size (0.8–2.25 μm) and acceleration pressure (10 and 18 MPa) were varied. After shooting, explants were left overnight on the osmoticum and were then transferred on to MS or MSH.

Clearing/GUS staining

For examination of particle penetration depth, immediately after shooting, embryos were fixed in a 25% propionic acid : 75% ethanol mixture for 24 h. Then they were washed in 70% ethanol for 4 h, changing the ethanol every hour, and cleared for 48 h in a solution containing 2 g chloral hydrate, 2 g phenol, 2 ml lactic acid, 2 ml diethyl phthalate, and 1 ml benzyl benzoate. Apices were examined with an inverted microscope (IM 35, Zeiss, Oberkochen, Germany) using interference contrast. Expression of the GUS gene was detected histochemically. Forty-eight or 72 h post-bombardment, explants were incubated overnight at 37 °C in a filter-sterilized GUS substrate solution according to Mendel *et al.* (1989). Explants were examined with the inverted microscope.

Results and discussion

Penetration of particles into a certain tissue depends upon their momentum as affected by their size and velocity. The apex of sugarbeet embryos was bombarded 24 h after seed isolation in order to test the ability of the microtargeting gun to deliver single particles into the subepidermal cell layer. Observation by light microscopy showed that particles of 0.8 and 1.2 μm in diameter could not penetrate into the apex using a pressure of 10 MPa. Particles of 1.8 μm in diameter could be found in *c.* 22% of the bombarded explants.

Using an acceleration pressure of 18 MPa, the first cell layer of the meristem (L1) was penetrated with a particle size of at least 1.2 μm. With 1.8 μm particles, the penetration rate of the first layer increased to nearly 42%, but the second cell layer was penetrated in less than 10% of the bombarded apices. To obtain penetration of the second layer in 50% of the apices, a further enlargement of particles to 2.25 μm was required; 72% of the apices contained particles in the first layer. The number of microprojectiles that reached the apex was adjustable within narrow limits, and particles were delivered individually. This is in contrast to previous studies with the helium gun PDS 1000-He (Mahn, 1995) where the distribution of particles over the target area was extremely uneven and particles tended to agglomerate. The momentum needed for penetration into the sugarbeet apex is substantially higher than that needed for wheat (Bilang *et al.*, 1993, Iglesias *et al.*, 1994, Leduc *et al.*, 1994). The use of commercial Aldrich gold-containing particles in a range between 0.7 and 4.9 μm improved penetration; 93% of the explants were hit in the first cell layer of the apex and 57% in the second. Thus, for the following experiments Aldrich particles and an acceleration pressure of 18 MPa were used.

In a first series of gene expression experiments, embryos 4–18 h ASI were bombarded immediately after embryo isolation. Their use would have the advantage that the group of initial cells is smaller in number than in older embryos. Hence, after a stable transformation of such a cell, the expected transformed sector should be larger. However, no GUS expression could be obtained using seedlings of this age. It may be that the metabolism of the cells in the apex was not completely reactivated after seed dormancy. This is supported by the findings of Lawrence *et al.* (1990) who reported that storage reserve mobilizing enzymes of sugarbeet seedlings were activated relatively slowly after sowing.

Consequently, for subsequent gene expression experiments 2-d-old seedlings were used. To promote the reactivation of cell metabolism, phytohormones, as used by Bidney *et al.* (1992) for enlargement of sunflower meristems, were applied for 24 h prior to bombardment. These hormones are known to enhance the mitotic activity of

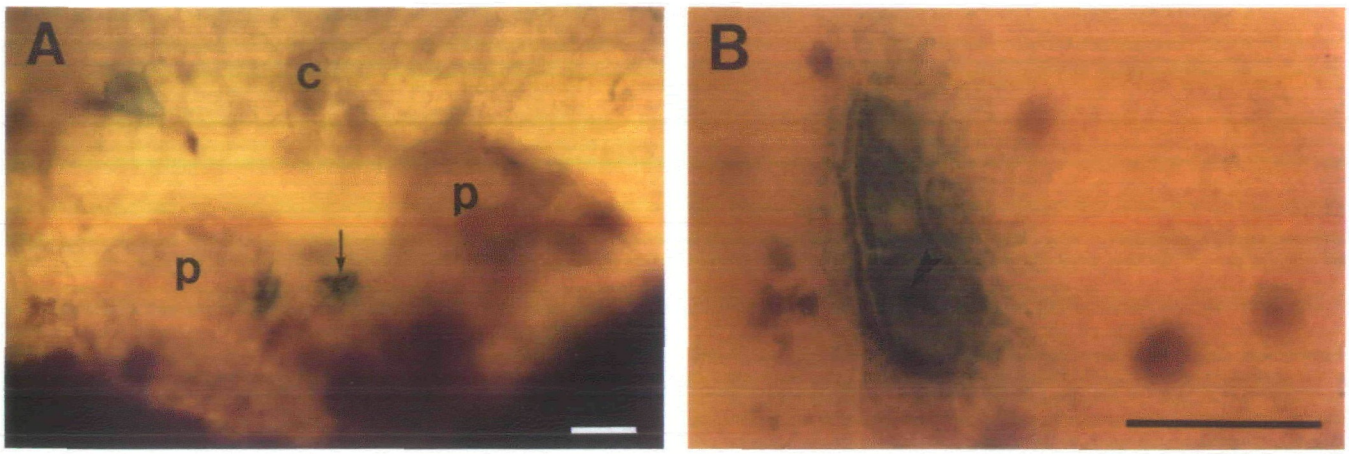

Plate 1. Transient expression of the GUS gene in sugarbeet apex cells (bars represent 50 μm). (A) Light micrograph of a sugarbeet apex with GUS-positive cells in the second cell layer of the meristem (arrow), the first cell layer of a primordium and the cotyledon. (B) Dividing GUS-positive cells 3 d after bombardment (arrowhead indicates particle). Abbreviations: p—primordium, c—cotyledon.

Table 1. *Effect of mannitol concentration, length of osmotic pretreatment and restriction size on transient GUS expression in bombarded sugarbeet apices*

Parameter varied		No. of experiments	No. of bombarded apices	Percentage of apices with blue cells (%)
Mannitol concentration	13%	7	410	2.4
	28%	5	226	4.0
Length of osmotic pretreatment	5 h	7	285	2.1
	8 h	8	387	3.6
Restriction size	140 μm	7	379	1.8
	200 μm	5	293	4.4

cells. Since it is generally believed that actively dividing cells are required for integration of foreign DNA into the genome, this could have an additional positive effect not only on transient expression, but also on stable integration of the foreign gene. Considering that the cells in the central zone of the embryo meristem contribute later in ontogeny to the upper part of the mature plant and the inflorescence (Jegla and Sussex, 1989), an increase in mitotic activity of those cells might even promote the recovery of chimeras with transgenic offspring. Using hormone-treated seedlings, 48 h ASI transient GUS expression was achieved in the first, as well as in the second, cell layer of the apex (Plate 1A). In slightly stained cells, particles, usually one per cell, could be seen. When the GUS assay was performed 3 d after bombardment, blue spots consisting of two dividing cells were found. Only one of these cells contained a particle. This indicates that cells survive the bombardment and maintain their meristematic ability (Plate 1B). An average of 3% of bombarded apices contained GUS-positive cells. Thirty per cent of apices with blue spots had GUS-expressing cells within the second cell layer. An extension of the osmotic pretreatment (8 h), increase of the osmotic pressure (28% mannitol) or use of a bigger restriction (200 μm) slightly increased the mean percentage of apices with GUS-expressing cells (Table 1). However, the effect was not statistically significant (Chi square test). No blue spots were observed after GUS assay of control explants shot with DNA lacking the GUS gene.

Investigations presented here could be the first step towards a gene transfer system for sugarbeet, as an alternative to those published until now. However, the rate of transient expression was still very low and needs to be increased before stable integration can be examined. Possible ways could be the use of the meristem of immature embryos as targets for particle bombardment or an enzymatic digestion of the outer cell walls of the meristems before bombardment.

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