Theor Appl Genet (1989) 77:469–472

Transfection of germinating barley seed electrophoretically with exogenous DNA

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Received September 27, 1988; Accepted October 10, 1988
Communicated by P.M.A. Tigerstedt

Summary. A method is described for transfection (genetic transformation) of barley caryopsis electrophoretically with DNA. β-Glucuronidase activity was detected after the electrophoretic transfection with plasmid pBI221 DNA carrying the cauliflower mosaic virus promotor and bacterial β-glucuronidase coding sequence. Electrophoretic transfection is evidently effective with pieces of callus and seeds of many plants.

Key words: *Hordeum vulgare* – Transformation – Transgenosis – Electrophoresis – Embryos of plants

Introduction

During the last few years, man has developed a variety of physical or chemically aided methods to introduce foreign nucleic acid into a plant cell. These involve macroinjection (Zhou et al. 1983; De la Peña et al. 1987), microinjection (Crossway et al. 1986; Reich et al. 1986; Spangenberg et al. 1986), laser microperforation (Weber et al. 1988), microprojectile bombardment (Klein et al. 1987), fusion with liposomes (Matthews and Cress 1981; Uchimiya and Harada 1981; Ahokas 1987; Gad et al. 1988), and electroporation (Fromm et al. 1986; Ou-Lee et al. 1986). Incubations with DNA under defined conditions have been reported to be effective (Krens et al. 1982; Lörz et al. 1985; Potrykus et al. 1985; Ohta 1986). Many of these methods need protoplasts as the target tissue. Below, I describe a method called electrophoretic transfection (ETR), which can be applied to germinating seeds of barley (*Hordeum vulgare*) and evidently to calli, as well as to seeds of various other species with a suitable embryo. The DNA reporter sequence used – *Escherichia coli* β-glucuronidase or GUS – (Jefferson et al. 1986) shows at least a transient expression in germinating target tissue.

Material and methods

Preparation of barley caryopses

Greenhouse-grown barley grains of two-rowed cv. Adorra were dehusked by hand so that the embryo-end was displayed, but the pericarp remained intact. The grains were washed in 50% H_2SO_4 for 1.5 h and rinsed in sterile distilled water with 25 changes and dried between sterile filter papers. Dry caryopses were aseptically prepared under a stereomicroscope with a needle. The pericarp above the coleoptile was removed, and a puncture was made through the base of the coleoptile towards the meristem. The caryopses were laid on the bottom of a plastic petri dish. Each caryopsis was supplied with 100 µl of sterile deionized water. The petri dishes were incubated on an ice bed in a closed styrox box. The wetted caryopses were used for ETR after 20–48 h incubation on ice.

Labelling of plasmid DNA and microscopic autoradiography

Plasmid DNA pBR322 (Promega) was NaOH denatured, annealed with clockwise and counterclockwise BamHI primers (Sigma). The primer extension was carried out with Klenow DNA polymerase fragment (Pharmacia) for 40 min in the presence of ^{3}H-thymidine 5′-triphosphate (TRK.576, Amersham) as the label after which the products were HindIII (BRL) digested. The DNA was purified with Nick-Column (Pharmacia), eluted with only 65% of the recommended volume, and ethanol precipitated. Agarose gel electrophoresis revealed three radioactive bands: circular dimer, linear monomer, and irreversibly denatured monomer. About 130 ng or 5×10^5 cpm per caryopsis were electrophoretically transfected. After the run, the caryopsis was immediately dipped 15 times in the electrode buffer (Tris-phosphate pH 8.2, 89 m*M* with EDTA, 2 m*M*) and fixed in acetic acid:ethanol (1:3). The dissected embryos were embedded in paraffin via an ethanol-butanol series and cut into 10 µm sections. After being deparaffinized via xylene, the sections were coated with AR.10 Stripping Plate film (Kodak) and were developed after an exposure for 8 or 12 days at −20°C.

In vivo labelling of proteins for SDS-PAGE

After an ETR without DNA or with HindIII linearized plasmid DNA of pBI221 (Clontech), a small well was dissected under the scutellum on the abaxial side of the caryopsis. The well was filled with 400 nCi (1.5 µl) of ^{14}C amino acids (CBF.25, Amersham) in water. The germination continued for 48 h on water agar, after which the germs carrying the scutellum were separated and frozen. Ten germs were homogenized in lysis buffer (Perbal 1984, p 542) and centrifuged 12,000 *g* for 10 min at +0–5 °C. The pellet was further dispersed in 50 µl of sample buffer (Laemmli 1970) with 3% SDS and boiled for 3 min. Samples of the pellet extracts containing the same amount of cpm were loaded on an SDS-PAGE slab gel (Laemmli 1970) with 9% separating gel. The radioactive part of the gel (Fig. 3) was enhanced with Enlightning (NEN) and exposed on Hyperfilm-βmax (Amersham).

Cytological detection of GUS activity

After ETR with linear pBI221 plasmid DNA, the caryopses were allowed to germinate for about 40 h. The germ, including the scutellum, was aseptically detached from each endosperm and was incubated in toto in 1 ml of 1.5 m*M* 5-bromo-4-chloro-3-indolyl-β-D-glucuronide or X-glur (Clontech) directly dissolved in sterile, 50 m*M* sodium phosphate buffer of pH 7.0. The sterile, aerated polypropylene tubes were slowly rolled with Mixer 820 (Swelab) at 30 °C. The bluish colour became visible to the eye in 15 min. After an incubation of 9 h, germs were dissected with a scalpel under stereomicroscope. Appropriate specimens were briefly immersed in 70% ethanol and mounted on a slide with 50% glycerol.

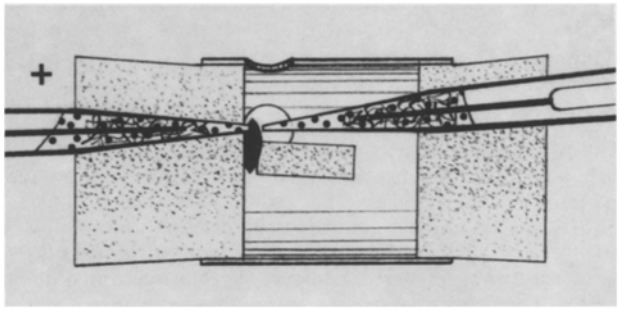

Fig. 1. The essential part of an electrophoretic transfection chamber with two electrode reservoirs. The positive pole is on the left. A barley caryopsis is depicted in black

ETR chamber and ETR operations

The ETR chamber used is depicted in Fig. 1 and was composed of two silicone stoppers, a piece cut from a 50-ml polypropylene tube (Nunc) with two cut apertures, two 1-ml polypropylene pipettor tips (Labsystems), and two Pt wires (Engelhard) with a diameter of 1 mm. Each of the Pt electrodes was attached as an extension with a banana plug which was firmly adapted by the wider end of the pipettor tip. A very loose plug of sterile Helmi wad of 90% viscose (Suomen Vanu) was placed in the pipettor tip to serve as a gas-bubble breaker. The points of the electrode housing were free from wad and electrode by about 15 mm (negative electrode) and by about 7 mm (positive electrode). The distance between the electrode points was about 3 cm. The pipettor tips acted as a buffer reservoir and were filled with a pipettor with 400 µl of buffer through the tip-point. The buffers used were 89 m*M* Tris-phosphate (pH 7.4 or 8.2) with EDTA (2 m *M*). After the buffer, the negative electrode was loaded with DNA in 1.2 – 4 µl of ⅔-strength reservoir buffer, immediately before running began. All the components in contact with seed and solutions were sterilized. Pipettor tips were disposed of after a single use.

Another version of the ETR chamber had a reservoir on the negative side only. In this single reservoir model, the caryopsis was in direct contact with the positive electrode point in a droplet of 10 µl of the buffer.

The punctured embryo projected towards the negative reservoir with the DNA sample in its tip (Fig. 1). The final contact with the embryo was arranged with a solution bridge from a distance of about 0.5 mm. The positive electrode tip or the contact electrode projected into the crease of the caryopsis under the embryo (Fig. 1). Power Supply 2197 (LKB) was run at constant current (0.1 mA) with 2 – 10 V cm^{-1}. The running time was 60 min if not indicated otherwise. Each chamber was run with its own power supply. The treated caryopsis were aseptically germinated in 1% water agar plates supplemented with

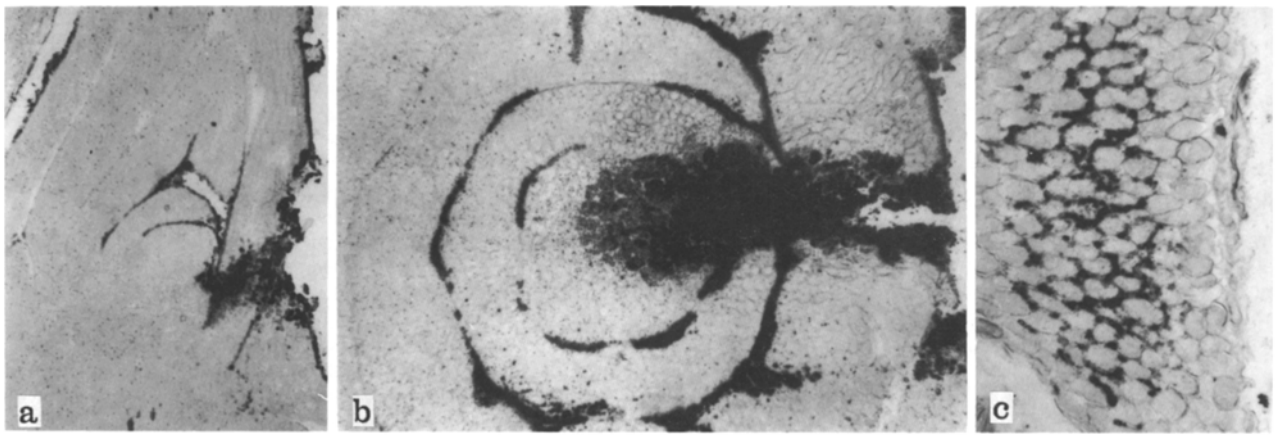

Fig. 2a–c. Autoradiograms of sectioned embryos after ETR with ^{3}H-labelled pBR322 DNA. The positive electrode has been on the left side. **a** Longitudinal section with radioactivity moving towards the inner meristem. Magn. ×75. **b** Transection of a meristem showing radioactivity also moving through cellular cytoplasms. Magn. ×190. **c** Radioactivity moving along cell walls of lower end of a mesocotyl. Magn. ×210. ETR times for **a** and **c**, – 60 min, for **b**, – 20 min

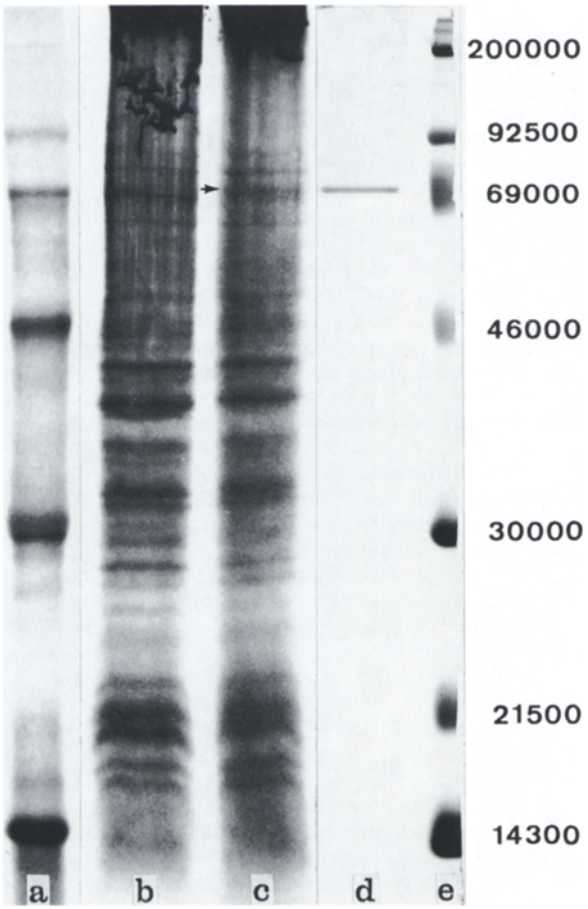

Fig. 3a–e. SDS-PAGE of in vivo-labelled proteins in mock transfected embryos **b** and embryos transfected with Hind III linearized pBI221 DNA **c**. The *arrow* indicates the band of the GUS mobility. Track **a**: radioactive molecular weight markers (CFA.626, Amersham). Track **d**: *E. coli* GUS protein (Clontech) with M_r of about 75,000. Track **e**: half-lane of unlabelled Rainbow molecular weight markers (RPN.756, Amersham). Track **a**, **b** and **c** were autoradiographied, **d** and **e** were stained with Brilliant blue R

$CaSO_4$ (0.01%). Germinability of 30%–60% was usually obtained with the single reservoir model, of 60%–90% with the double.

Results

Serving as a labelled DNA, tritiated pBR322 plasmid DNA was electrophoresed into barley embryo using a single reservoir chamber, and the transport was detected with microscopic autoradiography (Fig. 2). Even after a 20 min run, considerable transfer of radioactivity was evident. Three types of distribution of the label were observed: (A) spreading on all the epidermal areas of the leaf primordia, also inside the leaf whorls (Fig. 2a, b), (B) movement along cell walls (Fig. 2c), and (C) movement through cytoplasms (Fig. 2a, b). The puncture of the epidermis helps the penetration of DNA. A well-

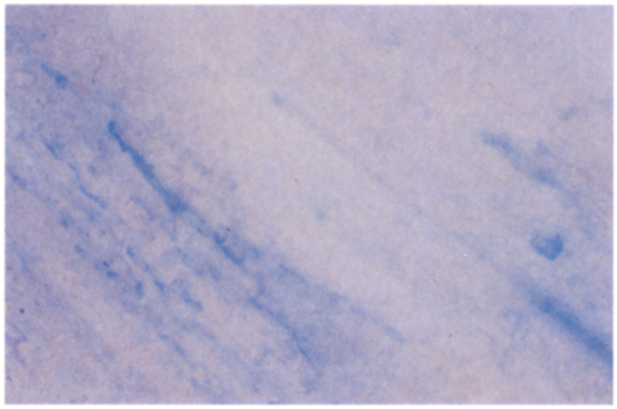

Fig. 4. Bluish end-product of histological GUS activity in abaxial epidermis (in focus) of the primary leaf on the site of ETR with Hind III linearized pBI221 DNA. Magn. × 195

projected puncture will admit the DNA into the meristem.

The plasmid pBI221 carrying the cauliflower mosaic virus 35S promotor and *E. coli* GUS coding sequence (Jefferson et al. 1987) was used to transfect barley caryopses (500 ng of DNA per caryopsis) with the single reservoir chamber. After ETR in two independent experiments, in vivo labelling revealed protein with an M_r of 74,400 (Fig. 3) in reference to the molecular weight markers used. The *E. coli* GUS protein also showed a mobility of about 75,000 on my gels (Fig. 3).

With X-glur as a cytological substrate, germinating unfixed barley embryos show endogenous activity after a mock transfection without DNA. The activity appears in some confined tissues of scutellum, coleoptile, leaf and root primordia. According to this observation, barley should be classified as a species with little GUS background activity. After an ETR treatment of barley caryopses with linearized pBI221 DNA, the GUS activity was also detected in cells and tissues not expressing endogenous activity. The epidermis of the primary leaf site exposed directly to ETR with pBI221 displayed GUS activity after 30–40 h of germination (Fig. 4). Mock treated germinants were not found to have such activity in epdermis.

Discussion

The GUS activity based on pBI221 transfection can evidently last for several days, though the expression of pBI221 after ETR is rather transient. The probability of a genomic integration of an introduced DNA might be raised by including a suitable anodic restriction endonuclease in the ETR sample. It is obvious that an ETR treatment can also disorganize chromosomes. With special care, almost all the germinating seeds that survived

ETR with pBI221 could be raised to maturity and the spikes showed the normal fertility.

Before ETR, the cell walls can also be loosened with enzymes. Five microliters of filter-sterilized 0.5% cellulase Onozuka R-10 (Serva) were pipetted on the embryo of wetted caryopsis and incubated for 2–4 h at room temperature. Before ETR, the caryopses were rinsed with sterile water. Cellulase pretreatment of less than 4 h did not markedly reduce germinability. Immature, soft grains might also be suitable for ETR. Calli can easily be treated with ETR using a block of low gelling temperature agarose as the source of DNA (Ahokas, in preparation).

Various barley tissues have been shown to produce DNase activity upon germination of the seed (Taiz and Starks 1977; Brown and Ho 1986; Prentice and Heisel 1986; Prentice 1987). The DNase activity in the wetted embryos at the beginning of ETR is expected to be low.

Barley was listed to possess no or little endogenous GUS activity by Jefferson (1987). According to the present observation, barley has an activity in some differentiating tissues with X-glur as the substrate. This little endogenous activity makes the spectrofotometric and fluorometric measurements doubtful. Previously, cytological GUS activity has been reported in plant tissues, in *Campanula* pollen and *Hyacinthus* pollen tube (Górska-Brylass 1965).

ETR studies of DNA's showing integration in some combinations are underway. With Ti plasmids, highly deformed germinating plumules of barley with final inviability may indicate expressions. The direct examination of opines with paper electrophoresis (Otten and Schilperoort 1978) is not relevant with barley germinants because of other endogenous fluorescent spots of similar mobility, a fact also encountered with maize tissue (Christou et al. 1986).

Acknowledgements This study was carried out under the auspices of the Academy of Finland and was supported with a grant from the Ministry of Agriculture and Forestry. I am obliged to Ms B. v. Schoultz (M.Sc.) for the paraffin sections.

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