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Transformation of haploid *Datura innoxia* protoplasts and analysis of the plasmid integration pattern in regenerated transgenic plants

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Abstract. We developed a highly efficient transformation protocol for the PEG-mediated direct transfer of plasmid DNA into protoplasts of haploid *Datura innoxia*. Vectors harbouring a neomycin phosphotransferase II gene or a hygromycin B phosphotransferase gene under the control of different promoters were used in the transformation experiments. Various amounts of plasmid DNA were applied without any carrier DNA to show the direct influence of the plasmid DNA concentration on the transformation efficiency. Approximately 95 % of the selected calli were regenerated to plants; 20 % of them remained haploid. Total DNA of different transgenic plants was analysed with regard to the integration pattern of the plasmid DNA. Plants carrying only one or two copies of the vector DNA were observed as well as individuals with multi-copy integration (up to ten or more copies).

Key words: *Datura* - haploids - direct gene transfer

Abbreviations. ATF/RTF: absolute/relative transformation frequency; BAP: 6-benzylaminopurine; CaMV: cauliflower mosaic virus; CTAB: N-cetyl-N,N,N-trimethylammonium bromide; HPT: hygromycin B phosphotransferase gene; PEG: polyethyleneglycol; MES: 2-(N-morpholino) ethanesulfonic acid; NPT II: neomycin phosphotransferase II gene

Introduction

Up to now, pioneer work and most successful plant transformation and regeneration experiments have been carried out using solanaceous species such as *Nicotiana tabacum*, *Petunia hybrida* and *Lycopersicon esculentum* (see in Vasil 1984). Efficient protocols in these plants result in a relative transformation rate of up to 2.0 % (Shillito *et al.* 1985). One may therefore conclude that the good *in vitro* characteristics common to many *Solanaceae* are a prerequisite for successful transformation. *Datura innoxia*, another species of this family, has been used with good results in tissue culture, protoplast isolation, regeneration experiments and somatic hybridization (Schieder 1975, 1978). However, no PEG-mediated transformation system for protoplasts has been reported until now. Such a system would be of interest, since haploids of this species have proven to be very stable when kept under greenhouse conditions. The goal of a transformation system for haploid plant material that is stable in tissue culture might be to establish an insertion mutagenesis system in which clones with dominant as well as recessive mutations can be detected without further progeny analysis.

Materials and Methods

Plant material. In 1972, Schieder obtained haploid plants of *Datura innoxia* by using anther culture, according to the technique of Guha and Maheshwari (1964). One of these lines (A7/1) has been kept under greenhouse conditions and propagated by cuttings for 20 years now. This plant material was used as the starting material for *in vitro* cultures. After surface sterilization with commercial bleach and three washes, the shoot tips were subcultered three times on Gamborg's B5 medium (1968) supplemented with 0.5 mg / l BAP (27 °C, 5,000 - 10,000 lux white light, 16 hrs photoperiod / day). The ploidy level in metaphase plates was checked to determine whether the chromosome numbers were stable 1 n = 12. The material was then used for the isolation of protoplasts.

Plasmids and molecular probes. Four different plasmids were used for transformation:

1) pHP23 (Paszkowski *et al.* 1988; 4.57 kbp), which carries the NPT II gene under control of the 35S promoter and terminator,
2) pKU2 (Baker *et al.* 1987; 7.52 kbp), which carries the NPT II gene controlled by the pTR 2' 1' promoter and ocs3' termination region,
3) pGL2 (Bilang *et al.* 1991; 4.46 kbp), which carries the HPT gene under the control of the 35S promoter and terminator,
4) pTT1 (13.56 kbp), a pGL2 derivative which carries the HPT gene under the control of the 35S promoter and terminator.

Three different probes were used for Southern hybridization:

P1) the NPT II gene with 35S promoter and terminator (1.9 kbp *Eco*RI fragment from pHP23),
P2) the coding sequence of the HPT gene (1.1 kbp *Bam*HI fragment from pGL2),
P3) pUC19 (2.7 kbp *Eco*RI fragment).

The fragments used in the hybridization experiments were labelled to a specific activity of 3×10^9 dpm / µg DNA with α-P^{32} dCTP according to Feinberg and Vogelstein (1983).

Isolation of protoplasts. The procedure is a modified version of the protocol published by Schieder (1975). Ten days after subculturing (= day 0), two grams of leaves were cut into small pieces and incubated in flasks with

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50 ml of enzyme solution (half-strength of that of Schieder 1984) on a roller apparatus (3 rpm; 27 °C). Sixteen hours later, the solution was filtered through a nylon membrane (100 µm pore size) and washed several times with North Sea water (from List / Sylt; adjusted to 700 mOsm with *A. dest*). Vital protoplasts were separated from the crude extract by centrifugation in 0.6 M sucrose (10 min, 80 x g). After a final wash with sea water, the protoplasts were suspended in starting solution for transformation (TS1: 154 mM NaCl, 125 mM $CaCl_2$ x 2 H_2O, 5 mM KCl, 5 mM glucose; 700 mOsm, pH 5.8).

Transformation protocol. After incubation for 20 min in TS1, protoplasts were pelleted (5 min, 160 x g) and resuspended in TS2 (TS2: 530 mM mannitol, 15 mM $MgCl_2$ x 6 H_2O, 0.1 % MES; 700 mOsm, pH 5.8). TS1 and TS2 are modifications of solutions W5 and MaMg formulated by Negrutiu *et al.* (1987). Cell density was adjusted to 1 x 10^6 protoplasts / ml, and aliquots of 1 ml were placed in 12 ml glass centrifuge tubes. 0 to 100 µg plasmid DNA (1 µg / µl) was added after 30 min. No carrier DNA or heat shock was applied. 10 min later 0.5 volumes of TS3 (TS3: 40 % PEG 6000 [Merck], 0.4 M mannitol, 0.1 M $Ca(NO_3)_2$ x 4 H_2O; pH 8.0) were added dropwise while gently rolling the tubes. After 30 min assays were filled with sea water and centrifuged (5 min, 120 x g). The pellets were resuspended in V47 medium (Binding 1974, supplemented with 0.4 mg / l BAP and 1,5 mg / l NAA; 700 mOsm, pH 5.8) and adjusted to a final density of 5 x 10^4 protoplasts / ml.

In vitro culture and regeneration of protoplast-derived transformants. Protoplasts (1 x 10^5 / 2 ml) were placed in a petri dish (Ø = 6 cm) and kept in the dark at 27 °C. On day 10, 0.3 % agarose in V47 medium was temperated to 40 °C, and 2 ml were mixed with the microcalli suspension. For selection, the antibiotic kanamycin (Boehringer Mannheim; final concentration 100 mg / l) or hygromycin B (Duchefa; final concentration 25 mg / l) was added together with the V47 medium. The soft agarose was spread on solid agarose medium (Gamborg's medium B5, 0.5 mg / l BAP; 9 cm dishes) on day 20. Four weeks later, small calli were transferred to fresh medium. Regeneration normally started during this first subculture.

Southern analysis. Total DNA of selected plants was isolated according to the method of Rogers and Bendich (1985). 10 µg DNA was digested with 30 units of the restriction enzymes *Eco*RI or *Eco*RV for 6 hrs at 37 °C and separated by electrophoresis (2.5 V / cm, 0.8 % agarose). Then, the DNA was transferred to Hybond N^+ membrane (Amersham) on a vacuum blotter. Filters were hybridized with 5 x 10^7 dpm / ml of the appropriate labelled fragment in 4 x SSC, 0.5 % skim milk powder and 0.5 % SDS at 65 °C for 16 hrs. After removing unspecific labels (stringent conditions: 2 x 15 min at 65 °C in 0.2 x SSC and 0.2 % SDS, constant agitation), filters were incubated together with Kodak X-Omat S film and intensifying screens (Du Pont) at -70 °C for 10 hrs to five days.

Results and Discussion

In vitro *culture aspects*

The haploid *Datura innoxia* has proven to be a plant with excellent *in vitro* capabilities. The yield of protoplasts per gram of fresh leaves is normally about 5 x 10^6 (n = 30, mean = 5.15, SE = ± 0.15). A density of 5 x 10^4 protoplasts / ml and 2 ml per petri dish (ø = 6 cm), which led to first cell divisions on day 3 or 4, was found to be optimal. Resistant calli were picked and subcultured around day 50. Nearly all of these calli regenerated shoots. Plants rooted on B5 medium containing charcoal (500 mg / l) flowered five to six months after protoplast isolation. No morphological differences between transgenic and control plants could be detected. About 20 % of the regenerants remained haploid after the transformation and regeneration procedure. Changes in ploidy level seem to occur during the first days after protoplast isolation. Some of the diploid plants were allowed to set seeds which were able to germinate. The seedlings matured and showed a normal phenotype.

Transformation of protoplasts

We have established a PEG-mediated transformation procedure using different plasmids in absence of carrier DNA and heat shock treatment, indicating that these treatments are not required for successful transformation. In order to enhance the transformation frequency, we adapted the method of Negrutiu *et al.* (1987). The selection of transformants was tested with different concentrations of the antibiotics kanamycin and hygromycin B, which showed that 100 mg / l and 25 mg / l, respectively, is sufficient to give a sharp selection with only minimal escapes (< 3 %). About 50 % of the protoplasts survived the transformation process and yielded a total of 15,000 calli (plating efficiency: 3 %).

Four plasmids of different size were tested using similar numbers of plasmid molecules in the standard assay (2.6 to 2.9 million copies per protoplast; Table 1a). Transformants were selected in all cases, but RTF varied between 0.02 and 0.48 %. Köhler *et al.* (1987), who transformed *Nicotiana tabacum* and *Vigna aconitifolia* with two different plasmids, also found that the plasmid influenced the transformation rate. They suggested that the different transformation rates could be due to different

Table 1: Transformation frequencies of haploid *Datura innoxia* protoplasts

a: Transformation rates with different plasmids using a similar number of plasmid molecules per protoplast

plasmid	plasmid size in kbp	amount of DNA	million copies / protoplast	resistant calli / 10^6 protoplasts*	RTF** in %	ATF** in %
pGL2	4.46	13 µg	2.8	18	0.12	0.0018
pHP23	4.57	13 µg	2.8	3	0.02	0.0003
pKU2	7.52	20 µg	2.6	72	0.48	0.0072
pTT1	13.56	40 µg	2.9	52	0.35	0.0052

b: Maximum transformation rates with different plasmids

plasmid	plasmid size in kbp	amount of DNA	million copies / protoplast	resistant calli / 10^6 protoplasts*	RTF** in %	ATF** in %
pGL2	4.46	30 µg	6.5	69	0.46	0.0069
pHP23	4.57	100 µg	21.1	253	1.69	0.0253
pTT1	13.56	40 µg	2.9	52	0.35	0.0052

* mean of at least three independent experiments (1 x 10^6 protoplasts each)
** relative and absolute transformation frequencies as defined by Negrutiu *et al.* (1987)

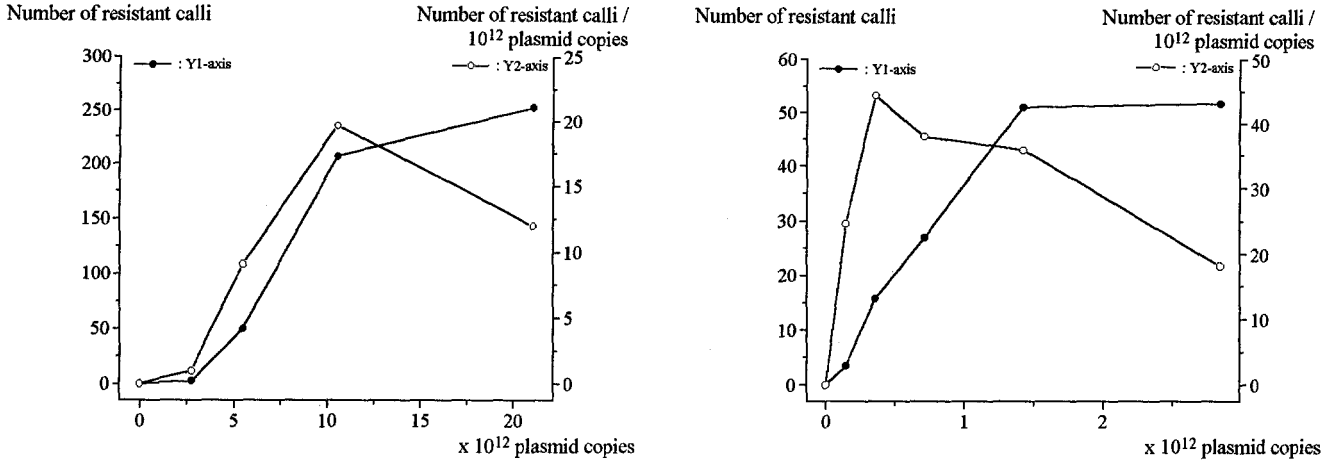

Fig. 1: Transformation of haploid *Datura innoxia* protoplasts with different amounts of plasmid DNA. Fig. 1a: pHP23 (left); Fig. 1b: pTT1 (right). All values are relative to 10^6 protoplasts.

i) integration rates, ii) expression rates and/or iii) DNA uptake by the protoplasts as a consequence of different plasmid structure and size. In contrast, Karesch *et al.* (1991) found that varying the amount of vector DNA (5 to 30 µg of pGL2) did not change the ATF in *Arabidopsis thaliana*. However, both authors used carrier DNA in their transformation assays, which might complicate the interpretation of the relation between the number of plasmid copies and the transformation frequency.

At about 15 x 10^6 plasmid copies of pHP23 per protoplast (equivalent to 70 µg; Fig. 1a) and about 1.4 x 10^6 copies of pTT1 per protoplast (equivalent to 20 µg, Fig. 1b), the curves reach a maximum similar to a saturation curve (solid circles in Fig. 1). The maximum RTF found in these experiments are given in Table 1b. The highest RTF that we obtained was 1.69 %, using the plasmid pHP23.

The other curves (open circles), which represent the relationship between the number of resistant calli at a constant amount of plasmid copies (10^{12}) and the number of plasmid copies used for transformation of 10^6 protoplasts, show that the optima in transformation efficiency are quite different for both plasmids.

Although PEG-mediated gene transfer is widely used, very little is known about the mechanisms responsible for the introduction of foreign DNA into plant cells. It has been suggested that the number of DNA strand breaks and that the activity of the DNA repair system could be the limiting step in the transformation process (Köhler *et al.* 1990; Benediktsson *et al.* 1991). The saturation of these enzymes can perhaps explain the course of the curve we obtained. Shillito *et al.* (1985) obtained similar results using the plasmid pABDI. They believe that this behaviour is due to a competence phenomenon between cells that are capable of being transformed, leading to a saturation effect. Our results also show that the level of saturation corresponds neither to the total amount of plasmid DNA nor to the number of plasmid copies per protoplast used for transformation. The saturation level has to be determined separately for each plasmid.

Hybridization analysis

A number of plants resistant to kanamycin (Fig. 2) or hygromycin B (Fig. 3) were selected arbitrarily for molecular analysis. The presence of marker genes, the integration of constructs into the plant genome and the approximate number of integrated plasmid copies was determined by Southern hybridization.

Total DNA was isolated from single kanamycin-resistant plants that had been transformed with the plasmid pHP23 (Fig. 2d) and separated by electrophoresis either undigested (Fig. 2a) or digested with the restriction enzyme *Eco*RI (Fig. 2b). After blotting the DNA, these filters were probed with the 1.9 kbp *Eco*RI fragment from pHP23 containing the complete plant NPT II cassette (P1).

In the case of the undigested plant DNA hybridization signals were within the high molecular DNA fraction. This demonstrates that the plasmid DNA was integrated into the plant genome. The *Eco*RI-digested DNA hybridized with the NPT II probe gave one strong signal at 1.9 kbp in all lanes (Fig. 2b, see arrow), representing multiple copies of the complete NPT II gene. All other signals indicate that integration also took place within the NPT II cassette. This hypothesis was proved by reprobing the blot with the linearized plasmid pUC19 (P3). The strong signals at 2.7 kbp (Fig. 2c, see arrow) show that, in this case, the bacterial vector remained intact. All signals not equal to 2.7 kbp indicate that integration also took place outside of the cassette.

Southern analysis of various hygromycin B-resistant regenerants transformed with pGL2 (Fig. 3e) revealed the presence of different numbers of the construct or parts of it in all plants tested (Fig. 3a-d). *Eco*RI-digested DNA of three different clones was probed with the 1.0 kbp *Bam*HI fragment (P2) containing the coding sequence of the HPT gene only. In general, the intact hygromycin gene with 35S promoter and 35S terminator is represented by two *Eco*RI fragments of 0.8 kbp and 0.95 kbp (Fig. 3a, see arrows). This is due to the presence of two flanking *Eco*RI

sites and one site within the HPT gene. One exception was plant no. 2. There was probably a minor mutation or modification in the region of one *Eco*RI site that did not alter hygromycin B resistance.

In the cases where the plant DNA was digested with the restriction enzyme *Eco*RV (that has a single cleavage site in the 35S promoter of the construct) and hybridized with the coding sequence of the HPT gene (P2), an intact HPT cassette produced one band (≥ 1.4kbp). However, one or two signals occured when the cassette was disrupted during integration into the genome (Fig. 3b).

*Eco*RI-digested plant DNA that was hybridized with linearized pUC19 as a probe gave a signal of 2.7 kbp when the HPT cassette was destroyed by illegitime recombination (Fig. 3c, see arrow). Hybridization signals not equal to 2.7 kbp indicate a recombination site outside of the HPT cassette.

*Eco*RV-digested plant DNA probed with pUC19 (P3) produced one or two signals per integrated plasmid copy.

As regards the hybridization pattern of kanamycin and hygromycin B-resistant plants, the site where the plasmid ring opens for illegitime recombination with the plant genome seems to be random. The integration into the kanamycin and hygromycin B cassette as well as into the bacterial vector part led us to the conclusion that there is no preferred site for recombination with the plant genome in the plasmids pHP23 and pGL2.

Our analyses also allowed for rough calculation of the copy number. In the case of regenerants 1 and 2 (Fig. 3), only a few copies of the plasmid pGL2 were integrated into the genome (probably two in plant 1 and one in plant 2). Several copies were, however, present in regenerant 3 (Fig. 3) and in all transformants with the plasmid pHP23 (Fig. 2).

The integration pattern was complex in most studied transgenics. This shows that the number of integrated copies in direct gene transfer experiments is often high (up to ten or more copies have been observed). This is in agreement with the results of other authors who used the direct gene transfer method (e. g. Shillito *et al.* 1985; Damm *et al.* 1989). The hybridization pattern can be reproduced as illustrated in lanes 1A and 1B in Fig. 2b and 2c. In this case, total DNA that was isolated from two individual plants regenerated from one clone was compared.

This procedure for transformation of haploid plant material opens the way to establish an insertion mutagenesis system for the isolation of dominant as well as recessive mutations in a one-step procedure without further analysis of the progeny.

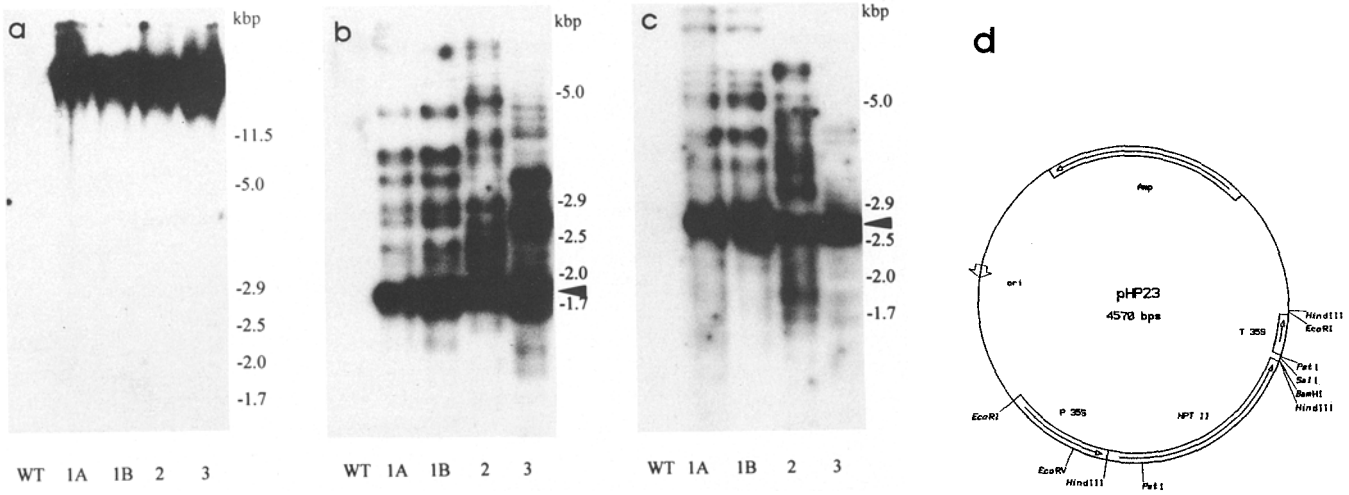

Fig. 2: Southern analysis of different *Datura innoxia* transformants. Total DNA of plants transformed with the plasmid pHP23 and selected on kanamycin-containing media.
Lanes 1A & 1B: two individual plants from one clone; lanes 2 & 3: two different clones; lane WT: untransformed control.

Fig. 2a: Undigested DNA, hybridized with the NPT II probe (P1), giving a signal in the high molecular weight fraction.

Fig. 2b: *Eco*RI-digested DNA, hybridized with the NPT II probe (P1), giving a signal at 1.9 kbp (see arrow) for the intact NPT II cassette.

Fig. 2c: *Eco*RI-digested DNA, hybridized with pUC19 (P3), giving a signal at 2.7 kbp (see arrow) in the case of the non-disrupted bacterial vector part.

Fig. 2d: Map of plasmid pHP23 (Amp: bacterial ampicillin resistance gene; ori: bacterial origin of replication; P 35S/T 35S: promoter/terminator of the 35S region of the CaMV; NPT II: neomycin phosphotransferase II gene).

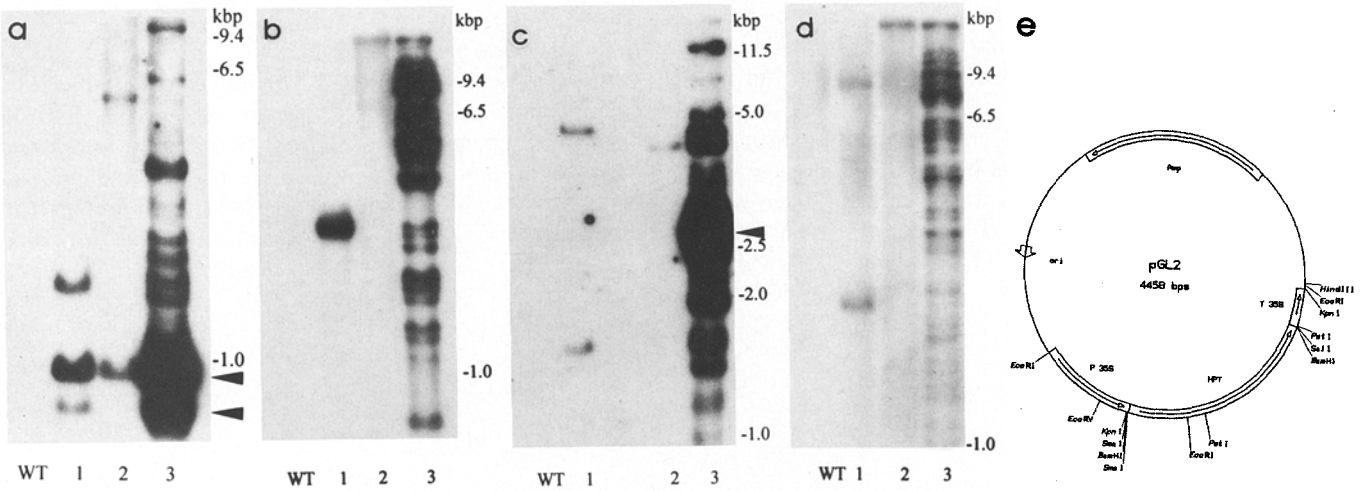

Fig. 3: Southern analysis of different *Datura innoxia* transformants. Total DNA of plants transformed with the plasmid pGL2 and selected on hygromycin B-containing media.
Lanes 1, 2 & 3: three different clones; lane WT: untransformed control.

Fig. 3a: *Eco*RI-digested DNA, hybridized with the *Bam*HI fragment of pGL2 (P2), giving two signals at 0.8 kbp and 0.95 kbp (see arrows) in case of the intact HPT cassette.

Fig. 3b: *Eco*RV-digested DNA, hybridized with the *Bam*HI fragment of pGL2 (P2), each integrated copy is giving one or two bands depending on the constitution of the HPT gene.

Fig. 3c: *Eco*RI-digested DNA, hybridized with linearized pUC19 (P3), giving a signal at 2.7 kbp (see arrow) in the case of the non-disrupted bacterial vector part.

Fig. 3d: Reprobing of 3b with linearized pUC19 (P3), showing the integration pattern in respect to the pUC part of the vector.

Fig. 3e: Map of plasmid pGL2 (Amp: bacterial ampicillin resistance gene; ori: bacterial origin of replication; P 35S/T 35S: promoter/terminator of the 35S region of the CaMV; HPT: hygromycin B phosphotransferase gene).

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