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Production of human papillomavirus type 16 E7 oncoprotein fused with β-glucuronidase in transgenic tomato and potato plants

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Abstract

The human papillomavirus type 16 (HPV 16) oncogene *E7* fused with the gene for β-glucuronidase (*gus*) was used in plant transformation experiments. The *E7* gene modified for lower cancerogenicity and fused with the 5´ end of the *gus* in cassettes with cauliflower mosaic virus 35S promoter and transcription terminator produced high contents of fusion proteins in potato protoplasts. Expression vectors harbouring E7 fusion cassettes were used for *Agrobacterium tumefaciens* LBA4404 mediated transformation of either potato (*Solanum tuberosum* L. cv. Bintje) or tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker). A fusion gene was found in all rooted regenerants using polymerase chain reaction with primers providing amplified fragments from *E7* and *gus* genes. GUS activity was revealed in all regenerants obtained. Nevertheless, the level of GUS expression in different constructs varied much more than in transient expression experiments with potato protoplasts. Especially, expression level in plants carrying vectors with the whole *E7* gene fused with *gus* was lowered by 2 - 3 orders of magnitude comparing with fusion of the first 41 codons of *E7* and *gus*. Southern hybridisation of 18 tomato and 23 potato regenerants revealed mostly multiple tandem integration of T-DNA into the plant genome and Western blot proved the presence of the fusion protein in 9 tomato and 11 potato plants out of 41 tested individuals.

Additional key words: E7/GUS fusion protein, HPV 16, *Lycopersicon esculentum*, *Solanum tuberosum*, transformation.

Introduction

Genital infection caused by human papillomavirus (HPV) is the most common sexually transmitted viral disease worldwide. The oncogenic HPV types were found to be associated with high-grade cervical lesions and carcinomas, and also with a proportion of carcinomas of the upper respiratory and digestive tracts, penis, vulva, and anus (Zur Hausen 1996). Promising results of immunization studies in animal models warrant the need for further efforts to develop vaccines against genital HPVs. It is generally agreed that the first generation of HPV vaccines will be HPV 16 specific because more than 50 % of cervical cancer cases are associated with this type of HPV. Since HPVs do not grow in tissue cultures, all current experimental HPV vaccines are prepared by recombinant DNA technology.

E7 protein is an HPV unstable oncoprotein exhibiting low immunogenicity. Point mutations resulting in substitution of three amino acids in the pRb-binding site of the HPV16 E7 protein were introduced into the gene sequence to obtain a safer E7GGG gene without compromising its immunogenicity in mice (Šmahel *et al.* 2003). To enhance E7GGG immunogenicity fusion genes of *E. coli* β-glucuronidase (GUS) and E7GGG or truncated E7GGG were constructed (Šmahel *et al.* 2004). The fusion genes show superior immunisation efficacy as compared with E7GGG and other E7 fusions.

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Abbreviations: 2,4-D - 2,4-dichlorphenoxyacetic acid; CaMV - cauliflower mosaic virus; ECL - enhanced chemoluminiscence; GUS - β-glucuronidase; HPV 16 - human papillomavirus type 16; MES - 2-(N-morpholino)ethanesulphonic acid; MOPS - 3-(N-morpholino)propanesulfonic acid; MUG - 4-methyl-umbelliferyl-β-D-glucuronide; NPTII - neomycin phosphotransferase II; ORF - open reading frame; PBS - phosphate buffered saline; PEG - polyethylene glycol; SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis.
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Since the gene coding for GUS is frequently used as a repoter gene in plant transgenosis, we employed the fusion genes for tomato and potato transformation and, subsequently, for estimation of expression levels of the transgenes in transgenic plants. Thus we could identify regenerants expressing the *E7GGG* gene to highest levels. The same strategy was used for the development of a foot and mouth disease virus peptide-based vaccine in transgenic alfalfa by Dus Santos *et al.* (2002).

In this study, we used a previously constructed *E7GGG/gus* fusion gene for the production of transgenic tomato and potato plants and screened a large number of transformants for their β-glucuronidase activity to identify plants exhibiting the highest level of the *E7GGG* gene product.

Materials and methods

Plasmid construction: MonoGUS plasmid with CaMV 35S promoter and a short polylinker *Sma*I(1)-*Bam*HI-*Sma*I(2) followed by *gus* gene and CaMV transcription terminator (Bonneville *et al.* 1989) was modified using procedures for the preparation of recombinant plasmids described by Sambrook *et al.* (1989). A 132bp *Pvu*II fragment of *E7GGGp* was excised from the plasmid pBSC/E7GGG (Šmahel *et al.* 2001) and cloned into the *Sma*I(1) site of MonoGUS after partial digestion with *Sma*I. The resulting fusion gene *E7GGGpgus* used in this study contains an ORF comprised of 41 codons from the N-terminus of *E7GGG*, 9 linker codons and 603 codons of *gus*. Alternatively, the whole *E7GGG* gene was amplified from the pBSC/E7GGG using primers E7-1: 5´-CCAGGATCCATCATGCATGGAGATACACC-3´ (forward) and E7-2: 5´-CAGCCATGGTGGATCCTGG TTTCTGAGAACAG-3' (reverse), digested with *Bam*HI (underlined sequences in primers) and cloned into the unique *Bam*HI site of MonoGUS. *In frame* fusions E7GGG/GUS with 8 linker codons between *E7GGG* and *gus* and E7GGG(2×)GUS containing two copies of *E7GGG* and additional 3 linker codons between *E7GGG* genes were selected. Finally, all these fusion genes together with CaMV 35S promoter and terminator (Fig. 1) were excised with *Eco*RI and cloned into the plant binary vector plasmid pCB1399 (Matoušek *et al.* 1989).

All plasmids were isolated by the "cleared lysate method" and purified by cesium chloride/ethidium bromide centrifugation (Sambrook *et al.* 1989). They were dissolved in water and adjusted to 2 mg DNA cm^{-3} before transfection.

Transient expression: Potato (*Solanum tuberosum* L. cv. Bintje) plants were used for protoplast isolation. The donor plants were cultivated *in vitro* on Schenk and Hildebrandt (1972; SH) medium and 3 weeks before isolation of protoplasts a 6-h photoperiod, a low irradiance (17 μmol m^{-2} s^{-1}) and a low temperature (10 °C) were applied. Leaf mesophyll protoplasts were isolated as described by Bříza and Machová (1991) with enzyme mixture consisting of 1.4 % cellulase R-10, 0.2 % macerozyme R-10, 5 mM $CaCl_2$, 0.5 M sucrose, 10 mM 2-(N-morpholino)ethanesulphonic acid (MES) and 2 mg dm^{-3} zeatin.

The transformation procedure mediated by polyethylene glycol (PEG) was a modified version of the protocol of Negrutiu *et al.* (1987). One sample contained 5×10^5 of freshly prepared potato protoplasts in 1 cm^3 SW_{11} medium (Bříza and Machová 1991). The protoplasts were incubated 5 min at 45 °C, immediately transferred on ice for 10 s and 50 μg of transforming DNA was added to each protoplast sample. After 5 min at room temperature 0.5 cm^3 of 40 % PEG 6000 solution was added, mixed by careful shaking and incubated at room temperature for 30 min. Three times 2 cm^3 wash solution G (0.812 g NaCl, 0.027 g KCl, 2 g glucose, 1.836 g $CaCl_2 . 2 H_2O$ in 100 cm^3 of water, pH 7.0) were added at 5 min intervals and carefully mixed after each dilution step. After centrifugation at 18 *g* for 5 min protoplasts were washed in W_5 medium (Menczel *et al.* 1981), centrifuged 5 min at 14 *g*, resuspended in 3 cm^3 SW_{11} medium and incubated at 25 °C in the dark for 24 h. Thereafter the protoplasts were collected and lysed in 0.05 cm^3 of GUS buffer (Jefferson 1987). GUS activity as well as the protein content was measured in 0.01 cm^3 of the homogenate. GUS activity was assayed with the 4-methyl-umbelliferyl-β-D-glucuronide (MUG) fluorescent substrate according to Jefferson (1987) and proteins were determined according to Bradford (1976).

Plant transformation and selection: *Agrobacterium tumefaciens* strain LBA4404 harbouring the particular binary vector was used for transformation of both potato and tomato. Transformation of potato (*Solanum tuberosum* L. cv. Bintje) plants was carried out as described previously (Pavingerová *et al.* 2001) using internode explants. Regenerated shoots were selected on MS medium (Murashige and Skoog 1962) supplemented with 100 mg dm^{-3} of kanamycin.

For tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) transformation, a modified cotyledon leaves method was used (Fillatti *et al.* 1987). Seeds were sterilised by wetting with 70 % ethanol for 2 min followed by immersion in 10 % commercial bleach *Domestos* for 30 min. The seeds were washed 4 times with sterile distilled water, planted on 0.8 % agar MS medium supplemented with 1 % glucose and 0.1 g dm^{-3} myo-inositol and cultivated in a growth chamber with a 16-h photoperiod, irradiance of 90 μmol m^{-2} s^{-1} and 25 °C for about 18 d. Cotyledons were sidewise-cut in two pieces and these were incubated with *A. tumefaciens* suspension for 20 min with occasional gentle shaking. After drying off the excess liquid of the bacterial

suspension on a sterile filter paper, the cotyledon explants were placed upside down onto Petri dishes with agar MS medium supplemented with 30 g dm^{-3} sucrose, 0.02 mg dm^{-3} 2,4-dichlorphenoxyacetic acid (2,4-D) and 0.1 mg dm^{-3} kinetin. The Petri dishes were placed in the dark at 25 °C for 3 d. Subsequently, the explants were transferred upside up onto agar Murashige and Skoog (MS) basal salt medium containing Nitsch's vitamins, 100 mg dm^{-3} myo-inositol, 1.5 % sucrose, 0.5 mg dm^{-3} folic acid, 1 mg dm^{-3} zeatin riboside (ZR), 0.1 mg dm^{-3} indole-3-acetic acid (IAA), 200 mg dm^{-3} timentin and 100 mg dm^{-3} kanamycin. The dishes were placed in a growth chamber with a 16-h photoperiod at 25 °C and the medium was changed every 4 weeks. Regenerated shoots were separated from calli forming on the explants and cultured on rooting MS medium with 0.5 % sucrose, 80 mg dm^{-3} timentin and 50 mg dm^{-3} kanamycin.

Molecular analysis of transgenic plants: The samples for polymerase chain reaction (PCR) were prepared from leaves of kanamycin resistant plants essentially following the protocol described by Klimyuk *et al.* (1993). *E7* or *E7p* genes were detected using primers 5'-AAGCTTACAGTCTCAGAAGACCAAAG-3' and 5'-CCACGGTGATATCGTCCAC-3' which amplified the DNA fragments comprising a part of CaMV 35S promoter, *E7* or *E7p* genes and part of *gus* gene. The sizes of amplified fragments were 1250 bp for plasmid E7/GUS, 1550 bp for plasmid E7/E7/GUS and 1150 bp for plasmid E7p/GUS. The PCR cycling conditions were as follows: 94 °C for 45 s, 55 °C for 30 s and 72 °C for 3 min for a total of 35 cycles.

Genomic DNA for Southern blot analysis was extracted from leaves as described by Tai and Tanksley (1991). About 15 μg of DNA were digested with *Eco*RI restriction enzyme, resolved overnight in 1 % agarose gel with TBE buffer (Sambrook *et al.* 1989) and transferred onto a nylon *Hybond-N*$^+$ membrane. Southern hybridizations were performed according to Church and Gilbert (1984). The membranes were probed with 306 bp *gus* gene or 699 bp *nptII* gene derived probes labelled with [α-^{32}P]dCTP (1.11×10^8 MBq $mmol^{-1}$) using a random priming kit *Rediprime*^{TM} *II* and were autoradiographed for 5 h using the phosphoimager *Storm* system (*Amersham Biosciences*, Little Chalfont, UK).

Northern blot hybridization with formaldehyde as a denaturing agent (Sambrook *et al.* 1989) was used for mRNA detection. Total RNA was isolated from 100 mg of leave tissue of each transformant using the *RNeasy Plant Mini Kit* (*Qiagen*, Hilden, Germany), 25 μg of each RNA were after electroforesis in 1 % agarose gel containing MOPS gel running buffer with 1.2 M formaldehyde transferred onto a nylon *Hybond-N*$^+$ membrane and probed with 306 bp long *gus* gene DNA probe labelled with [α-^{32}P]dCTP (1.11×10^8 MBq $mmol^{-1}$) by random priming using the *Rediprime*^{TM} *II* kit. Hybridization buffer, temperature, membrane washing conditions and detection conditions were the same as for Southern hybridization.

For Western blotting leaf tissue (about 25 mg) was lysed in a buffer (0.075 cm^3) composed of 4 % sodium dodecyl sulphate (SDS), 20 % glycerol, 10 % mercapto-ethanol, 2 mM EDTA, 100 mM Tris-HCl (pH 8). Tissue homogenates were centrifuged at 4 °C and 13 000 *g* for 1 min and supernatants were transferred to fresh tubes and kept at -70 °C until use. Boiled lysates (3 min) were analysed by 10 % SDS-PAGE electro-phoresis. The proteins separated were electroblotted onto nitrocellulose membranes (*Amersham Biosciences*). Remaining binding sites were blocked in 10 % skim milk for 2 h at room temperature. Membranes were incubated with mouse monoclonal anti-E7 antibody (*Zymed*, San Francisco, USA) diluted 1:500. Incubation proceeded for 1 h at room temperature under constant agitation, and then overnight at 4 °C. Membranes were then washed with 0.1 % *Tween 20* in phosphate buffered saline (PBS) for 5 × 5 min and treated with peroxidase-labelled secondary anti-mouse antibody (*Amersham Biosciences*) for 1 h under agitation. Both primary and secondary antibodies were diluted with 10 % skim milk in PBS. The blots were again washed in 0.1 % Tween 20 in PBS for 7 × 5 min. Immunocomplexes were visualized using the *ECL plus* system (*Amersham Biosciences*).

Detection of GUS activity: The *gus* gene expression in transgenic plant tissues was quantified by fluorogenic assay (Jefferson 1987). Briefly, about 4 mg of leaves was homogenised in 0.075 cm^3 GUS extraction buffer (50 mM $NaPO_4$, pH 7, 10 mM mercaptoethanol (β-ME), 1 mM Na_2EDTA, 0.1 % Triton X-100), 0.0045 cm^3 of the extract was added to 0.075 cm^3 MUG (1 mM MUG in extraction buffer) and the samples were incubated at 37 °C. The reaction was stopped at different times with 0.2 M Na_2CO_3, 0.02 cm^3 of sample was added to 1.5 cm^3 0.2 M Na_2CO_3 and fluorescence was quantified using a *Hoefer* (San Francisco, USA) *DyNAQuant 200* fluoro-meter at 365 nm excitation wavelength and 445 nm emission wavelength. Protein concentration was measured according to Bradford (1976).

Results

Transient expression of fusion cassettes in potato protoplast: A modified *E7GGG* oncogene (Šmahel *et al.* 2001) showing higher immunogenicity but lower cancerogenicity comparing with the original *E7* gene was used for construction of three different fusion cassettes: complete *E7GGG*, tandem repeat of two copies of *E7GGG*, and the truncated *E7pGGG* with the first 41 codons, were fused with the *gus* gene. The original expression cassettes pCB1155, pCB1160 and pCB1133 were constructed in a pUC18 derived plasmid MonoGUS

(Bonneville *et al.* 1989) and fusions were made on N-terminus of GUS. Whole cassettes with CaMV 35S promoter, fusion gene and transcription terminator were then integrated into the plant binary vector pCB1399 (Matoušek *et al.* 1989) to yield binary vectors pCB3132, pCB3133 and pCB3134 (Fig. 1).

Table 1. Transient expression of *gus* gene in potato protoplast after 24 h cultivation in the dark at 25 °C. 50 μg of DNA was used for transfection. GUS activity [μmol(MU) g^{-1}(protein) min^{-1}] or in % of that in MonoGUS. Expression cassette symbols: ⇒ - CaMV 35S promoter; → - pmas1' promoter; MonoGUS - plasmid expressing high levels of GUS in plant cells (Bonneville *et al.* 1989) used as standard; transcription terminators are not shown. Other symbols are explained in the text.

DNA	Expression cassette	GUS activity	
MonoGUS	⇒GUS	281	100 %
pCB3132A	⇒E7/GUS←reverse orient.	88	31 %
pCB3132B	→⇒E7/GUS forward orient.	344	122 %
pCB3134A	⇒E7/E7/GUS←reverse orient.	46	16 %
pCB3134B	→⇒E7/E7/GUS forward orient.	121	43 %
pCB3133A	⇒E7p/GUS←reverse orient.	91	32 %
pCB3133B	→⇒E7p/GUS forward orient.	375	133 %

Expression potency of each plasmid construct was ascertained by measurement of transient expression in potato protoplasts. Very high activity of GUS was achieved with all structures tested (Table 1). E7/GUS fusions showed activities comparable with the original GUS even if the expression level somewhat dropped with more E7 codons added to the N-end of GUS. E7/GUS fusion proteins of appropriate size could be detected with E7 antibodies in protoplast lysates. On the other hand, unfused E7 protein is very unstable and could not be detected after transfection with various expression cassettes (not shown).

Plant binary vectors with reverse orientation of the cassette (A-vectors) produced somewhat less GUS than similar pUC cassettes (not shown) as they were 3 times larger and less plasmid molecules were applied during transformation. Nevertheless, binary vectors with forward orientation of the cassette (B-vectors) showed consistently higher activity compared to similar pUC cassettes and about four times higher GUS activity than A-vectors. Two promoters, active 35S and "silent" mas1', initially derived from the vector pAP2034 (Velten and Schell 1985) appeared in structures B in tandem but are separated by transcription terminator. Still, the pmas1' promoter very probably supports the recruitment of transcription factors by p35S. Only B-vectors were used for plant transformation.

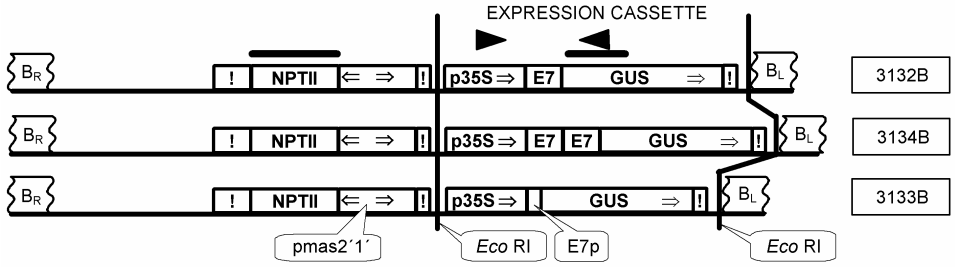

Fig. 1. T-DNAs of binary vectors harbouring E7/GUS (3132B), E7/E7/GUS (3134B) and E7p/GUS (3133B) fusion genes. Only B-vectors with forward orientation of expression cassette are shown, reverse orientation vectors that have expression cassette inverted were also prepared. *Arrowheads* represent primers used for the PCR analyses, *black lines* show positions of Southern probes. p35S - CaMV promoter; *nptII* - neomycinphoshotransferase II gene; E7 - E7 oncogene; E7p - truncated E7 oncogene; pmas2'1' - T-DNA 2'mannopine gene promoter; GUS - β-glucuronidase gene; B_R and B_L - right and left borders of T-DNA; ! - terminators (from CaMV for fusion genes and from T-DNA 2' mannopine gene for *nptII*).

Production and analysis of transgenic potato and tomato plants: Binary vectors were transformed into *Agrobacterium tumefaciens* strain LBA4404 and successfully transferred into tomato and potato plants. Regenerated plants rooting on kanamycin containing medium were analysed by PCR and all of them carried appropriate fusion transgenes (Fig. 2). Activity of β-glucuronidase was revealed in almost all regenerants obtained (Table 2); nevertheless, the level of *gus* expression of different constructs varied much more than in transient expression experiments with potato protoplasts. The cassette with the highest expression in both plant species harboured the fusion *E7p/gus*. The expression levels in plants transformed with other vectors were 1 - 3 orders of magnitude lower. The contents of mRNA estimated by Northern hybridisation showed the same effect, at least in tomato (Fig. 4). Southern hybridisation of 18 tomato and 23 potato transformants showed many multiple tandem integrations of T-DNA into the plant genome (Fig. 3). At the same time, many transformants contained incomplete fusion genes (Fig. 3). Fusion proteins of appropriate size were detected by Western hybridisation analyses in 9 tomato and 20 potato plants out of 39 tested individuals (Fig. 5). Plants with the highest fusion protein content were transferred into a greenhouse and vegetatively propagated.

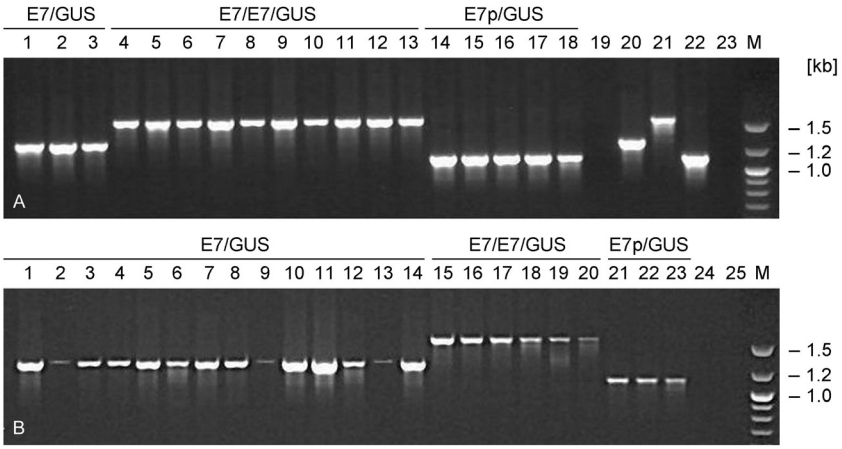

Fig. 2. PCR detection of T-DNAs carrying fusion cassettes E7/GUS, E7/E7/GUS or E7p/GUS in rooted regenerants of tomato (*A*) and potato (*B*). Expected fragment size was about 1150 bp for E7p/GUS, 1250 bp for E7/GUS and 1550 bp for E7/E7/GUS fusion cassettes. *A: lanes 1 - 3* - transformed tomato plants with cassette E7/GUS; *lanes 4 - 13* - plants harbouring E7/E7/GUS; *lanes 14 - 18* - plants with E7p/GUS; *lane 19* - nontransformed tomato; *lane 20 - A. tumefaciens* with binary vector 3132B (E7/GUS); *lane 21 - A. tumefaciens* with binary vector 3134B (E7/E7/GUS); l*ane 22 - A. tumefaciens* with binary vector 3133B (E7p/GUS); *lane 23* - negative control; *lane M* - DNA size marker (100 bp ladder, NEB). *B: lanes 1 - 14* - transformed potato plants with cassette E7/GUS; *lanes 15 - 20* - plants with E7/E7/GUS; *lanes 21 - 23* - plants with E7p/GUS; *lane 24* - nontransformed potato; *lane 25* - negative control; *M* - DNA size marker (100 bp ladder, NEB).

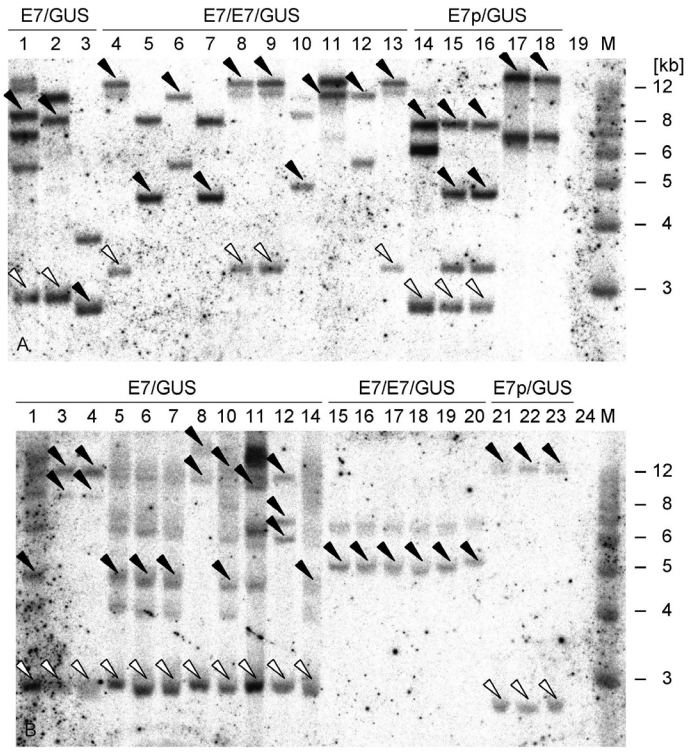

Fig. 3. Southern hybridisation analysis of transformed tomato (*A*) and potato (*B*) plants carrying fusion cassettes E7/GUS, E7/E7/GUS or E7p/GUS. Expected band size was about 3 kb for E7/GUS, 3.3 kb for E7/E7/GUS and 2.8 kb for E7p/GUS when the *gus* gene derived probe was used. For the *nptII* probe, size of bands should be more than 4.5 kb. Firstly, *gus* gene derived probe was used - bands with appropriate size are indicated by *white arrowheads* whereas bands showing different sizes are not marked. Consecutively, the *nptII* gene derived probe was applied onto membrane without previous *gus* probe stripping and all newly appearing bands were marked by black arrowheads. *A: lanes 1 - 3* - transformed tomato plants with cassette E7/GUS; *lanes 4 - 13* - plants harbouring E7/E7/GUS; *lanes 14 - 18* - plants with E7p/GUS; *lane 19* - nontransformed tomato; *lane M* - DNA size marker (1 kb ladder, *Gibco-BRL*). *B: lanes 1 - 14* - transformed potato plants with cassette E7/GUS; *lanes 15 - 20* - plants with E7/E7/GUS; *lanes 21 - 23* - plants with E7p/GUS; *lane 24* - nontransformed potato; *lane M* - DNA size marker (1 kb ladder).

Table 2. Fluorogenic assay of the *gus* gene expression in transgenic plant tissues. The plant codes starting with T denote tomato, those starting with P denote potato. Tomato plants No. 1 - 3 and potato plants No. 1 - 14 harbour fusion cassette E7/GUS, tomato plants No. 4 - 13 and potato plants No. 15 - 20 carry cassette E7/E7/GUS while tomato plants No. 14 - 18 and potato plants No. 21 -23 bear E7p/GUS. [a] nontransformed tomato (cv. Moneymaker) and/or potato (cv. Bintje); [b] estimated on the basis of the finding that 5 ng of pure β-glucuronidase show activity of 1 nmol(MU) min^{-1} (Jefferson 1987).

Fusion cassette	Plant number	Plant code	GUS activity [μmol(MU) g^{-1} (protein) min^{-1}]	Fusion protein content [% of TSP][b]	E7 or E7p protein content [% of TSP]
E7/GUS	1	T 54	0.42	0.0002	0.0000
	2	T 99	18.50	0.0107	0.0015
	3	T 100	0.11	0.0001	0.0000
E7/E7GUS	4	T 03	0.28	0.0002	0.0000
	5	T 16	0.10	0.0001	0.0000
	6	T 38	0.77	0.0005	0.0001
	7	T 43	0.12	0.0001	0.0000
	8	T 47	0.48	0.0003	0.0001
	9	T 48	0.94	0.0006	0.0002
	10	T 65	2.55	0.0017	0.0004
	11	T 74	0.19	0.0001	0.0000
	12	T 96	2.54	0.0017	0.0004
	13	T 98	0.89	0.0006	0.0001
E7p/GUS	14	T 05	541.79	0.2905	0.0195
	15	T 20	1446.62	0.7751	0.0521
	16	T 34	404.13	0.2165	0.0145
	17	T 92	0.58	0.0003	0.0000
	18	T 93	0.20	0.0001	0.0000
	19	MM[a]	0.10	0.0000	0.0000
E7/GUS	1	P 81/03	20.51	0.0119	0.0016
	2	P 81/10	48.03	0.0279	0.0038
	3	P 81/12	23.74	0.0138	0.0019
	4	P 81/13	33.67	0.0195	0.0027
	5	P 81/16	33.30	0.0193	0.0027
	6	P 81/18	36.89	0.0214	0.0030
	7	P 81/23	16.14	0.0095	0.0013
	8	P 81/41	49.02	0.0284	0.0039
	9	P 81/47	22.94	0.0133	0.0018
	10	P 81/49	23.05	0.0134	0.0018
	11	P 81/55	53.84	0.0312	0.0043
	12	P 81/56	74.61	0.0433	0.0060
	13	P 81/57	140.75	0.0816	0.0113
	14	P 81/62	21.23	0.0123	0.0017
E7/E7GUS	15	P 82/05	25.94	0.0173	0.0043
	16	P 82/07	3.28	0.0022	0.0005
	17	P 82/23	8.99	0.0060	0.0015
	18	P 82/26	16.93	0.0113	0.0028
	19	P 82/28	5.27	0.0035	0.0009
	20	P 82/30	6.72	0.0045	0.0011
E7p/GUS	21	P 83/04	288.74	0.1548	0.0104
	22	P 83/08	375.17	0.2011	0.0135
	23	P 83/20	204.24	0.1095	0.0074
	24	Bi[a]	0.11	0.0000	0.0000

Discussion

Our previous paper showed that some fusions of human papillomavirus gene for protein E7, modified for low oncogenicity, with the *gus* gene result in highly enhanced stability of E7 in human cells and immunogenicity as well (Šmahel *et al.* 2004). Expression in plant protoplasts was studied here with the result that *E7/gus* fusions are strongly expressed from plant expression cassettes to steady-state levels reaching up to 0.5 % of the cell protein. This makes them promising candidates for plant-derived vaccines against human papillomavirus.

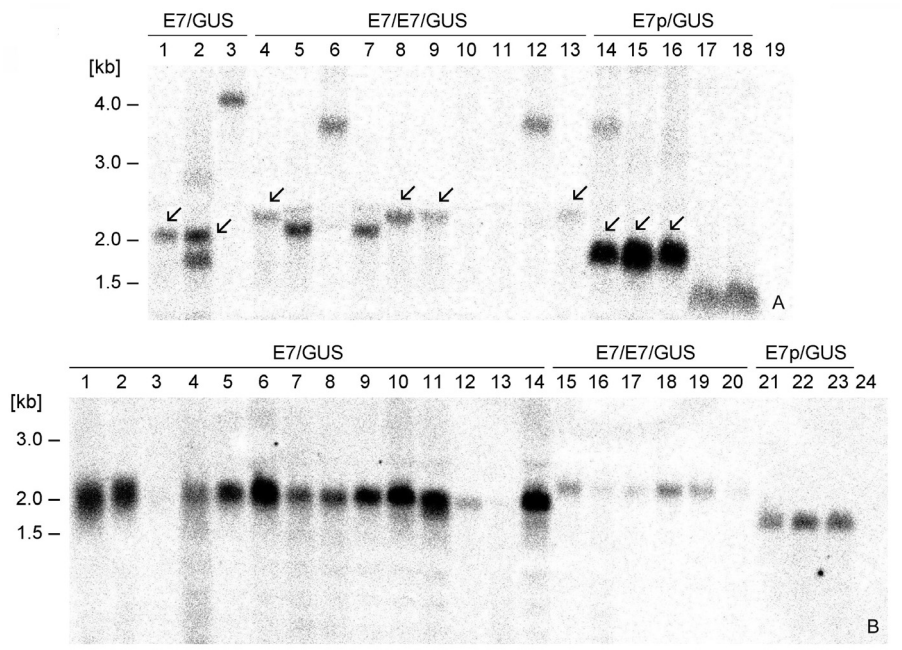

Fig. 4. Northern hybridisation analysis of transformed tomato (*A*) and potato (*B*) plants carrying fusion cassettes E7/GUS, E7/E7/GUS or E7p/GUS. Expected size of mRNAs was about 1920 bases for E7p/GUS, 2100 bases for E7/GUS and 2400 bases for E7/E7/GUS. *A: lanes 1 - 3* - transformed tomato plants with cassette E7/GUS; *lanes 4 - 13* - plants harbouring E7/E7/GUS; *lanes 14 -18* - plants with E7p/GUS; *lane 19* - nontransformed tomato. The *arrows* indicate appropriate size bands. *B: lanes 1 - 14* - transformed potato plants with cassette E7/GUS; *lanes 15 - 20* - plants with E7/E7/GUS; *lanes 21 - 23* - plants with E7p/GUS; *lane 24* - nontransformed potato. All lanes show corresponding band sizes.

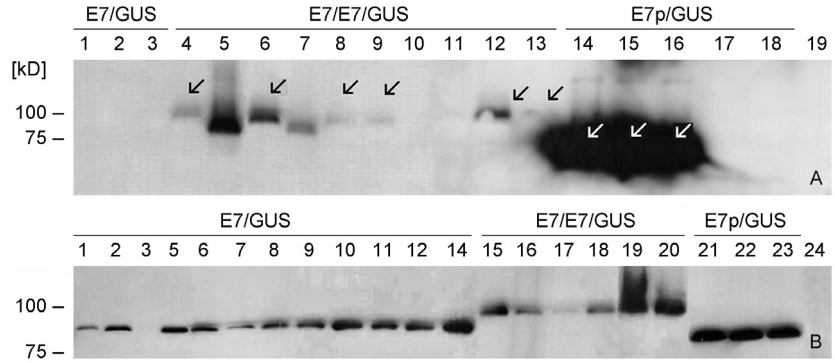

Fig. 5. Western blotting analysis of transformed tomato (*A*) and potato (*B*) plants carrying fusion cassettes E7/GUS, E7/E7/GUS or E7p/GUS. *A: lanes 1 - 3* - transformed tomato plants with cassette E7/GUS; *lanes 4 - 13* - plants harbouring E7/E7/GUS; *lanes 14 - 18* - plants with E7p/GUS; *lane 19* - nontransformed tomato. The *arrows* show bands with appropriate sizes. *B: lanes 1 - 14* - transformed potato plants with cassette E7/GUS; *lanes 15 - 20* - plants with E7/E7/GUS; *lanes 21 - 23* - plants with E7p/GUS; *lane 24* - nontransformed potato. All lanes show corresponding band sizes.

Agrobacterium-mediated transformation of tomato and potato with three different E7/GUS constructs yielded after much effort only 11 independent tomato and 5 potato transformants. After probing with the *gus*-gene probe and right-part T-DNA *nptII* probe, the Southern hybridization analysis indicates in potato one single insertion of E7p/GUS and one transformant with E7/E7/GUS structure, probably deleted on the GUS transcription stop side, but still producing small amount of mRNA and protein. E7/GUS transformed potatoes were produced probably by 3 different integration reactions and represent single, double and triple insertions (Fig. 3). Only the single insertion produces larger amounts of GUS mRNA and protein (Figs. 4, 5) but its structure inferred from Southern blot is unclear.

In tomato, one right-border *nptII* fragment in all but one tomato plant indicates single insertion. On the other hand, probing with *gus*-probe reveals very complicated patterns. Only 5 out of 11 plants have a characteristic *gus* band of about 3kb and only these plants show GUS activity. Nevertheless, all of them have another, unexpected *gus* band of variable size but always larger;

other 6 plants have only large *gus* bands.

Some of our one-large-*gus*-band plants show with *gus*-probe mRNA smaller than GUS mRNA, and sometimes even smaller, inactive, but immunogenic protein as well. This proves incomplete transfer close to the left border of the T-DNA resulting in deletion of the part of *gus* ORF. In our constructs with the *gus* gene close on the left part of T-DNA and the adjacent selectable *nptII* gene to the right, left border deletions extending to the T-DNA can be expected according to the literature (Peerbolte *et al.* 1986, Spielmann and Simpson 1986). Nevertheless, surprisingly, all regenerated tomato plants have such left border deletion. Right border *Eco*RI fragment is in all but one plant larger than 5 kbp which indicates its relative intactness. According to some authors (Deroles and Gardner 1988), right-border deletions should be as common as the left-border deletions.

In 4 plants with two *gus* fragments and one *nptII* fragment, tandem insertion of two copies head to head with partial *gus* deletion in one of the copies is assumed. It is again very unusual that such complicated structure arose in nearly one half of all independent transformants. We infer that the full length E7/GUS fusions are toxic to the plant cell and single T-DNA insertions, providing usually highest expression of the transgene (Hobbs *et al.* 1990), are contra-selected during regeneration of the transformants. Plants with a single transgene, expressing high levels of transgenic proteins and potentially promising for immunization, were always of the E7p/GUS type, expressing first 41 codons of *E7* fused with *gus*. Unfortunately, the most important antigenic determinants of E7 are not present on this N-terminal fragment.

Relative instability of the full length E7/GUS protein compared to E7p/GUS may represent another mechanism of plant cell protection. In potato plants, similar levels of E7/GUS and E7p/GUS mRNA lead to much lower steady-state level of E7/GUS protein compared to E7p/GUS. Relative E7/GUS protein instability was revealed in human cells as well (Šmahel *et al.* 2004).

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343rd volume of scientific and medical publishers Humana Press series Methods in Molecular Biology opens after more than 10 years a field concerning *Agrobacterium*-mediated transformation of plants. When published in 1995 the first edition of *Agrobacterium* Protocols could bring information about a modest amount of plant species successfully transformed using *A. tumefaciens*. Over against today, *Agrobacterium* is routinely used for transgenosis of plenty plant species, even monocotyledon species that seemed to be recalcitrant for transformation by this bacteria in the beginning of transgenosis era.

Volume 1 consists of 39 chapters divided into 6 parts. First part (named *Agrobacterium* Handling) deals in 6 chapters with basic *Agrobacterium* manipulation techniques as well as with strategies for construction of transformation vectors. Seven chapters of second part (Model Plants) bring description of transgenosis of three model plant species, *Arabidopsis thaliana, Medicago truncatula* and *Nicotiana tabacum*. Of course, plenty laboratories all over the world are able to transform these species but protocols from leading experts may enhance their skills. Remaining parts of the volume report protocols for transgenosis of 25 plant species: the readers may find members of cereal crops (barley, maize, rice, rye, sorghum, wheat), industrial plants (canola, cotton, mustard, sunflower), legume plants (alfalfa, chickpea, clover, peas, peanut, pigeonpea, soybean, tepary bean), and vegetable plants (*Brassica oleracea*, cucumber, eggplant, lettuce, tomato).

Each chapter is written by the leader in the field in the same format characteristic for the Methods in Molecular Biology series and offers a detailed manual of the transformation protocol. The heart of each chapter is the method section with step-by-step procedure description starting from the preparation of initial material and ending with the harvest of transgenic plants. All users of this book will certainly appreciate the notes sections that bring additional information on potential difficulties in the protocols and alternative materials or methods.

The book is the fountainhead of recent practical knowledge concerning *Agrobacterium*-mediated transformation and therefore, we may suppose it will become the core manual in all plant genetic and breeding laboratories dealing with transgenosis.

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