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Use of phosphomannose isomerase-based selection system for *Agrobacterium*-mediated transformation of tomato and potato

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Abstract

Two selection systems for *Agrobacterium tumefaciens* mediated transformation of tomato and potato were compared. In the tomato (*Lycopersicon esculentum* cv. Moneymaker), the highest transformation rate, 4.2 %, of cotyledon explants on mannose-selection medium was obtained when mannose/sucrose concentration in the regeneration medium was 5/15 g dm^{-3}. The best transformation efficacy with the commonly used concentration of 100 mg dm^{-3} kanamycin as a selection agent was 9 %. In the potato (*Solanum tuberosum* cv. Bintje), the highest transformation frequency was 53.3 % when mannose concentration in the regeneration medium was 5 g dm^{-3} during the first 3 weeks after transformation and 10 g dm^{-3} afterwards. The optimum concentration of sucrose was 20 g dm^{-3}. The transformation efficiency using kanamycin as a selection agent at a concentration 100 mg dm^{-3} was 33.3 % with potato. Our results demonstrate that the transformation efficiency using mannose selection is 1.6-fold higher for potato and about 2 times lower for tomato comparing with the ordinary protocol using kanamycin.

Additional key words: *Lycopersicon esculentum*, *npt II*, *pmi*, *Solanum tuberosum*, transformation efficiency.

Introduction

Transformation represents a key tool for both basic and applied plant research from the date when Fraley *et al*. (1983) reported their first successful stabile transformation of a plant nucleus. For successful plant transformation the development of a reliable system for transgenic tissue selection is highly important. The traditional and frequently used plant selectable marker genes include the *nptII* gene granting resistance to antibiotics like kanamycin, neomycin and G-418, the *hph* gene conferring antibiotic hygromycin resistance, and the *bar* gene rendering resistance to the herbicides containing phosphinothricin as an active compound. Unfortunately, antibiotic resistance markers are not appropriate for all plant species, for example for monocots (Wilmink and Dons 1993), and they are not favourably accepted by the public despite the fact that the safety of these markers has been thoroughly tested and proven over several years. Similarly, the use of markers based on herbicide resistance is currently a major public concern. In addition, the selective agent may adversely affects the transformed plant cells bringing about a decrease in the regeneration of transformed cells by the accumulation of toxic compounds from killed nontransformed cells (Hansen and Wright 1999).

To date, a number of marker genes have been employed for the development of alternative selection methods. Such selection systems were developed by Joersbo and Okkels (1996) with benzyladenine-N-3-glucuronide, Haldrup *et al*. (1998) with D-xylose, Kunze *et al*. (2001) with 2-deoxyglucose, Erikson *et al*. (2004) with D-amino acids, Erikson *et al*. (2005) with D-serine, You *et al*. (2003) with a ferredoxin-like protein gene, or Ebmeier *et al*. (2004) with the *E. coli* threonine deaminase gene as selectable markers. The phosphomannose isomerase (*pmi*) gene was originally used as a selectable marker by Joersbo *et al*. (1998) for the

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Abbreviations: MS - Murashige and Skoog (1962) medium; NPT II - neomycin phosphotransferase II; PMI - phosphomannose isomerase; RM - regeneration medium; SE - selection efficiency; TE - transformation efficiency.
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transformation of sugar beet. In the following years, PMI was shown to be an useful marker in the *Agrobacterium* transformation of a number of plant species such as cassava (Zhang *et al*. 2000), maize (Negrotto *et al*. 2000, Wright *et al*. 2001, Reed *et al*. 2001), *Arabidopsis* (Todd and Tague 2001), wheat (Wright *et al*. 2001, Reed *et al*. 2001, Gadaleta *et al*. 2006), rice (Lucca *et al*. 2001), sweet orange (Boscariol *et al*. 2003), hemp (Feeney and Punja 2003), pearl millet (O'Kennedy *et al*. 2004), bentgrass (Fu *et al*. 2005), papaya (Zhu *et al*. 2005), sorghum (Gao *et al*. 2005), almond (Ramesh *et al*. 2006), onion (Aswath *et al*. 2006), cucumber (He *et al*. 2006) and Chinese cabbage (Ku *et al*. 2006). In tomato, Sigareva *et al*. (2004) employed the mannose selection procedure for the transformation of cotyledon petiole, hypocotyls and leaf explants of three cultivars.

The cells of the majority of plant species take up mannose and convert it by endogenous hexokinase to mannose-6-phosphate. This inhibits glycolysis, depletes the cell of inorganic phosphate and induces the endonucleases to degrade DNA (Stein and Hansen 1999). PMI catalyses the reversible isomerization of mannose-6-phosphate to fructose-6-phosphate which is an intermediate of glycolysis and positively influences the growth of transformed cells. In the absence of PMI the mannose-6-phosphate accumulates and the cells starve and cease growing.

The PMI is common in bacteria, yeast and mammals (including humans) but in plants, there are only a few species like soybeans and several other legumes where the enzyme has been found (Lee and Matheson 1984). Therefore, the selection system employing the *E. coli manA* gene (Miles and Guest 1984) coding for PMI is highly versatile. In addition, the end product of the selection is harmless; the safety assessment for PMI (Reed *et al*. 2001) revealed that purified PMI protein has no adverse effects in a mouse toxicity test and does not change glycoprotein profiles in PMI-transformed plants.

This report brings the results of comparison between the selection systems based on mannose and kanamycin in tomato cv. Moneymaker and potato cv. Bintje.

Materials and methods

Plasmid construction and bacterial strain: The binary vector pCB3160 used for plant transformation was constructed by inserting the *nptII* coding sequence from plasmid pGA472 (An *et al*. 1986) into a polylinker sequence near the right border of pNOV2819 vector obtained from *Syngenta Seeds AG*, Basel, Switzerland (Fig. 1). The *nptII* gene is driven by the nopaline synthase promoter and the *pmi* gene by the cestrum yellow leaf curling virus promoter, short version (CMPS), and both are followed by the nopaline synthase terminator (tNOS). The *Agrobacterium tumefaciens* strain LBA4404 was employed in these experiments and the pCB3160 was transfected into bacterial cells using the freeze-thaw method of Holsters *et al*. (1978). Spectinomycin resistance gene of pNOV2819 was used for bacterial selection. The bacterial culture (10 cm^3) in LK medium (Langley and Kado 1972) with spectinomycin (50 mg dm^{-3}) was centrifuged and the bacterial cells were resuspended in 9 cm^3 of inoculation medium (10 mM $MgSO_4$).

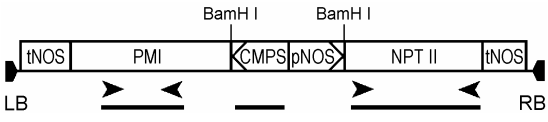

Fig. 1. Structure of the T-DNA of pCB3160. CMPS - cestrum yellow leaf curling virus promoter, short version; PMI - phosphomannose isomerase coding sequence; tNOS - nopaline synthase terminator; NPT II - neomycin phosphotransferase II coding sequence; pNOS - nopaline synthase promoter; RB - right border; LB - left border. The arrowheads represent primers used for the PCR analyses, the black lines show the positions of Southern or Northern probes.

Plants and transformation: Seedlings from tomato (*Lycopersicon esculentum* Mill., cv. Moneymaker) were used for transformation using the modified cotyledon leaves method (Fillatti *et al*. 1987) as described previously (Bříza *et al*. 2007). During selection, the explants were placed bottom down on microagar (0.6 %; m/v) regeneration medium (RM) containing Murashige and Skoog's (MS) basal salts, Nitsch's vitamins, 100 mg dm^{-3} myo-inositol, 15 g dm^{-3} 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 (or 400, see Table 2) mg dm^{-3} *Timentin* and mannose (10, 7.5, 5, 2.5, 1.25 or 0.625 g dm^{-3}) or 100 mg dm^{-3} kanamycin. The dishes were placed in a growth chamber with 16-h photoperiod (irradiance of 90 μmol m^{-2} s^{-1}) and temperature of 25 °C and the medium was changed every 4 weeks. Regenerated shoots were cut off from calli and cultured on a rooting MS medium with 5 g dm^{-3} sucrose, 80 (or 160, see Table 2) mg dm^{-3} *Timentin*, 2 g dm^{-3} *Gerlite* (*Duchefa,* Haarlem, The Netherlands) and 50 mg dm^{-3} kanamycin or mannose at the same concentration as in the RM media.

The potato (*Solanum tuberosum* L. cv. Bintje) plants were propagated on the MS medium including vitamins (0.5 mg dm^{-3} nicotinic acid, 0.5 mg dm^{-3} pyridoxine, 0.1 mg dm^{-3} thiamine, 100 mg dm^{-3} myo-inositol, 2.0 mg dm^{-3} glycine) and 20 g dm^{-3} sucrose. The internodes were sub-cultured on the MS medium including vitamins with 1 mg dm^{-3} zeatin, 0.02 mg dm^{-3} α-naphtaleneacetic acid (NAA), 0.02 mg dm^{-3} gibberelic acid (GA_3) and 20 g dm^{-3} sucrose. The internodes were used for transformation via *Agrobacterium tumefaciens* as described previously (Pavingerová *et al*. 2001). For

mannose selection, the explants were transferred to the selection MS medium with D-mannose immediately after their co-cultivation with *Agrobacterium*. The mannose concentration was increased in a stepwise manner during selection, starting in first experiment at 1.0 g dm^{-3} for 1 or 2 subculture periods, then increased to 5.0 g dm^{-3} for the next 1 - 3 subculture periods and finally to 10.0 g dm^{-3} for the last period. The second experiment was also started at 1.0 g dm^{-3} but then the mannose concentration was increased to 2.5 g dm^{-3} for 1 or 2 subcultures, to 5.0 g dm^{-3} for 1 subculture and finally to 10.0 g dm^{-3} for the last period. The third experiment was started at 2.5 g dm^{-3} mannose for 1 or 2 subcultures, then increased to 5.0 g dm^{-3} for the next 1 - 3 subcultures and finally to 10.0 g dm^{-3}. The fourth experiment was started at 5.0 g dm^{-3} mannose for 1 - 4 subcultures followed by a concentration of 10.0 g dm^{-3} for the last period. The fifth experiment was started at 3.5 g dm^{-3} mannose for the 2 subcultures followed by subculture at 10.0 g dm^{-3} for the last period. Each subculture period lasted 3 weeks. Regenerated shoots were rooted on MS medium containing 10.0 g dm^{-3} mannose or 100 mg dm^{-3} kanamycin (after selection on MS medium with 100 mg dm^{-3} kanamycin).

Effect of mannose on shoot formation and rooting of explants: To estimate the optimal combination of mannose/sucrose concentration in selection media, the mannose inhibition of shoot formation and the rooting of explants were evaluated. To evaluate the inhibition of shoot formation, non-transformed tomato cotyledon leaves were plated on *Gelrite* (0.225 % m/v, *Duchefa*) R media containing MS basal salts, Nitsch's vitamins, 100 mg dm^{-3} myo-inositol, 0.5 mg dm^{-3} folic acid, 2 mg dm^{-3} zeatin riboside (ZR), and different concentrations of mannose and sucrose (Table 1). Nine Petri dishes, each with 10 explants, were used for every mannose/sucrose combination. The dishes were cultured under a 16-h photoperiod and 25 °C for 4 weeks and the number of explants with shoots was determined. The rooting inhibition of tomato was evaluated after 4 weeks cultivation of non-transformed growth apexes on R media lacking folic acid and ZR.

To evaluate the sensitivity of the potato internodes to mannose, the MS medium containing 0, 20 or 50 g dm^{-3} sucrose was supplemented with different concentrations of mannose (0, 5, 10 20 g dm^{-3}). The explants were cultured under 16-h photoperiod and 25 °C for 4 weeks and thereafter, the numbers of internodes with regenerated shoots were determined.

Molecular analysis of transgenic plants: PCR assays were done as reported by Klimyuk *et al*. (1993) using leaves from mannose or kanamycin resistant plants. A fragment of the *nptII* gene was amplified using primers NPT-1 (5'-ACGCAGGTTCTCCGGCCGCTTG-3') and NPT-2 (5'-GAAGCGGTCAGCCCATTCGCCG-3') resulting in a product size of 699 bp, whereas the presence of the *pmi* gene was confirmed by the amplification of the 514 bp fragment with the primers PMI-1 (5'-ACAGCCACTCTCCATTCA-3') and PMI-2 (5'-GTTTGCCATCACTTCCAG-3') designed by *Syngenta*. In tomato, both PCR for *nptII* and for *pmi* were performed as duplex reactions with primers amplifying a 141 bp fragment of tomato chromosome 11 (primers 2995AL 5'-CGAGAGAGATTCAAGAATAGACCC-3' and 2995AR 5'-TATAACCAAATGCAACTC CGTCTT-3', Carroll *et al*. 1995). Amplification of this fragment in duplex reactions acted as a positive control of PCR implementation in the tube. 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 *Bam*HI restriction enzyme, electrophoresed overnight in 1 % agarose gel with tris-borate-EDTA (TBE) buffer (Sambrook *et al*. 1989) and transferred onto nylon *Hybond-N*$^+$ membrane. Southern hybridisations were performed according to Church and Gilbert (1984). The membranes were probed with the 514 bp fragment of the *pmi* gene, the 699 bp of the *nptII* gene or the 395 bp of CMPS promoter derived probes labelled with [α-^{32}P] dCTP (1.11×10^8 MBq $mmol^{-1}$) using a random priming kit, Rediprime™ II, and were autoradiographed for 5 h using a phosphoimager *Typhoon* system (*Amersham Biosciences*, Little Chalfont, UK).

To perform Northern blot hybridisation, the total RNA was isolated from 100 mg of leaf tissue of selected transformants using a *RNeasy Plant Mini Kit* (*Qiagen*, Hilden, Germany). 25 μg of each RNA were electrophoresed in a 1 % agarose gel in MOPS running buffer with 1.2 M formaldehyde, transferred onto nylon *Hybond-N*$^+$ membrane and probed with 514 bp long *pmi* gene DNA probe labelled with [α-^{32}P] dCTP (1.11×10^8 MBq $mmol^{-1}$) using a random priming kit *Rediprime*™ II from *Amersham Biosciences*. The hybridisation buffer and temperature as well as conditions during membrane washing and detection were the same as for the Southern hybridisation.

Results and discussion

Effect of mannose on shoot formation and rooting of explants: In tomato, 8 different combinations of sucrose/mannose in shoot inducing medium were used to evaluate the effect of mannose on the organogenesis of cotyledon explants (Table 1). After 4 weeks cultivation we found that 84 % of the explants produced shoots on medium R2 (5 g dm^{-3} mannose) comparing with 93 % on medium without mannose. The inhibition of organogenesis on medium containing 10 g dm^{-3} mannose was complete regardless of the sucrose content. Similar

Table 1. Effects of mannose and sucrose on callus and shoot induction on tomato cotyledon explants (R1 - R8) and potato internode explants (P1 - P10) after 4 weeks of cultivation.

Medium	Mannose [g dm^{-3}]	Sucrose [g dm^{-3}]	Number of explants	Explants with calli number	[%]	Explants with shoots number	[%]
R1	0	20	90	86	96	84	93
R2	5	15	90	86	96	76	84
R3	10	10	90	10	11	0	0
R4	10	5	90	8	9	0	0
R5	10	0	90	3	3	0	0
R6	15	5	90	1	1	0	0
R7	15	0	80	0	0	0	0
R8	20	5	75	0	0	0	0
P1	0	20	20	20	100	20	100
P2	5	20	20	8	40	7	35
P3	10	20	20	0	0	0	0
P4	0	50	20	19	95	19	95
P5	5	50	20	6	30	5	25
P6	10	50	20	0	0	0	0
P7	20	50	20	0	0	0	0
P8	5	0	20	3	15	2	10
P9	10	0	20	0	0	0	0
P10	20	0	20	0	0	0	0

Table 2. Transformation efficiency of tomato cotyledon explants using mannose or kanamycin selection. *One asterisk* denotes media with high *Timentin* concentration (400 mg dm^{-3}), *two asterisks* media with 100 mg dm^{-3} kanamycin instead of mannose.

Medium	Mannose [g dm^{-3}]	Number of explants	Explants with shoots number	[%]	Number of shoots	Number of rooting shoots	PCR positive plants	Independent transform.	TE [%]
RM1	10.0	260	2	1	11	0	0	0	0
Control	10.0	40	0	0	0	0	-	-	-
RM2	7.5	260	8	3	27	6	5	1	0.4
Control	7.5	40	0	0	0	0	-	-	-
RM2a	7.5*	182	26	14	50	1	0	0	0
Control	7.5*	60	0	0	0	0	-	-	-
RM3	5.0	100	2	2	2	0	0	0	0
Control	5.0	50	3	6	4	0	-	-	-
RM3a	5.0*	120	68	57	112	6	5	5	4.2
Control	5.0*	60	9	15	12	0	-	-	-
RM4	2.5	220	74	34	109	6	0	0	0
Control	2.5	50	6	12	10	1	-	-	-
RM5	1.25	224	88	39	163	8	6	4	1.8
Control	1.25	50	23	46	38	19	-	-	-
RM6	0.62	100	14	14	18	0	0	0	0
Control	0.62	50	18	36	31	11	-	-	-
RM-Kn	100.0**	200	27	14	47	27	26	18	9.0
Control	100.0**	20	0	0	0	0	-	-	-

results in tomato were described by Sigareva *et al*. (2004) when the addition of sucrose to the selection medium modulated the inhibitory effect of mannose on shoot formation. In our study, the protective effect of sucrose was also appreciable when explants producing calli were viewed. In this case, we demonstrated sucrose concentration dependency – the lower the sucrose concentration the lower the ratio of cotyledons with

induced calli. An analogous outcome was observed by Joersbo *et al*. (1999) when they tested the phytotoxic effect of mannose in the presence of four different non-toxic saccharides on the dry mass of sugar beet cotyledonary explants after 3 weeks of growth – the higher the sucrose concentration the higher the alleviation of mannose toxicity. The inhibition of callogenesis was almost absolute on media with 15 or 20 g dm^{-3} mannose combined with low sucrose content, we found only one explant with callus out of 245 cotyledon leaves (Table 1). On the other hand, no significant effect of sucrose on mannose toxicity was observed on potato explants – the medium containing the mannose/sucrose content of 5/20 g dm^{-3} revealed even lower callus and shoot growth inhibition than the medium with 5/50 g dm^{-3} (Table 1). Complete inhibition of callus production and shoot regeneration from internodes was shown for a mannose concentration of 10 g dm^{-3} regardless of sucrose content.

Tomato transformation: Based on a previous evaluation of toxic effects of mannose, 10 or 7.5 g dm^{-3} mannose (combined with 15 g dm^{-3} sucrose in all experiments) were used for the first transformation experiment. Since only one transgenic (*i.e.* PCR positive) plant was derived (Table 2) lower mannose concentrations were used in the following experiments. Besides, a higher *Timentin* concentration was applied (400 mg dm^{-3}) in two experiments in media with high mannose (5 and 7.5 g dm^{-3}) because 200 mg dm^{-3} *Timentin* was not able to entirely suppress *Agrobacterium* growth during selection.

Transformation efficiency (expressed as the ratio of transgenic plants to total number of plated explants) higher than 1 % was only observed on medium with 5 or 1.25 g dm^{-3} mannose (Table 2). In the experiment with medium RM2, a total of 8 (3 %) explants formed 27 shoots and out of them 6 were able to root and normally grow on the medium with the same mannose concentration. A PCR assay confirmed that 5 of them harboured the *pmi* gene. All were derived from one explant and a Southern hybridisation of the DNA of 3 surviving plants using a *pmi* gene derived probe proved their clonal origin (Fig. 3*A*, *lanes* 22 - 24). Therefore, we concluded that only one transgenic event occured in this experiment. A transformation efficiency of 1.8 % was reached on RM5 medium with 1.25 g dm^{-3} mannose. In this case, 88 (39 %) out of 224 explants were able to regenerate 163 shoots in total but only 8 shoots rooted on the rooting medium with 1.25 g dm^{-3} mannose followed by medium containing 5 g dm^{-3} mannose. A PCR assay (Fig. 2) proved the presence of the *pmi* transgene in 6 plants and Southern hybridisation revealed 4 independent transformation events (Fig. 3*A*,*B*, *lanes* 31 - 36). Control explants (*i.e*. without *Agrobacterium* co-cultivation) showed the same level of organogenesis as the explants culture after *Agrobacterium* transformation, the shoots partially rooted on 1.25 g dm^{-3} mannose but they did not root on a medium with 5 g dm^{-3} mannose.

The highest frequency of transformation (4.2 %) was achieved using RM3a medium with 5 g dm^{-3} mannose and high (400 mg dm^{-3}) *Timentin* concentration. A total of 68 (57 %) explants regenerated 112 shoots, 6 of them rooted on 5 g dm^{-3} mannose and 5 were shown to bear the *pmi* gene by PCR (Fig. 2). Southern blot demonstrated independent transformation events in all 5 plants (Fig. 3*A*,*B*, *lanes* 26 - 30). Out of 60 control explants only 9 (15 %) of them regenerated, producing a total of 12 shoots, none of which rooted.

Transformation efficiency described by Sigareva *et al*. (2004) depended on genotype, on type of plasmid construct and on explant type. For cotyledon explants the authors reported that the efficiency of transformation ranged from 2.9 to 13.2 %.

The plasmid construct allowed use of either mannose or kanamycin as a selection agent to compare the transformation efficiency of different selection systems. From Table 2 it is apparent that kanamycin selection was about 2 times more efficient than mannose selection. Organogenesis on kanamycin was obvious on 27 (14 %)

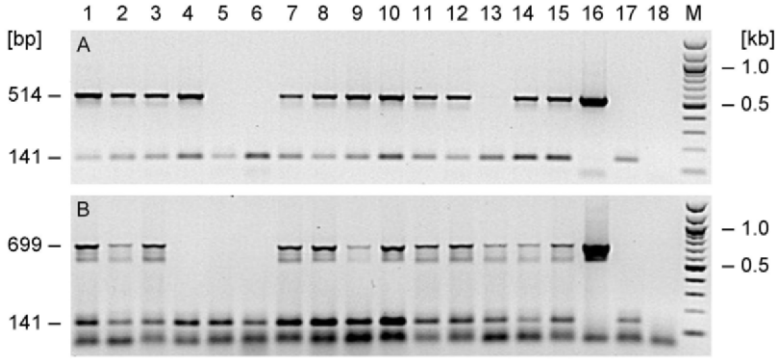

Fig. 2. Example of ethidium bromide stained products of PCR analyses for detection of *pmi* gene (*A*) or *nptII* gene (*B*) in rooted regenerants of tomato. Duplex reactions were carried out by amplifying a 514-bp fragment of the *pmi* gene (*A*) or a 699-bp fragment of the *nptII* gene (*B*) and a 141-bp fragment from tomato chromosome 11 as a positive control of PCR implementation. *Lanes 1 to 4* - plants from experiment RM5; *lane 5* - plant from experiment RM2; *lanes 6 to 11* - plants from experiment RM3a; *lanes 12 to 15* - plants from RM-Kn experiment; *lane 16* - *A. tumefaciens* harbouring binary vector pCB3160; *lane 17* - non-transformed tomato; *lane 18* - negative control; *lane M* - DNA marker (100-bp ladder, *New England Biolabs*, Hitchin, UK).

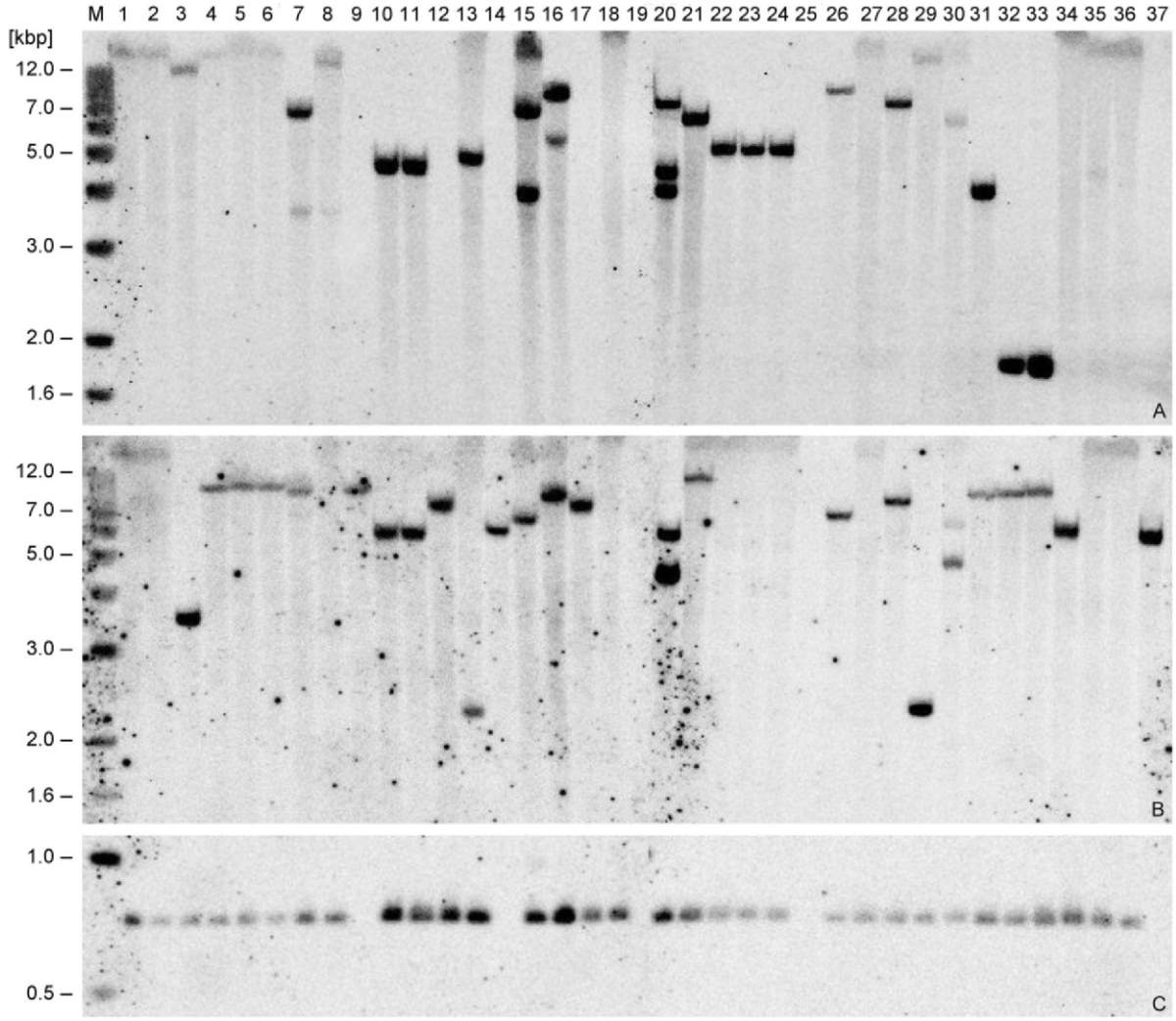

Fig. 3. Southern hybridisation analysis of transformed tomato plants carrying the T-DNA of pCB3160. DNAs digested with *Bam*H I were hybridised in successive steps with a 514 bp *pmi* probe (*A*), a 699 bp *nptII* probe (*B*) and a 395 bp *CMPS* promoter probe (*C*) with stripping between hybridisations. The minimal expected band size was about 1.6 kb for the *pmi* probe, 2.25 kb for the *nptII* probe and about 0.7 kb for the *CMPS* probe. *All panels: Lane M* - DNA size marker (1 kb ladder, *Gibco-BRL,* Paisley, UK); *lanes 1 to 18, 20, 21 and 37* - plants from experiment RM-Kn; *lane 19* - non-transformed tomato; *lanes 22 to 24* - plants from experiment RM2; *lane 25* - rooted PCR negative plant from experiment RM2a; *lanes 26 to 30* - plants from experiment RM3a; *lanes 31 to 36* - plants from experiment RM5.

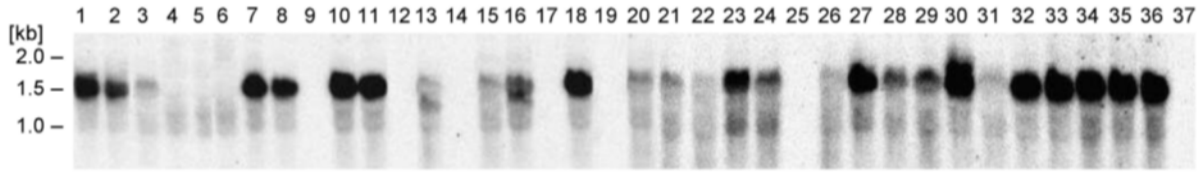

Fig. 4. Northern hybridisation analysis of transformed tomato plants carrying the T-DNA of pCB3160. About 25 μg of total RNA were probed with 514 bp *pmi* gene derived probe. The expected size of mRNA was about 1500 b. Lanes are the same as in Fig. 3.

of the 200 explants used, 47 shoots were recovered in total, 27 out of them rooted on a medium with 100 mg dm^{-3} kanamycin and 26 plants were positive (Fig. 2) when tested by PCR for the *nptII* gene. Southern hybridisation of 21 DNAs (Fig. 3*A*,*B*, *lanes* 1 - 18, 20, 21, 37) revealed the clonal origin of some regenerants (*lanes* 1, 2, 4 - 6, 10, 11, 14, 37). Since 2 out of 5 Southern blot non-tested plants arose from different explants, and 3 remaining regenerants originated from the same explants as plants tested in lanes 8 or 10/11, we can conclude that minimal number of independent transformants obtained after kanamycin selection was

18 plants. All control explants died during the first month of culture on kanamycin containing medium.

Almost all transgenic plants harboured one copy of T-DNA with the exception of the plants in *lanes* 15, 16, 20 and possibly *lanes* 7, 8 and 30 (Fig. 3*A*). As regards the incomplete transfer of T-DNA, large left-part T-DNA (*i.e*. *pmi* gene as well as CMPS promoter) was not inserted into the host genome in plants in *lanes* 9, 14 and 37 (the plant from RM2a experiment in *lane* 25 was an escape, *i.e.* a plant rooting on selection medium but negative in PCR assays both for *nptII* and *pmi*) whilst only the *pmi* gene is missing in plants in *lanes* 12 and 17. Right-part T-DNA was probably not revealed in *lane* 8 and possibly in *lanes* 22 - 24 containing plants of clonal origin. Taking these findings into account, it is not surprising that Northern blot (Fig. 4) showed no *pmi* specific mRNA in *lanes* 9, 12, 14, 17, and 37 as well as, of course, in *lanes* 19 (non-transformed tomato) and 25 (escape). Probably due to the gene silencing phenomenon a quite different content of *pmi* mRNA was found in plants in *lanes* 22 - 24 which have clonal origin.

Table 3. Effect of gradually increase of mannose concentration on transformation and selection efficiency from potato internodal explants. Selection efficiency (SE) is expressed as the ratio of transgenic shoot numbers to the total number of shoots surviving selection and transformation efficiency (TE) is the ratio of transgenic plant numbers to the total number of plated explants (wk - week).

Exp.	Mannose concentration [g.dm^{-3}] start	3rd wk	6th wk	9th wk	12th wk	SE [%]	TE [%]
1	1.0	5.0	10.0	10.0	10.0	3.0	3.3
	1.0	5.0	5.0	10.0	10.0	15.7	10.0
	1.0	5.0	5.0	5.0	10.0	31.2	16.6
2	1.0	2.5	5.0	10.0	10.0	7.1	3.3
	1.0	2.5	2.5	5.0	10.0	0	0
3	2.5	5.0	10.0	10.0	10.0	72.7	26.6
	2.5	5.0	5.0	10.0	10.0	8.0	6.6
	2.5	5.0	5.0	5.0	10.0	33.3	13.3
4	5.0	10.0	10.0	10.0	10.0	26.6	53.3
	5.0	5.0	10.0	10.0	10.0	20.0	22.2
	5.0	5.0	5.0	10.0	10.0	10.0	8.8
	5.0	5.0	5.0	5.0	10.0	33.3	31.2
5	3.5	3.5	10.0	10.0	10.0	100.0	5.8

Potato transformation: Transformation efficiency (TE) along with selection efficiency (SE) was studied in a series of mannose concentrations from 1 to 10 g dm^{-3}. SE was calculated as the number of transgenic shoots among the total number of shoots surviving selection. In order to eliminate the non-transgenic escapes, the mannose concentration was increased in the course of selection (Table 3).

TEs were very low with the initial concentration of mannose 1 g dm^{-3} even when it was increased to 2.5, 5 and 10 g dm^{-3} during 3 weeks. However, when followed by prolonged culture on mannose 5 g dm^{-3}, higher TE as well as SE was induced. A stronger starting selection pressure (2.5 g dm^{-3}) induced higher TE and SE. The highest TE (53.3 %) was achieved on selection medium containing 5 g dm^{-3} mannose for three weeks followed by a medium with 10 g dm^{-3} mannose for the next 4 sub-cultures (Table 3). This is a very high efficiency of transformation, when compared with reported data for other plant species. For example, TE reached 25 % in wheat (Wright *et al*. 2001), 30 % for maize (Negrotto *et al*. 2000), 31 % for hemp (Feeney and Punja 2003), 41 % for rice (Lucca *et al*. 2001), 23 % for cucumber (He *et al*. 2006), 23 or 27 % for onion (Aswath *et al*. 2006), 6.8 % for almond (Ramesh *et al*. 2006) but only 0.72 % for pearl millet (O'Kennedy *et al*. 2004), 0.94 % for sugar beet (Joersbo *et al*. 1998), 1.14 % for durum wheat (Gadaleta *et al*. 2006) or 3 - 24 % for sweet orange (Boscariol *et al*. 2003). When compared with kanamycin selection (TE = 33.3 %, Table 4) the transformation efficiency of optimal mannose selection procedure was about 1.5-fold higher.

The presence of *pmi* and/or *nptII* genes was tested by PCR in all shoots growing on MS medium with 10 g dm^{-3} mannose. The highest proportion of regenerated shoots harbouring the *pmi* gene was obtained again in experiment 4 (22 %) whilst the average proportion of such regenerants in all 5 experiments reached 15 % (Table 4). Surprisingly, 7 PCR samples of plants growing on mannose media in the experiments 3 and 4 showed only the *npt II* gene fragment but no the *pmi* gene fragment. The reason for such findings could be the way of PCR sample preparation when no DNA isolated from the whole plant but directly pieces of plant tissue are used as a template for PCR assay. In that event, the result of PCR assay of regenerated plant and its phenotype (*i.e*. ability to grow on selection medium) could be different when assayed plant is chimeric, *i.e*. when it consists of transgenic tissue harbouring either whole T-DNA or truncated T-DNA with one out of two transgenes.

Table 4. Presence of *pmi* and *nptII* transgenes in regenerated potato shoots growing on MS medium with 10 g dm^{-3} mannose (experiments 1 - 5) or 100 mg dm^{-3} kanamycin as assayed by PCR.

PCR analysis	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Total	Kn100
pmi^{+}/ *nptII*$^{+}$	2	1	4	20	6	33	21
pmi^{+}/ *nptII*$^{-}$	5	0	2	4	2	13	0
pmi^{-}/ *nptII*$^{+}$	0	0	4	3	0	7	2
pmi^{-}/ *nptII*$^{-}$	61	23	38	81	49	252	47

Selected transgenic plants from experiment 5 harbouring T-DNA of pCB3160 were analyzed by Southern hybridisation (Fig. 5*A*) using *Bam*HI digested genomic DNA or by Northern hybridisation (Fig. 5*B*) with the different probes (Fig. 1). It is apparent that out of 8 transgenic plants 5 plants arose through independent

transformation events. Five plants also revealed transcription products of the *pmi* transgene.

In the present study we developed an alternative transformation selection system for potato cv. Bintje. The procedure allows us to achieve a transformation efficiency that is almost 2-fold higher than with a selection system based on the *nptII* gene and kanamycin. As regards tomato, our findings are consistent with that of Sigareva *et al*. (2004). Our value of TE for tomato cotyledon explants fall into the published interval and was about 2 times lower comparing with the ordinary protocol using kanamycin.

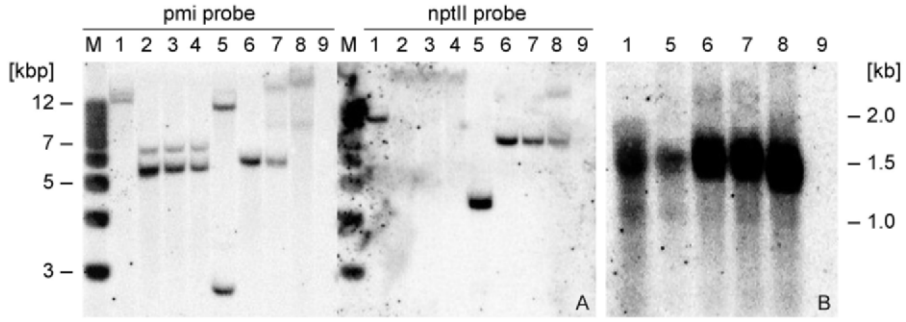

Fig. 5. Southern (*A*) or Northern (*B*) hybridisation analyses of transformed potato plants carrying the T-DNA of pCB3160. *A*: DNAs digested with *Bam*HI were hybridised successively with a 514 bp *pmi* probe and a 699 bp *nptII* probe with stripping between hybridisations. The minimal expected band size was about 1.6 kb for the *pmi* probe and 2.25 kb for the *nptII* probe use. *B*: About 25 µg of total RNA were probed with a 514 bp *pmi*. The expected size of mRNA was about 1500 bases. *Lane M* - DNA size marker (1 kb ladder, *Gibco-BRL*); *lanes 1 to 8* - plants from experiment 5; *lane 9* - non-transformed potato.

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