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Genotype-independent leaf disc transformation of potato (*Solanum tuberosum*) using *Agrobacterium tumefaciens*

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Summary. Leaves of the in vitro grown potato cultivars 'Bintje', 'Berolina', 'Desiree', and 'Russet Burbank' were wounded and co-cultivated with *Agrobacterium* strains having chimeric *bar* and *nptII* genes on a disarmed T-DNA. Each leaf from these cultivars formed numerous calli on kanamycin-containing medium, and almost all calli regenerated shoots. For 'Russet Burbank', it was necessary to include $AgNO_3$ in the medium to obtain efficient shoot regeneration. The transformed plants have one to a few copies of the T-DNA, show NPT-II and PAT activities, and are resistant to high doses of the commercial preparation of phospinotricin (glufosinate). Almost no somaclonal variation was detected in transgenic plants.

Key words: *Solanum tuberosum* – Transformation – *Agrobacterium* – Ethylene – Phosphinotricin

Introduction

Due to its exceptional qualities (high productivity, and high carbohydrate, protein, and vitamin content), the potato has become one of the major food crops, and its use is increasing even now far more rapidly than that of any other crop. For these reasons, there is a growing interest in further improving the qualities of the potato by using, in addition to the classic breeding techniques, techniques such as protoplast fusion and genetic engineering. Recently, three methods have been published on the transformation of potato using *Agrobacterium* based vectors. Ooms et al. (1987) described the transformation of the cvs 'Maris Bard' and 'Desiree' by infecting stems of in vitro grown plants with a mixture of two *Agrobacterium* strains: one contained an engineered *nptII* gene (neomycin phosphotransferase) in a disarmed T-DNA, the other *Agrobacterium* was a shoot-inducing strain. However, from the six transformed plants obtained, four were highly aneuploid, and one contained 47 instead of the normal 48 chromosomes. Sheerman and Bevan (1988) and Stiekema et al. (1988) used tubers as starting material. However, the reactivity of tuber slices in tissue culture varies greatly during the year, and the material is not convenient to work with.

For these reasons, I adapted the leaf disc transformation method as described by Horsch et al. (1985). The chimeric *nptII* and chimeric *bar* genes are used as marker genes. The *bar* gene codes for the enzyme phosphinotricin acetyltransferase (PAT) which inactivates the herbicide phosphinotricin (glufosinate) by acetylating it (Murakami et al. 1986). Phosphinotricin is a glutamate analog that inhibits glutamine synthetase. The inhibition results in the accumulation of NH_4^+ which is toxic for the plant cell. For more detailed information about the isolation and characterization of the *bar* gene and its expression in plants, see Thompson et al. (1987) and De Block et al. (1987).

The method described here gives rooted transgenic shoots in about 7–10 weeks. Each leaf gives rise to numerous transformed calli, and each callus regenerates shoots. Rooted transgenic shoots were obtained from all four cvs tested ('Berolina', 'Desiree', 'Bintje' and 'Russet Burbank'). Almost no somaclonal variation was detected in these transgenic plants.

Materials and methods

Agrobacterium strains

The *Agrobacterium* strains with their features are summarized in Table 1.

Table 1. *Agrobacterium* strains and their characteristics

Agrobacterium strain	Vector type	Ti-vir functions	Promotor expressing	
			nptII	*bar*
C58C1 Rif^R (pGV2260)[a]	Control	B6S3	–	–
C58C1 Rif^R (pMP90)[b]	Control	C58	–	–
C58C1 Rif^R (pGSFR1161)[c]	pGV2260 co-integrate	B6S3	TR1′[g]	TR2′[g]
C58C1 Rif^R (pGSFR1280)[d]	pGV2260 co-integrate	B6S3	nos[h]	35S[i]
C58C1 Rif^R (pMP90) (pGSFR760A)[e]	Binary vector	C58	TR1′	TR2′
C58C1 Rif^R (pMP90) (pGSFR780A)[f]	Binary vector	C58	nos	35S

[a] Deblaere et al. (1985)
[b] Koncz and Schell (1986)
[c] See Fig. 5A
[d] De Block et al. (1987)
[e,f] Have the same T-DNA as pGSFR1161 and pGSFR1280 respectively, but are binary vectors based on pGV941 (Deblaere et al. 1987)
[g] Velten and Schell (1985)
[h] Herrera-Estrella et al. (1983)
[i] Odell et al. (1985)

Plant materials

Sterile plants of cvs 'Bintje', 'Desiree', 'Berolina' and 'Russet Burbank' were propagated in vitro by transferring the top shoots or 1-cm-long pieces of stem explants together with an auxillary bud to S1 medium. The shoots were grown at 23 °C with a daylength of 16 h under 3000 lux light intensity (a mixture of "lumilux white" and "natura" from Osram, FRG).

Media

S1: B5 medium (Gamborg et al. 1968) with 20 g/l sucrose and supplemented with 150 mg/l $CaCL_2 \cdot 2H_2O$, 0.4% agarose, pH 5.8.
S2: MS medium (Murashige and Skoog 1962) with 30 g/l sucrose and supplemented with 0.5 g/l MES pH 5.5, 20 g/l mannitol.
S3: MS medium without sucrose and supplemented with 200 mg/l glutamine, 0.5 g/l MES pH 5.7, 0.5 g/l PVP, 20 g/l mannitol, 20 g/l glucose, 40 mg/l adenine-SO_4, 0.5% agarose, 1 mg/l trans-zeatin, 0.1 mg/l NAA, 1 g/l carbenicillin or 0.5 g/l cefotaxime.
S4: S3 supplemented with 10 mg/l $AgNO_3$.
S5: S3 medium without NAA and an one-half concentration of antibiotics.
S6: S5 supplemented with 10 mg/l $AgNO_3$.
S7: S5 supplemented with 0.01 mg/l GA3; 250 mg/l carbenicillin or 150 mg/l cefotaxime.
S8: S5 supplemented with 0.1 mg/l GA3 and 10 mg/l $AgNO_3$; 250 mg/l carbenicillin or 150 mg/l cefotaxime.
Antibiotics, hormones, and $AgNO_3$ were added after autoclaving. $Ag_2S_2O_3$ was added as 10 mg/l $AgNO_3$ + 117 mg/l $Na_2S_2O_3 \cdot 5H_2O$. Min A medium is as described (Miller 1972).

Transformation, selection, and regeneration

Leaves (3–10 mm) from 3- to 4-week-old shoots were cut at the base. The leaves were not further wounded. About 10 wounded leaves were floated upside down on 10 ml of infection medium S2 contained in a 9 cm Petri dish. To each Petri dish, 30 µl of *Agrobacterium*, which had been grown in Min A medium to late log, was added. The plates were then incubated at low light intensity (500 lux). After 2 days, the leaves were washed with S2 medium containing 1 g/l carbenicillin or 0.5 g/l cefotaxime, patted dry on filter paper, and placed upside down on medium S3 containing 50 to 100 mg/l kanamycin-SO_4 for 'Berolina', 'Bintje' and 'Desiree', and S4 with 100 mg/l kamanycin-SO_4 for 'Russet Burbank'. The Petri dishes were sealed with tape that allows gas diffusion ("urgo pore" tape, Urgo, Chenove, France) and were incubated at high light intensity (3000 lux, a mixture of "lumilux white" and "natura" from Osram, FRG). After 1 week, the leaves were transferred to fresh medium. After 2 more week, many small calli had formed at the wounded edges of the leaves, and the leaves were transferred to selective medium S5 for 'Berolina', 'Bintje' and 'Desiree', and S6 for 'Russet Burbank'. After 2–3 more weeks, those leaves with calli were transferred to medium S7 for 'Berolina', 'Bintje' and 'Desiree', and S8 for 'Russet Burbank'. From this point on, 250-ml glass jars were used.

When using cefotaxime in the medium, it is important to transfer the leaves to fresh medium every 10 days. After 2 weeks, the first shoots (0.5 cm high) could be isolated and transferred to rooting medium S1 containing 100 mg/l carbenicillin or cefotaxime. Normally, the shoots rooted in about 1 week. When they did not root after 2 weeks, a thin slice was cut away from the stem base: most of these recut shoots rooted after 1 week. All shoots were harvested within a period of 3 weeks. In order to avoid isolating identical shoots, two shoots from the same or closely linked calli were never taken.

Enzyme assays

NPTII activity was detected by the in situ gel assay of Reiss et al. (1984) as modified by Schreier et al. (1985). PAT activity was detected by the thin layer chromatography method as described by De Block et al. (1987).

Leaf assays

Leaves from the in vitro grown selected plants were cut at the base and placed on selective S3 medium without carbenicillin or cefotaxime. To select for resistant leaves, 50 mg/l kanamycin-SO_4 or 20–50 mg/l phosphinotricin was used. The resistant leaves formed a large amount of callus after 2–3 weeks, while the sensitive controls never formed callus under these selective concentrations.

Herbicide applications

Plants were placed in 1 m^2 and sprayed with a 1% aqueous solution of the commercial preparation Basta® containing 20% D,L-glufosinate (phosphinotricin) (Hoechst AG, FRG) from all four sides using a Badger (Badger Air-Brush Co./IL, USA) air-brush line.

Plant DNA isolation and Southern analysis

DNA was isolated from 200 to 500 mg stem and leaf tissue as described by Dellaporte et al. (1983). The DNA was electrophoresed in a 0.8% agarose gel, transferred to nylon Hybond-N filters, and hybridized with nick-translated DNA as described in the Amersham manual for the use of Hybond-N filters. A purified *BamHI* fragment containing the *bar* gene from the plasmid pGSFR280 (De Block et al. 1987) was used as a probe.

Cytological analysis

Root tips from in vitro grown plants (on S1 medium) were incubated in 1-bromonaphtalene prefixative (2 ml 1-bromonaphtalene in 100 ml distilled water) for 2–3 h at 20°–23 °C. The prefixative was removed, and the root tips were rinsed 3 times with water. The root tips were then placed for 12–24 h in Carnoy's fixative (95% ethyl alcohol, glacial acetic acid, and chloroform 6:1:3). To soften the cell wall and to enhance staining, the fixed material was incubated in 2 N HCl at 58°–60 °C for 7 min. After hydrolysis, the root tips were washed with 45% acetic acid. The root tips should remain in 45% acetic acid for at least 15 min. Squash preparations were made in propionocarmine. Chromosome counts were obtained from about 5–10 well-spread cells from at least two separate roots.

Results

Infection

The infections were done as described in the Materials and methods. All *Agrobacterium* strains contained both the *nptII* and the *bar* gene, which were put under the control of different promoters (Table 1).

The addition of hormones to the infection medium (1 mg/l trans-zeatin and 0.1 mg/l NAA) was deleterious for leaves of 'Russet Burbank': they died within a few days after transfer to callus-inducing medium. For 'Berolina', 'Bintje', and 'Desiree', the addition of hormones to the infection medium had no influence on transformation and regeneration frequency. Consequently, hormones were omitted from the infection medium.

The leaves were wounded by cutting them at the base (Fig. 1), since this had been found to be the most reactive part of the leaf. Extensive wounding lowered the transformation and regeneration frequency.

Basic medium and hormones

Table 2 summarizes the efficiency of callus formation on kanamycin-containing medium from 10 independent transformation experiments with cvs 'Berolina', 'Bintje', and 'Desiree'. These three cultivars reacted very similarly on the different media. Callus formation on B5 medium was similar to that on MS medium, but calli on MS medium regenerated faster and produced more shoots than those on B5 medium. The use of BAP instead of trans-zeatin had no effect on callus formation, but shoot regeneration was more effective with trans-zeatin. As an auxin source, NAA was clearly superior to IAA: on IAA fewer and smaller calli formed, and these were more difficult to regenerate than those formed on NAA.

No difference was found in transformation frequencies when either carbenicillin (1000 mg/l) or cefotaxime (500 mg/l) was used to kill the *Agrobacterium* strain.

The use of Ag^+

The addition of Ag^+ had a dramatic effect on callus formation of 'Russet Burbank' leaves (Table 2). Whereas the other cultivars showed a less pronounced reaction, 'Russet Burbank' leaves formed yellow-brownish and non-regeneratable callus on medium without Ag^+ (Fig. 2). When Ag^+ was added to kanamycin-containing medium, callus and shoot formation was as efficient as that shown by the other three cultivars. The stimulating effect of Ag^+ on shoot regeneration has been described by Purnhauser et al. (1987). However, the addition of $AgNO_3$ to the media prior to autoclaving, as these authors have done, is toxic to potato tissues. Hence, we added $AgNO_3$ after autoclaving. It has been suggested that Ag^+ blocks ethylene action by binding to the ethylene receptor(s) (Aharoni et al. 1979; Beyer et al. 1976). The stimulating effect it has on callus formation and shoot regeneration from 'Russet Burbank' leaves can be explained by its anti-ethylene properties. For this reason also, the Petri dishes were sealed with "urgo pore tape" in order to allow sufficient gas-exchange during the callus induction period. Light conditions enriched for red were used, since red light is thought to lower the sensitivity of plant tissue to ethylene (Decoteau and Craker 1987). Some reports propose that Ag^+ in the form of $Ag_2S_2O_3$ is less toxic (Cameron and Reid 1981) and more effective because of better transport (Veen and Van De Geijn 1978) than Ag^+ as $AgNO_3$.

We also found that $Ag_2S_2O_3$ is more effective than $AgNO_3$ in callus and shoot induction, but these shoots were difficult to root, and many died shortly after transfer to rooting medium. Shoots from 'Berolina', 'Desiree', and 'Bintje' that arose on $AgNO_3$ medium were more difficult to root than those from Ag^+-free medium. Therefore, we used $AgNO_3$ for 'Russet Burbank' and omitted Ag^+ for 'Berolina', 'Desiree', and 'Bintje'. It is

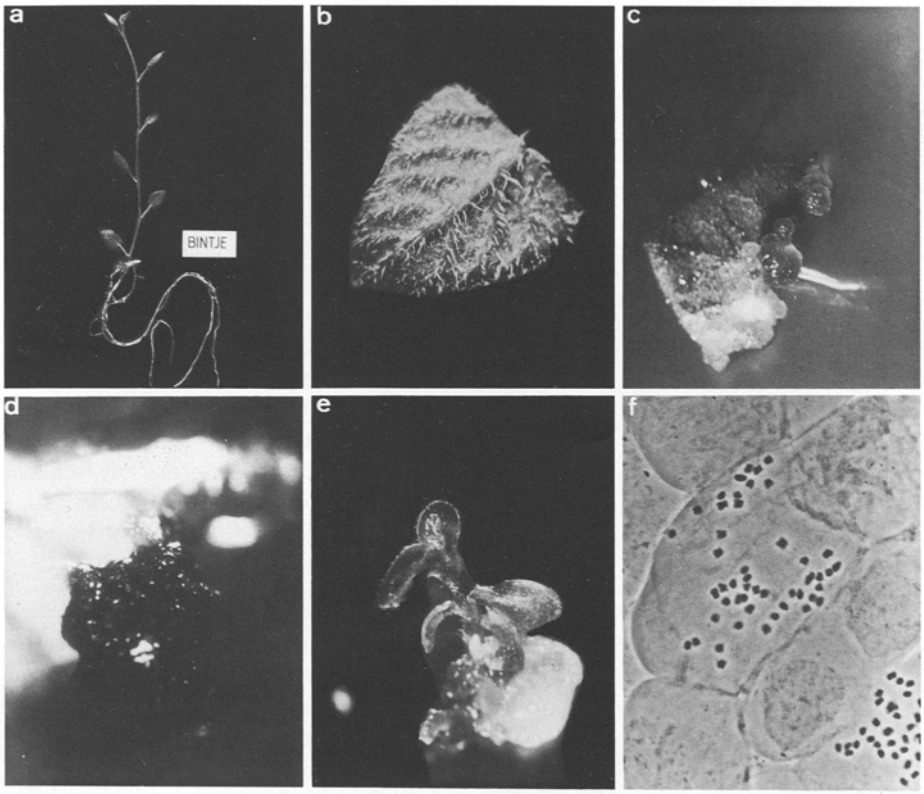


Fig. 1 a–f. Transformation of potato (cv 'Bintje') using the *Agrobacterium* strain C58C1 Rif^R (pMP90) (pGSFR780A). The selection was done on 100 mg/l kanamycin-SO_4. **a** 'Bintje' grown on S1 medium, 3 weeks after subculture; **b** wounding of the leaf. The leaf was cut at the base; **c** callus formed on selective medium S5. The picture was taken 3 weeks after infection; **d** meristems starting to form on selective S7 medium 1 month after infection; **e** shoots formed on selective S7 medium 6 weeks after infection; **f** root-tip squash preparation of a transformed plant showing the normal 48 chromosomes per cell

important to note that if $AgNO_3$ is added to the medium, 100 mg/l kanamycine-SO_4 should be used. It was observed that in the presence of $AgNO_3$, 50 mg/l kanamycin-SO_4 does not provide sufficient selection and "escapes" were found (data not shown).

Regeneration of transformants

After 2 weeks, the leaves were transferred from medium S3 and S4 to medium without auxins (S5 and S6). Two to three weeks later, the callus-forming leaves were transferred to medium S7 for 'Berolina', 'Desiree', and 'Bintje' and S8 for 'Russet Burbank'. The addition of $AgNO_3$ at this step was absolutely required to obtain shoot regeneration for 'Russet Burbank'. Direct transfer from auxin-containing medium (S3 and S4) to GA3-containing medium (S7 and S8) slowed down shoot formation significantly.

After 1–2 weeks, some calli started to form shoots. After 1 month, nearly every callus had regenerated shoots. Shoots of about 5 mm were separated and transferred to rooting medium S1 containing 100 mg/l carbenicillin or cefotaxime. About 75% of the shoots formed roots within 2 weeks. Those shoots that did not root after 2 weeks were cut again, and the majority then rooted after 1 more week.

Enzyme analysis

Figure 3 shows an NPTI-II and PAT assay of leaf material from plants selected on 50 mg/l kanamycin. PAT assays were done on 50 selected plants, and all contained high enzyme activities.

The use of phosphinotricin resistance as a selectable marker

Two transformation experiments were done with potato cvs 'Bintje' and 'Desiree' using either kanamycin 50 mg/l or phosphinotricin (5 and 10 mg/l) as selective agents. The *Agrobacterium* strain used contained the *nptII* gene under control of the nos promoter and the *bar* gene under control of the 35S promoter (pGSFR780A, Table 1). In both experiments, a transformation frequency of 100% was obtained when kanamycin was used, while a frequency of only 20% was obtained when phosphinotricin was used for selection. The omission of glutamine in the

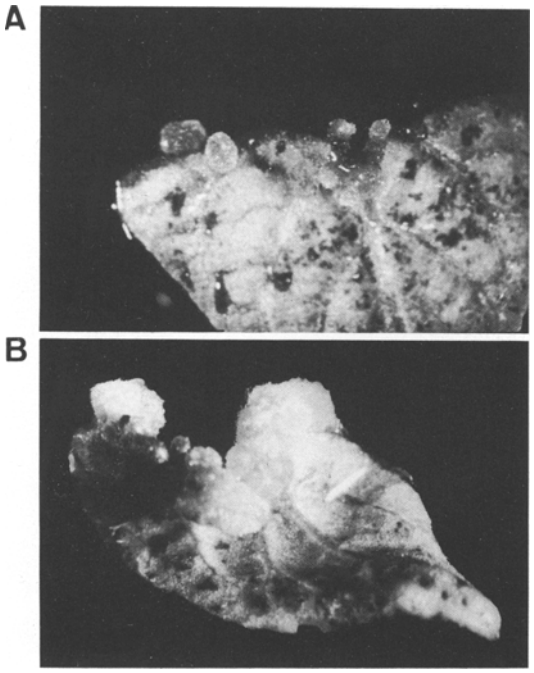


Fig. 2 A and B. The influence of $AgNO_3$ on callus formation of 'Russet Burbank'. Leaves were infected with the strain C58C1 Rif^R (pMP90) (pGSFR780A). Transformed calli were selected on 100 mg/l kanamycin-SO_4. The picture was taken 3 weeks after infection. **A** No $AgNO_3$ added to the medium; **B** 10 mg/l $AgNO_3$ added to the medium

Table 2. Percentage of leaves forming callus on medium containing 100 mg/l kanamycin – SO_4 [a]

Basic medium	Conditions		Callus formation (%)	
	Hormone (mg/l)	Additives	Berolina/ Bintje/ Desiree	Russet Burbank
B5	1 zeatin 0.1 IAA	–	40	10
	1 zeatin 0.1 NAA	–	60	60
MS	1 zeatin 0.1 IAA	–	40	10
	1 zeatin 0.1 NAA	–	70	50 [d]
	1 zeatin 0.1 NAA	$AgNO_3$ [b]	80	100 [e]
	1 zeatin 0.1 NAA	$Ag_2S_2O_3$ [c]	70	100 [e]
	1 BAP 0.1 NAA	–	70	60 [d]
	1 BAP 0.1 NAA	$AgNO_3$ [b]	80	100 [e]

[a] Average of 10 experiments scored after 3 weeks
[b] 10 mg/l $AgNO_3$
[c] 10 mg/l $AgNO_3$ + 117 mg/l $Na_2S_2O_3 \cdot 5\ H_2O$
[d] Calli are brownish, and there are only a few calli/leaf
[e] Calli are healthy, and the leaves are covered with calli

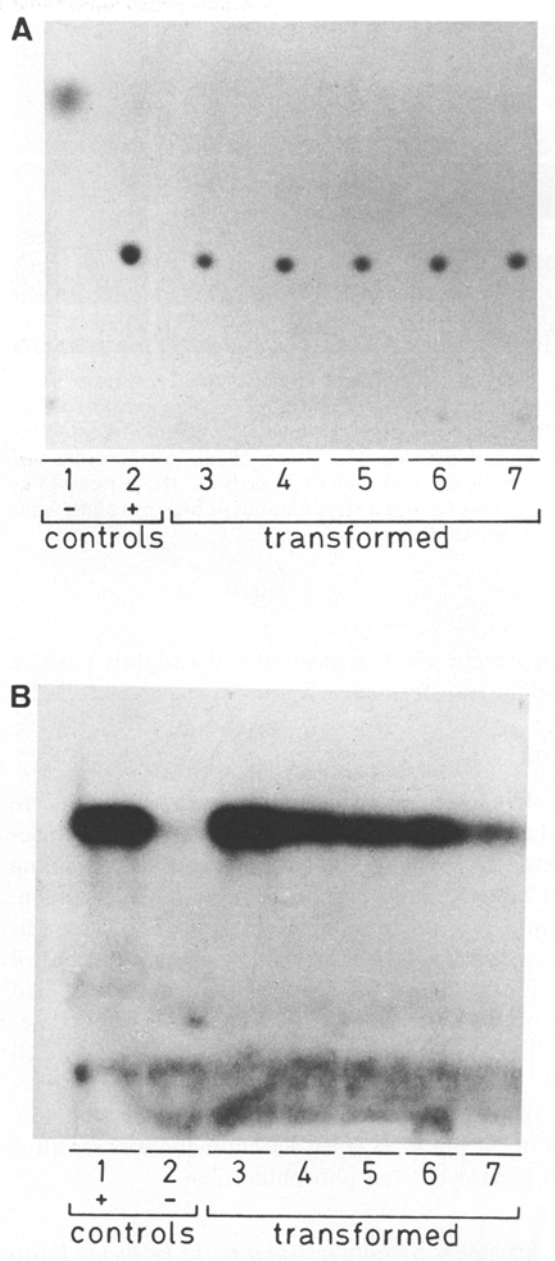


Fig. 3. **A** Detection of PAT-activity by thin layer chromatography. The extracts were prepared from leaf and stem tissue and diluted 100 fold. The reaction was performed, and 6 µl was put on a silica gel thin-layer chromatography plate for ascendent chromatography. [^{14}C]-labelled substrate and reaction products were visualized by autoradiography. *Lane 1*, extract from untransformed plants; *lane 2*, extract of untransformed plants to which PAT-enzyme was added; *lanes 3–7*, extracts from 5 independent transgenic plants transformed by the *Agrobacterium* strain C58C1 Rif^R (pMP90) (pGSFR760A): *lane 3*, 'Berolina'; *lane 4*, 'Desiree'; *lane 5* and *6*, 'Bintje'; *lane 7*, 'Russet Burbank'. **B** Detection of NPTII-activity by the in situ gel assay of Reiss et al. (1984). *Lane 1*, transformed tobacco plant expressing the *nptII* gene under the control of the TR1 promotor; *lane 2*, untransformed potato plant; *lanes 3–7*, 5 independent transgenic potato plants transformed by the *Agrobacterium* strain C58C1 Rif^R (pMP90) (pGSFR760A). The same plants are shown in the same order as in **A**

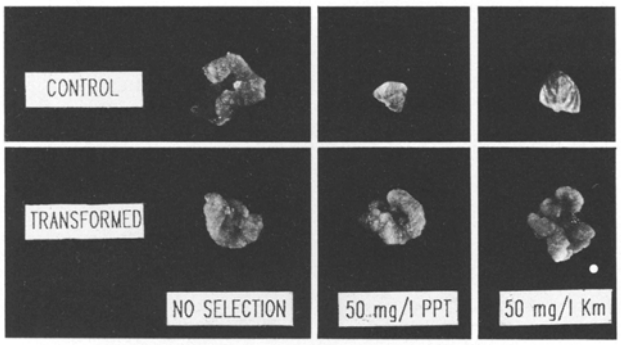
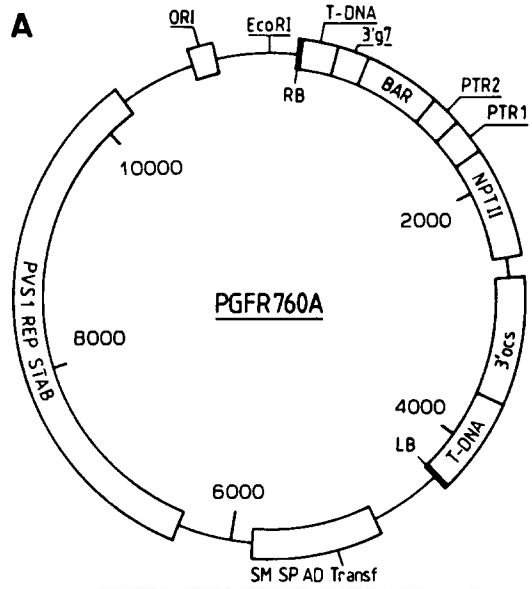


Fig. 4. Leaves of control and transgenic potato plants cv 'Bintje' transformed by C58C1 Rif^R (pGSFR1280) were placed on S3 medium (without carbenicillin or cefotaxime) containing 50 mg/l phosphinotricin (*PPT*) or 50 mg/l kanamycin-SO_4 (*Km*). On both the Km and PPT medium, the leaves of the transgenic plants formed a large amount of healthy callus, while the control leaves died



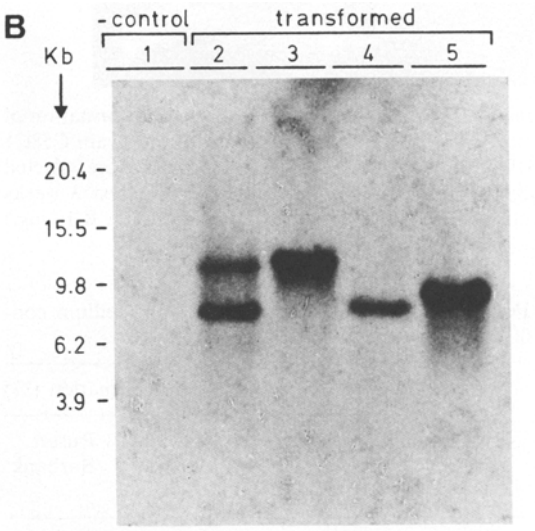


Fig. 5. **A** Schematic representation of pGSFR760A indicating the unique *EcoRI* site outside the T-DNA. The *nptII* gene is under the control of the TR1′ promotor, while the *bar* gene is under the control of the TR2′ promotor. Both genes are inserted between the T-DNA border repeats (*RB*, right border; *LB*, left border). The *nptII* and *bar* genes are followed by fragments encoding termination and polyadenylation signals derived from the octopine synthase gene (3′ocs) and the T-DNA gene 7 (3′g7) (Velten and Schell 1985), respectively. **B** Southern blot analysis of 4 transgenic potato plants transformed with the vector pGSFR760A. The plant DNA was digested with *EcoRI* and hybridized with a ^{32}P-labelled purified fragment containing the *bar* gene. *Lane 1*, untransformed control plant cv 'Berolina'; *lanes 2–5*, transgenic potato plants. From left to right: cv 'Berolina', 'Desiree', 'Bintje', and 'Russet Burbank'

phosphinotricin selective medium had a slightly positive effect on the transformation frequency, raising it to 30%.

Leaf assays

Shoots that regenerated after kanamycin selection were assayed for expression of both NPT-II and PAT by placing leaves from these shoots on medium containing 50 mg/l kanamycin-SO_4 or 20–50 mg/l phosphinotricin.

Twenty mg/l kanamycine-SO_4 and five mg/l phosphinotricin prevents all callus growth on leaves from control plants (Fig. 4). Three hundred selected shoots that had been subcultured twice on carbenicillin- or cefotaxime-free medium were tested (100 'Bintje', 100 'Berolina', 50 'Desiree' and 50 'Russet Burbank'): 299 shoots were resistant to both kanamycin and phosphinotricin, and only 1 shoot of cv 'Bintje' escaped selection and was sensitive to both kanamycin and phosphinotricin.

Transgenic plants are fully resistant to the herbicide Basta

Four transgenic plants of each cultivar expressing the *bar* gene under the control of the TR2′ promotor were transferred to soil and grown in the greenhouse. Control potato plants of the four cultivars were effectively killed with doses equivalent to 1 l basta/ha. All 16 transgenic plants assayed were fully resistant to 20 l Basta/ha.

Southern hybridization analysis

Total DNA of nine transgenic plants (3 'Berolina', 3 'Bintje', 2 'Desiree' and 1 'Russet Burbank') transformed with PGSFR760A was digested with *EcoRI*, for which there are no recognition sites in the T-DNA of this plasmid (Fig. 5A). The digested DNA was electrophoresed on a 0.8% agarose gel, transferred to Hybond filters, and hybridized with a ^{32}P-labelled purified fragment containing the *bar* gene. Giving the size of the T-DNA (4-Kb) and judging by the number of bands, 3 plants contained 2 copies of the T-DNA, while the other 6 contained only 1 copy (Fig. 5B).

Somaclonal variation

Potato is extremely sensitive to somaclonal variation in tissue culture. Many authors (Creissen and Karp 1985; Karp et al. 1982; Pijnacker and Ferwerda 1987; Ramulu et al. 1983; Secor and Shepard 1981) have studied chromosome variation in protoplast-derived potato plants. A high degree of aneuploidy (up to 50%) was observed.

The chromosomes of 40 transgenic plants (10 of each variety) were counted. Only one plant of cv 'Berolina' had an abberant chromosome number of 47 instead of 48. However, this doesn't mean that the cytogenetically normal plants are free of somaclonal variation. To study this, six transgenic plants were propagated and transferred to the field (3 'Berolina', 2 'Bintje' and 1 'Desiree'). They all showed normal morphology and yield (De Greef et al. in preparation). More transgenic plants will be transferred to the field to study the occurrence of somaclonal variation more extensively.

Discussion

The transformation method for potato as described here is highly efficient and reproducible, and genotype independent.

As described in the Materials and methods, the four *Agrobacterium* strains used in the transformation experiments (Table 1) contained either co-integrate plasmids (with the vir-functions of the octopine Ti-plasmids) or binary vector plasmids (with the vir-functions of the nopaline Ti-plasmids). In each case, the *nptII* gene from the bacterial transposon Tn5 was fused to the promoter of the nopaline synthase gene (pr_{nos}) or to the promoter of the T-DNA gene encoding the enzyme involved in the first step of the mannopine agropine biosynthetic pathway (pr_{TR1}). The *bar* gene was under the control of the TR2′ or 35S (of cauliflower mosaic virus) promoter. There were no significant differences in transformation and regeneration frequencies resulting from the different *Agrobacterium* strains used.

The adaptation of the method made for var 'Russet Burbank' can probably be extended to other potato varieties in which excess ethylene production in tissue culture (characterized by the formation of brown-yellow, friable callus that is difficult to regenerate) is a problem. It is known that potato plants in vitro can produce a large amount of ethylene (Hussey and Stacey 1981). $AgNO_3$ cannot be replaced by $Ag_2S_2O_3$ because the shoots that arise on $Ag_2S_2O_3$-containing medium often die shortly after their transfer to the rooting medium. The tissue that grows on $AgNO_3$-containing medium must be incubated under a high light intensity as Ag^+ is toxic when the tissue is incubated at low light intensity. This phenomenon has been described previously (Curtis 1982, 1987). Ag^+ cannot be replaced by the anti-ethylene agents AVG (Yu and Yang 1979) and salicylic acid (Leslie and Romani 1986). Both compounds had no effect on callus and shoot formation of 'Russet Burbank' leaves (data not shown). The absence of any inhibitory effects of AVG and salicylic acid on ethylene production by *Persea americana* fruit tissue and *Medicago sativa* tissue cultures has been observed by Baker et al. (1982) and Meijer and Brown (1988). Thus, it can be that ethylene production by potato leaves is not inhibited by these compounds. The specific property of Ag^+ to bind to certain ethylene receptors is most likely responsible for its stimulating effect on 'Russet Burbank' leaf regeneration.

The transformed plants have a limited number of T-DNA insertions (1 or 2) and are cytogenetically and phenotypically normal. This lack of somaclonal variation is probably due to the mild hormone treatment and the short regeneration time (from leaf explant to rooted plant takes only 7–10 weeks). Kanamycin resistance and, to a lesser extend, phosphinotricin resistance can be used as selective markers. A weak expression of the *nptII* gene (fused, for example, to the pr_{nos}) is sufficient to allow a highly efficient and clear selection. The fact that phosphinotricin resistance is an inefficient selective marker can be explained by the high sensitivity of potato leaves to phosphinotricin. The leaves of non-transformed plants turn brown (the cells lyze) in a few days on low concentrations of phosphinotricin (5 mg/l). The few transformed cells that are present in a just-infected leaf have probably no chance to divide because they are killed by the toxic compounds released by the large number of dying (lyzed) non-transformed cells. This hypothesis is confirmed by the fact that leaves of transgenic plants expressing the *bar* gene are resistant to high levels of phosphinotricin (up to 50 mg/l). Moreover, these plants are resistant to high concentrations (20 l/ha) of the commercial herbicide Basta.

The important points to observe when applying this method are:

- wound leaves just at the base.
- do not add hormones to the infection medium.
- limit time (2 weeks) on the auxin-containing medium.
- dilute produced ethylene by working with ventilated plates (e.g., sealed with urgo pore tape) and large glass vials.
- add $AgNO_3$ when leaves and callus produce too much ethylene.

This transformation method allows an easy genetic manipulation of potato for fundamental and applied research. We now intend to extend this method to other potato cultivars.

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