REGENERATION AND TRANSFORMATION OF POLISH CULTIVARS OF POTATO

ANNA NADOLSKA-ORCZYK, LIDIA MIŁKOWSKA, ANDRZEJ PAŁUCHA¹,
PAWEŁ CZEMBOR and WACŁAW ORCZYK

Department of Plant Tissue Culture,
Plant Breeding and Acclimatization Institute,
Radzików, 05-870 Błonie, Poland

¹Institute of Biochemistry and Biophysics,
Polish Academy of Sciences,
Pawieńskiego 5a, 02-106 Warsaw, Poland

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ABSTRACT

The article presents the results of regeneration and transformation experiments of 12 Polish cultivars of potato. The cultivars Brda, Bzura, Elipsa and Irga regenerated the highest number of shoots from leaf explants (60% to 100% of explants regenerated 4.9 to 16.5 shoots per explant). The cultivars Brda, Bzura, Elipsa and Irys were the source of the best regenerating tuber explants (66% to 100% of explants regenerated 6.2 to 11.9 shoots from one explant). Both types of explants (from leaves and tubers) were used for transformation experiments. *Agrobacterium tumefaciens* strains used for transformation contained binary vector system: LBA 4404 (pAL 4404:pBI 121) and C58C1 (pGV2260:pVU104). There was a strong correlation between regeneration ability of tested cultivars and transformation efficiency. GUS-positive, kanamycin resistant and well rooted plants from leaf explants in cultivars Bzura, Brda and from tuber explants in Bzura and Elipsa were obtained. Northern blot analysis confirmed the presence of β -glucuronidase mRNA in transgenic plants.

KEY WORDS: in vitro regeneration, transformation, *Agrobacterium*, *Solanum tuberosum*.

INTRODUCTION

Improvement of potato cultivars via traditional breeding is complicated because of their tetraploidy, heterozygosity and incomplete fertility. On the other hand potato belongs to *Solanaceae* family which is easy regenerable in tissue culture and susceptible to *Agrobacterium* infection – the most widely used technique of transformation. A number of procedures concerning regeneration and transformation of different genotypes of potato have been developed. Regeneration from leaf explants was reported by Hulme et al. (1992), Wenzler et al. (1989), Visser et al. (1989) and from tuber explants by Stiekema et al. (1988). Transformation methods based mainly on *Agrobacterium tumefaciens* were presented by De Block (1988), Newell et al. (1991), Ooms et al. (1987), Sheerman and Bevan (1988), Stiekema et al. (1988), Tavazza et al. (1988), Wenzler et al. (1989). Transformation with *Agrobacterium rhizogenes* was described by Hanisch ten Cate et al. (1988), Ottaviani et al. (1990) and Visser et al. (1989b). These methods have been used for introduction of foreign, agronomically important traits (genes) like virus resistance, insect resistance, increased starch content in the tubers (see review, Fraley 1992) into potato plants.

One of the general rules of plant tissue culture is the high dependence of regeneration and transformation ability on

plant genotype. In order to establish conditions suitable for regeneration and transformation of chosen Polish cultivars additional tests were needed.

In this paper we described the procedures and results of shoot regeneration from leaf and tuber explants of 12 Polish cultivars of potato as well as transformation of both types of explants with *Agrobacterium tumefaciens* and regeneration of transgenic plants.

MATERIALS AND METHODS

Plant material

Regeneration ability was tested using leaf and tuber discs from 12 potato cultivars: Bronka, Ruta, Brda, Atol, Sokół, Irys, Irga, San, Bryza, Stobrawa, Elipsa and Bzura provided by The Potato Research Institute from Młochów.

Culture conditions

The basal culture medium contained MS salts and vitamins (Murashige and Skoog, 1962) plus 3% sucrose and 0.2% gelrite. Leaf explants were taken from well expanded leaves of plants growing in sterile conditions (glass jars) on 1/2 strength of MS medium. For primary culture of the explants, MS medium was supplemented with:

- 0.1 mg/l NAA + 2 mg/l BAP + 10 mg/l GA₃ (modified medium according to Wenzler et al. 1989),
- 2 mg/l BAP + 5 mg/l GA₃ + 1.5% sucrose (modified medium according to Visser et al. 1989).

After 4 weeks the explants were transferred on basal medium supplemented with 2 mg/l BAP plus 5 mg/l GA₃. Five leaf explants (about 10 x 10 mm) were cultured per 80 mm Petri dish.

Tuber discs were taken from surface-sterilized tubers by immersing them in 70% ethanol followed by 20 min. in HgCl₂ (0.1%) and rinsed free times with sterile distilled water. Primary culture of tuber explants was performed on MS basal medium (Murashige and Skoog, 1962) supplemented with:

- 0.01 mg/l NAA + 1 mg/l zeatin (Stiekema et al. 1988),
- 0.1 or 0.01 mg/l NAA and one of the following cytokinines: 1, 2 or 5 mg/l BAP or 1 mg/l 2iP or 1 mg/l kinetin or 0.5 mg/l zeatin. Subculture medium contained 0.25 mg/l BAP plus 0.1 mg/l GA₃ (Stiekema et al. 1988).

Transformation experiments.

Transformation experiments were performed with tuber discs taken from cultivars Bzura and Elipsa and leaf explants from Brda, Bzura, Sokół and Irys. Leaf explants were cultured according to modified procedure by Wenzler et al. (1989) and tuber discs according to Stiekema et al. (1988).

Two strains of *Agrobacterium tumefaciens* containing binary vector system: LBA 4404 (pAL 4404:pBI 121) and C58C1 (pGV2260:pVU104) were kindly provided by Dr. J.J.M. Dons from CPRO-DLO, Wageningen, The Netherlands. The first strain LBA 4404 (Hoekema et al. 1985) was a derivative of Ach5 strain harboring the helper plasmid pAL 4404 and binary plasmid pBI121 (Jefferson et al. 1987). The second one was a disarmed nopaline strain with the pGV 2260 vir plasmid (Deblaere et al. 1985) and pVU 1011 binary plasmid (Dr. P. van den Elzen; cited by Visser et al. 1989a). The pBI 121 binary plasmid (Vancanneyt et al. 1990) contained the npt-II gene (controlled by Pnos promoter) and the intron containing β -glucuronidase gene, controlled by the CaMV35S promoter. The pVU1011 disarmed plasmid harbored hygromycin phosphotransferase (hpt) gene fused to the CaMV35S promoter and the nopaline synthetase terminator. It allowed the selection of kanamycin and hygromycin resistant plants.

The bacteria were grown 2 days at 26°C in the dark on gyratory shaker set on 200rpm in LB liquid medium supplemented with appropriate antibiotics. For bacterial inoculation two-days precultured leaf and tuber explants were used. They were immersed in bacterial suspension for 5 minutes, blotted dry on sterile filter paper and placed back on the medium. After two or four days the explants were transferred to the selection medium containing 300-500 mg/l of carbenicillin or cefotaxime as well as 50 mg/l kanamycin sulphate. As a reference to results of transformation two control experiments were conducted. Positive control – culture of explants which were not subjected to *Agrobacterium* infection on antibiotic free media was carried out in the same time and conditions as in the transformation experiment. Negative control – culture of explants not inoculated with *Agrobacterium* was performed using leaf explants of Atol, Brda and Irys on media with 50, 100 and 150 mg/l kanamycin. After 3 weeks of primary culture explants were transferred to secondary medium. Small shoots (about 1-2 cm) were cut and cultured on 1/2 MS medium (the same as for plants of potato) supplemented with kanamycin. The experiments were repeated 2-3 times. The number of explants used in each experiment varied from 20 to 25.

GUS assay and RNA analysis.

Expression of GUS (β -glucuronidase) in 57 putative transgenic potatoes was determined by fluorimetric assay (Jefferson, 1987). The protein concentration in plant extracts were estimated by the method of Bradford (Bradford 1976).

RNA was extracted from GUS positive and from untransformed plants by powdering leaf tissue in liquid nitrogen and homogenization in extraction buffer/ phenol:chloroform:isoamyl alcohol followed by LiCl precipitation and ethanol precipitation (Verwoerd et al. 1989). Ten to fifty μ g of total glyoxal denaturated RNA was loaded on 1% agarose gel. Electrophoresis was performed at 100 mA for 4 hours. The RNA was transferred to Hybond N⁺ membrane under alkali conditions and hybridized with radiolabelled EcoR I – Hind III fragment from pBI 121 containing Pnos promoter, GUS coding region and terminator.

RESULTS AND DISCUSSION

The best shoot regeneration from leaf explants (Fig. 1a) was achieved on modified media according to Wenzler et al. (1989). Tuber explants regenerated the highest number of shoots on media according to Stiekema et al. (1988) (Fig. 1b). The percentage of regenerating leaf and tuber explants and a mean number of shoots per explant in twelve cultivars of potato on these media were presented in Table 1. The highest regeneration frequency from leaf explants were found for Brda, Bzura, Elipsa and Irga (60%-100% explants regenerated from 4.9 to 16.5 shoots per explant). Cultivars Brda, Bzura, Elipsa and Irys regenerated the highest number of shoots from tuber disks (66-100% explants regenerated 6.2-11.9 shoots per explant). In both cases cultivars showed similar regeneration ability independently on the type of explants. Low regeneration frequency obtained on other tested media (data not shown) made them unsuitable for further transformation experiments.

Two *Agrobacterium tumefaciens* strains, the disarmed nopaline strain C58C1 and the most commonly used octopine LBA4404 strain were used for transformation. The procedure was similar to the typical one published by Horsch et al. (1985) and routinely used also for potato transformation. To simplify the method, we did not use a feeder layer. The second modification was two day preculture of explants. Negative control showed, that 50 mg/l kanamycin concentration was sufficient for effective selection. There was no callus formation nor shoot regeneration in explants from three tested cultivars.

Level of β -glucuronidase (GUS) activity (which corresponded to expression of introduced gene) was measured in 57 plants regenerated and rooted on kanamycin-containing medium. Fifty one of them were GUS-positive, showing from 2.5 to 100 times higher GUS activity compared to the control plants. Only six plants (derived from one clone) were GUS-negative – the same as control plants. Such low number of GUS-negative plants regenerated on selection medium supported the suitability of kanamycin used as a selective factor for regeneration of transgenic potato. The broad range of GUS expression could be explained by the presence of more than one (per genome) copy number of introduced gene, position effect occurring when the promoter of introduced gene was located closely to plant regulatory sequences (Jones et al. 1987) as well as by specific methylation which could be responsible for stable alteration of structure and expression of the gene (Keshet et al. 1985, Ottaviani et al. 1993).

Figs 1, 2. Shoot formation from leaf explants (1) and tuber explants (2) of cv. Bzura in control experiment (without *Agrobacterium* infection and selection) (A); and after infection and culture on selection medium (B); 8 weeks of culture.

Kanamycin resistant and GUS positive plants (these transformed with strain pAL 4404:pBI 121) were regenerated from tuber explants of Bzura (Fig. 2a) and Elipsa and from leaf explant of Bzura (Fig. 2b) and Brda (Table 2). Sixty three putative transgenic plants were obtained in four single transformation experiments (two experiments with two repeats; about 25 explants each) from Bzura leaf explants using LBA 4404 strain. The other 23 putative transformants were regenerated from Brda leaf explants in one of two repeats of the experiment. There were no plants from leaf explants of Sokół, Irys and Elipsa (for 50 explants tested) after transformation with LBA 4404 strain. For tuber explants a slightly better effect was obtained with the C58C1 strain compared to

LBA 4404. However 29 plants from tuber explants of Bzura and only one from Elipsa were recovered. Northern blot analysis confirmed the presence of β -glucuronidase mRNA transcript in transgenic plants (Fig. 3).

The results showed that the transformation efficiency was strongly dependent on the regeneration ability of tested cultivars. Based on this we could expect that increasing number of explants in transformation experiment increased the probability of obtaining transformants.

Results of transformation published by other authors indicated that transformation efficiency could be increased by selection of better responding type of explants, choosing proper bacterial strain and transformation protocol (Godwin et al.

TABLE 1. Callus formation and shoot regeneration from leaf and tuber explants. The results are expressed as the percentage of responding explants and as the number of shoots regenerated from one explant (in brackets).

Cultivar	Leaf explants			Tuber explants
	% ekspl. forming callus after 4 weeks of culture	% ekspl. regenerating shoots (no. of shoots per explant) after		% of explants regenerating shoots (no. of shoots per explant) after 6 weeks
		4 weeks	8 weeks	
Bronka	100 +	0	0	0
Ruta	80 +	0	0	0
Brda	88 +	56 (7).6	88 (16)	100 (11)
Atol	100 +	40 (3.5)	65 (4.9)	15 (1.2)
Sokół	100 ++	0	26 (2)	0
Irys	100 +	0	9.5 (11)	66 (9.6)
Irga	100 ++	22 (4.5)	60 (12)	18 (1.3)
San	100 ++	18 (2.5)	42 (4.2)	–
Bryza	100 +	0	8 (4)	0
Stobrawa	25 +	0		33 (1.7)
Elipsa	100 +++	100 (6.1)	62 (16.6)	80 (6.5)
Bzura	100 +++	90 (5.3)	100 (13.4)	100 (6.2)

+ – weak callus growth

++ – good callus growth

+++ – intensive callus growth

1992), activating virulence of bacteria with acetosyringone (Stachel et al. 1985, Godwin et al. 1991), applying 5-azacytidine as a demethylating agent (Mandal et al. 1992) and/or using other chemicals enhancing transformation efficiency (James et al. 1993).

Frequency of transformation experiments of potato reported in other papers varied very much, and also it was very stron-

gly dependent on the plant genotype. Wenzler et al. (1989) compared transformation of four potato genotypes. Cultivar Russet Burbank did not regenerate any transformants, Superior regenerated 9 and Desiree 20 shoots from 100 leaf explants, while the line FL1607 routinely gave 400-500 regenerated shoots from the same number of explants.

TABLE 2. Results of plant regeneration in four independent transformation experiments. Number of plants regenerated and rooted on kanamycin containing medium are given in brackets.

Cultivar expl. type	Experiment 1	Experiment 2	Cultivar expl. type	Experiment 3	Experiment 4
Bzura tuber expl.	C58C1 (26) LBA4404 (0)	C58C1 (0) LBA4404 (3)	Brda leaf expl.	LBA4404 (0)	LBA4404 (23)
Elipsa tuber expl.	C58C1 (1) LBA4404 (0)	C58C1 (0) LBA4404 (0)	Sokół leaf expl.	LBA 4404 (0)	LBA 4404 (0)
Bzura leaf expl.	C58C1 (0) LBA4404 (0)	C58C1 (0) LBA4404 (0)	Irys leaf expl.	LBA4404 (0)	LBA4404 (0)
Bzura leaf expl.	LBA4404 (9)	LBA4404 (54)

Fig. 3. Northern blot analysis of total RNA extracted from control (lanes 1,7) and transformed plants cv. Bzura (lines 2, 3, 4, 5 and 6) hybridized with radiolabelled GUS probe. Twenty μg of total RNA from non transformed plant were loaded on lines 1 and 7. Lines 2, 3, 4, 5 and 6 were loaded with 10, 20, 30, 40 and 50 μg of total RNA respectively.

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ZDOLNOŚĆ POLSKICH ODMIAN ZIEMNIAKA DO REGENERACJI I TRANSFORMACJI

STRESZCZENIE

Określono zdolność polskich odmian ziemniaka do regeneracji i transformacji w kulturach *in vitro*. Spośród dwunastu testowanych odmian – odmiany: Brda, Bzura, Elipsa i Irga charakteryzowały się najwyższą zdolnością do regeneracji pędów z eksplantatów liści (60%-100% eksplantatów regenerowało od 4,9 do 16,5 pędów z jednego eksplantatu). Odmianami charakteryzującymi się formowaniem największej liczby pędów z eksplantatów bulw były: Brda, Bzura, Elipsa i Irys (66%-100% eksplantatów regenerowało 6,2-11,9 pędów z jednego eksplantatu). Do transformacji użyto dwóch szczepów *Agrobacterium tumefaciens* zawierających binarny system wektorów: LBA 4404 (pAL4404:pBI121) i C58C1 (pGV2260:pVU104) oraz obydwie typy eksplantatów z czterech odmian. Stwierdzono silną zależność pomiędzy zdolnością do regeneracji i wydajnością transformacji u badanych odmian. Po transformacji eksplantatów liści otrzymano GUS-pozytywne, odporne na kanamycynę i dobrze ukorzeniające się na pożywce z kanamycyną rośliny odmian Brda i Bzura. Po transformacji eksplantatów bulw otrzymano podobne rośliny u odmian Bzura i Elipsa. Obecność transkryptów mRNA β -glukuronidazy w transgenicznym roślinach potwierdzono za pomocą hybrydyzacji typu Northern blot.

SŁOWA KLUCZOWE: regeneracja *in vitro*, transformacja, *Agrobacterium*, *Solanum tuberosum*.