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IN VITRO PROPAGATION AND AGROBACTERIUM- MEDIATED TRANSFORMATION OF POTATO CV. DESIREE

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Shoot cultures of cv. Desiree were established from five different sources and were maintained on MS medium, without phytohormones. The addition of adenine sulfate provided higher number of internodes. One of five clones was virus-free, according to ELISA test. That clone, designed as PKB, was used for transformation. Transformed roots were obtained by inoculating shoot segments with *Agrobacterium rhizogenes* A4M70 GUS. Hairy roots appeared in 90% of explants. The transformation was confirmed by assaying the activity of β- glucuronidase enzyme.

Key words: *Solanum tuberosum* L., potato, micropropagation, *Agrobacterium rhizogenes,* β-glucuronidase.

Ključne reči: *Solanum tuberosum* L., krompir, mikropropagacija, *Agrobacterium rhizogenes*, β-glukuronidaza.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most important food crops in the world. Some genetic characteristics of potato including polyploidy, self-incompatibility and high heterozigosity make application of classical breeding methods difficult in this species. Techniques of genetic engineering via *Agrobacterium* mediated transformation offer interesting oportunities according to which certain useful traits can be directly introduced into economically important potato cultivars.

It is well known that soil bacteria *Agrobacterium tumefaciens* and *A. rhizogenes*, can induce appearance of crown galls and hairy roots in many plant species. Bacteria can transfer specific regions of their Ti plasmids, (T-DNA) into the genome of plant cells (Hoemaka et al., 1984). This natural gene-transferring system, was exploited to transform many plant species, including potato (Zambryski et al. 1983., An et al., 1986). In recent years, many reports have been dedicated to transformation of various potato cultivars.

Ooms et al., (1983) were first to report transformation of potato cv. Moris Bard. They studied the appearance of tumors which appeared after wounding and inoculation of potato shoots with *A. tumerfaciens*. Galls (tumor) tissue could regenerated shoots and roots. Shoots manifested lysopine dehydrogenase activity (LpDH) which is a positive sign of transformation. When these shoots were grafted uppon stems of normal plants they could form stolons and within three months tubers. Among the shoots regenerated from galls authors detected a number of shoots with abnormal morphology and chromosome number. In the further study, the same authors (Ooms et al., 1987), refined transformation method with *A. tumefaciens* and obtained morphologicaly normal transgenic plants of cv. Desiree.

Ooms et al., (1985) also studied transformation of cv. Desiree with *A. rhizogenes* and showed that Ri plasmids also can be used as a vector to introduce genes via Ri T-DNA into potato. Shoots inoculated with the bacteria developed abundant roots which were further cultured and studied. Shoots were regenerated from callus which developed on root cultures. Transformed plants distincly differed from untransformed potatoes. Growth of the transformed plants was more vigorous but the final size of plants and tuber yield were similar.

Shahin and Simpson (1986) developed a transformation system in which leaf discs were cocultivated with disarmed (non oncogenic) LBA4404 *A. tumefaciens* contained binary vector pARC8 with Nos/Npt gene. Transformed nature of plants was assayed by neomycin phospho-transferase II activity.

Tuber slices were used as explants for potato transformation with *A. tumefaciens* in work of Sheerman and Bevan (1988). Shoots appeared within 4 weeks without intervening callus stage. Shoots were first rooted and then screened for transformants on medium supplemented with kanamycine. They worked with Desiree cultivar and out of 200 independant transformants only one was morphologicaly different from parental types. At the same time Stiekama et al., (1988) also presented a study on transformation of Bintje and Desiree using disarmed binary *A. tumefaciens* LBA 4404/ pBi121 vector system. Explants for inoculation with bacteria were tuber discs from which shoots were efficiently regenerated and rooted. In this case, transgenic plants were assayed with GUS test.

Besides the experiments of potato transformation with *A. tumefaciens* strains, different *A. rhizogenes* strains were used too. De Vries-Uijtewaal et al., (1988)

studied transformation of monohaploid and diploid genotypes with *A. tumefaciens* LBA1020 and *A. rhizogenes* LBA 9402, both containing the Ri1855 plasmid. Transformation efficiency was generaly higher in diploids than monohaploids. Hanish ten Cate et al., (1988) obtained transformed root lines of Bintje and Desiree with the LBA 9402 and AR 15834 using leaf segments and tuber discs as explants. Shoots were induced from roots but even more from compact green callus adjoining roots. However only 10% of transformed root lines could regenerate shoots. Transgenic Ri-Desiree plants were all uniform and corresponded to the normal phenotype whilst Ri-Bintje plants showed a pattern of phenotypic variation.

Beside those authors, Visser et al., (1989) transformed potato using a binary vector in virulent *A. rhizogenes* strains (AM8703/ pRi1855 and pBi121). The transformation efficiency was much higher with *A. rhizogenes* then *A. tumefaciens*.

De Block (1988) transformed Desiree, Bintje, Berolina and Russet Burbank by co-cultivation of leaves in bacterial suspension of C58C1 strain carring *nptII* and *bar* genes. The *bar* gen codes for the enzyme phosphinotricin acetyltransferase (PAT) which inactivates herbicide phosphinotricin (glufosinate). Thus transformed plants were resistant to commercial herbicide Basta at high concentration (20 l/ha). Almost recently Figuera Filho et al., (1994) reported upon transformation of several Brazilian potato cultivats with *A. tumefaciens* carrying the pGV1040bar confering the resistance to phosphinotricin herbicides.

Newell et al., (1991) transfered potao virus X and Y coat protein genes into Russet Burbank, using *A. tumefaciens* pMON 9809 vector containing the PVX coat protein gene. The levels of PVX coat protein in transformed shoots were detected by an ELISA assay.

One of the last reports on potato transformation was Nadolska-Orczyk et al., 1995 who reported successfull transformation of 12 polish potato cultivars. The authors used well known strains of *A. tumefaciens* LBA 4404/ pBi121 and C58C1/ pVU104, carrying the NPT and GUS genes. They regenerated plants from leaf explants, which rooted well.

In our country Desiree is the leading potato cultivar presenting 80% of the total potato production. We therefore decided to use cultivar Desiree as a standard model system in our investigation on potato. In this paper we present preliminary research on the use of *in vitro* methods and *Agrobacterium* mediated transformation in the breeding of new, improved potato cultivars.

For transformation studies we used *Agrobacterium rhizogenes*, strain A4M70GUS, which induces hairy roots. This strain carries gene coding for enzyme β-glucuronidase, whose activity can be detected histochemically in the transformed tissues.

MATERIAL AND METHODS

Plant culture: Shoot cultures of cv Desiree were established from etiolated tuber sprouts. Surface sterilization was performed for 20 minutes in 10% solution of commercial bleach containing 4-6% NaOCl. MS (Murashige & Skoog, 1962) medium which was used in all experiments was supplemented with 3% sucrose. Subculturing was performed by segmentation at regular 3-4 week intervals. Segments containing single axillary bud were at least 5 mm long. Cultures were maintained in 100 ml wide neck Erlenmayer flasks or 150 ml blod transfusion bottles. Both types of culture vessels were stopped with cotton wool plugs. Clone PKB which we used for transfor-

mation studies was virus-free according to ELISA test (Sigma enzyme immunoassay kit for potato virus A, M, S, X, Y, detection). Test was performed in Center for potato, Guča by Dr D. Milošević.

Conditions in the growth room: Photoperiod was 18/6 hours light to darknes, provided by cool white fluorescent lamps, irradiance 5.0-7.2 Wm^{-2} and temperature 25 $\pm 2^{o}C$.

The transformation procedure: The *A. rhizogenes*, A4M70GUS (Tepfer, M. and Delbart, C. F. 1987) was maintained on agar (1,5%) solidified YEB medium (Van Larebeke et al., 1977), with antibiotic neomycine. The density of bacterial suspension was about 10^8 bacteria/ml. The 2 cm long shoot segments were inoculated by wounding with a sterile needle, shortly dipped in the bacterial suspension. The explants were then left on the same media in the growth room, and after 2 days transferred to a medium supplemented with cefotaxime, 100 mg/l. Culture were screened after four weeks. About 50 pieces of potato shoots were inoculated in one experiment.

GUS assay: Roots were cut from the stems and β-Glucuronidase enzyme activity was detected, using X-gluc at pH 7.0, after overnight incubation at 37°C (Jefferson et al., 1987).

RESULTS AND DISCUSSION

Establishment and maintenance of shoot cultures

Shoot cultures of cv. Desiree were established from five different sources. Only one of them, designated clone PKB, was found to be virus free. This clone was used in further experiments, while the others was eliminated. Clone PKB from the beginning had more sturdy shoots than the other four introductions.

In a preliminary study on the maintenance of potato shoot cultures we showed that addition of cytokinins to the medium offers no improvement in standard growth parameters. Thus on the hormone free medium the mean number of internodes per explant was 8.6 ± 0.2 and the shoot length 81.6 ± 2.9 mm. Only the addition of adenine sulfate was benefitial providing higher number of internodes per explant 9.7 ± 0.2 and somewhat longer shoots 89.4 ± 2.5 mm at 100 mg/l. Growth of shoot cultures on the hormone free medium was stable and here was no visible variation in growth pathern of successive subcultures.

In transformation studies on potato in which shoots were used as explants for inoculation (Ooms et al., 1983, 1985, 1987 and others) shoots were previously cultured on the hormone free medium. In contrast, the use of leaf and tuber tissue requires presence of both cytokinin and auxin type growth regulators.

Transformation

The appearance of hairy roots after transformation was fast. First root could be observed in the inoculation zone within ten days. This hairy roots could be easily distinguished from adventitious roots which developed on the cut end of shoot explants. Production of roots was not accompanied by formation of callus.

After four weeks on the cefotaxime supplemented transformation medium development of hairy roots was registered in 90% explants i.e 45 out of 50 inoculated shoots.

To investigate the ability of hairy roots for individual growth they were excised and re-cultured on the same type medium. Roots not only elongated well but also produced numerous laterals which enabled us to establish true root cultures. Spontaneous regeneration of shoots was not recorded.

Rapid growth associated with high branching of roots obtained after inoculation of plants with *A. rhizogenes* is a good preliminary indication of successfull transformation (Dobigny, A. et al., 1995). The hairy roots tested for GUS activity were coloured uniformly blue (Fig. 1, Fig. 2). This can be accepted as a proof for positive transformation of potato roots.

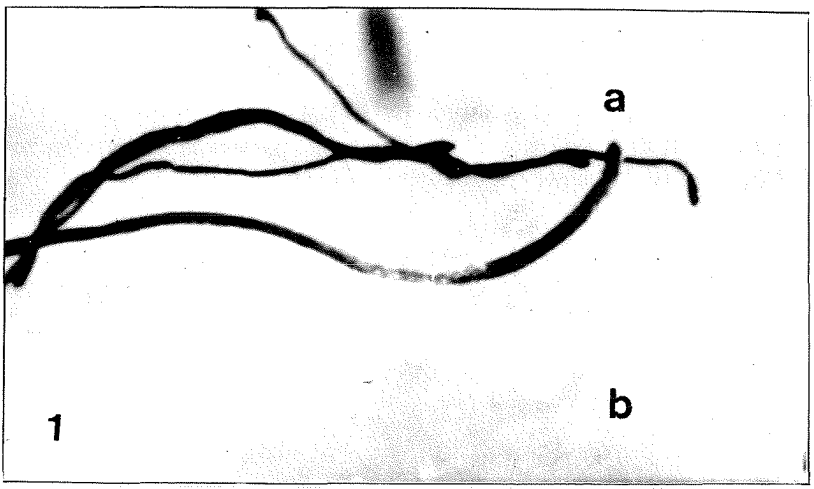

Fig. 1. – Detection of β-glucuronidase activity by histological assay: transformed root becomes blue (a), while control root remains white (b).

Fig. 2. – Phenotypes of root lines: most of the transformed root are highly branched and exhibited a high growth rate

GUS positive roots were fixed in FAA fixative and prepared for hystological investigation which we plan to perform in due course.

The *A. tumefaciens* strain A4 is agropine type, whose Ri plasmid contains two fragments of T-DNA, a TL-DNA carrying the *rol* genes and a TR- DNA carrying genes encoding for opine and auxine synthesis. So, the presence of TL- DNA in transformed tissues can not be detected only by opine marker, and that is the reason we did not performed that analysis.

In this study, a protocol for genetic transformation in potato using a *A. rhizogenes* A4 M70GUS was successfully developed. This transformation method can be used as a routine method for introducing foreign genes into local potato cultivars.

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REFERENCES

An, G., Watson, B.D. & Chiang, C. C. (1986): Transformation of tobacco, tomato and potato and *Arabidopsis thaliana* using a binary Ti vector system. – Plant. Phyiol. *81*: 301-305.

DeVries - Uijtewaal, E., Gilissen, L. J. W., Flipse, E., Sree Ramulu, K. & De Groot, B. (1988): Characterization of root clones obtained after transformation of monohaploid and diploid potato genotypes with hairy root inducing strains of *Agrobacterium*. – Plant Science, *58*: 193-202.

De Blosk, M. (1988): Genotype-independent leaf disc *transformation* of potato (*Solanum tuberosum*) using *Agrobacterium tumefaciens*. – Theor. Appl. Genet. *76*: 767-774.

Dobigny, A., Ambroise, A., Haicour, R., David, C., Rossignol, L. & Sihachakr, D. (1995): Transformation of potato using mannopine and cucumopine strains of *Agrobacterium rhizogenes*. – Plant Cell, Tissue and Organ Culture *40*: 225-230.

Figuera Filho, S. E. Figueiredo, L. F. A. & Monte - Neshich, D. C. (1994): Transformation of potato (*Solanum tuberosum*) cv. Mantiqueira using *Agrobacterium tumefaciens* and evaluation of herbicide resistance. – Plant Cell Reports *13*: 666-670.

Hanischten Cate, Ch. H., Ennik, E., Roest, S., Sree Ramulu, K., Dijkhuis, P. & de Groot, B. (1988): Regenaration and characterization of plants from potato root lines transformed by *Agrobacterium rhizogenes*. – Theor. Appl. Genet. *75*: 452-459.

Hoekama, A., Hooykaas, P. L. & Schilperoort, R. A. (1984): Transfer of the octopine T-DNA segment to plant cells modified by different types of *Agrobacterium* Tumor or Root inducing plasmids. Generality of virulence systems. – Journal of Bacteriology *158*: 383-385.

Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. (1987): GUS fusions: Ω-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. - EMBRO J. *6*: 3901-3907.

Murashige, T. & Skoog, F. (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. – Physiol. Plant. *15*: 473-497.

Nadolska - Orczyk, A., Milkowska, L., Palucha, A., Czembor, P. & Orczyk, W. (1995): Regeneration and transformation of polish cultivars of potato. – Acta Soc. Bot. Poloniae *4*: 335-340.

Newel, C. A., Rozman, R., Hinchee, M. A., Lawson, E. C., Haley, L., Sanders, P., Kaniewski, W., Tumer, N. E., Horsch, R. B. & Fraley, R. T. (1991): *Agrobacterium* - mediated transformation of *Solanum tuberosum* L. cv. Russet Burbank. – Plant Cell reports *10*: 30-34.

Ooms, G., Karp, A. & Roberts, J. (1983): From tumour to tuber; tumour cell characteristics and chromosome numbers of crown gall-derived tetraploid potato plants (*Solanum tuberosum* cv. „Maris Bard"). – Theor. Appl. Genet. *66*: 169-172.

Ooms, G., Karp, A., Burrell, M. M., Twell, D. & Roberts, J. (1985): Genetic modification of potato development using Ri T-DNA. – Theor. Appl. Genet. *70*: 440-446.

Ooms, G., Burrell, M. M., Karp, A., Bevan, M. & Hille, J. (1987): Genetic transformation in two potato cultivars with T-DNA from disarmed *Agrobacterium*. Theor. Appl. Genet. *73*: 744-750.

Shahin, E. A. & Simpson, R. B. (1986): Gene transfer system for potato. – Hort. Science *21*: 1199-1201.

Stiekama, W. J., Heidekamp, F., Louwerse, J. D., Verhoeven, H. A. & Dijkhuis, P. (1988): Introduction of foreign genes into potato cultivars Bintje and Desiree using an *Agrobacterium tumefaciens* binary vector. – Plant Cell Reports *7*: 47-50.

Sheerman, S., Bevan, M. W. (1988): A rapid transformation method for *Solanum tuberosum* using binary *Agrobacterium tumefaciens* vectors. – Plant. Cell Reports *7*: 13-16.

Tepfer, M. & Delbart, C. F. (1987): *Agrobacterium rhizogenes* as a vector for transformating higher plants. – Microbiol. Sci. *4*: 24-28.

Van Larebeke, N., Genetello, C. H., Hernalsteens, J. P., De Picker, A., Zaenenl., E., VanMontagu, M. & Schell, J. (1977): Transfer of Ti plasmids between *Agrobacterium* strains by mobilisation with the conjugative plasmid RP4. – Molec. Gen. Genet. *152*: 119-124.

Visser, R. G. F., Jacobson, E., Witholt, B. & Feenstra, W. J. (1989): Efficient transformation of potato (*Solanum tuberosum* L.) using a binary vector in *Agrobacterium rhizogenes*. – Theor. Appl. Genet. *78*: 594-600.

Zambryski, P., Joos, H., Genetello, C., Leemans, J., VanMontagu, M. & Schell, J. (1983): Ti plasmid vector for the introducing of DNA into plant cells without alteration of their normal regeneration capacity. – EMBO, J. 2: 2143-2150.

Rezime

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IN VITRO PROPAGACIJA I TRANSFORMACIJA KROMPIRA CV. DESIREE POMOĆU *AGROBACTERIUM RHIZOGENES*

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Kulture pupoljaka cv. Desiree uspostavljene su iz pet različitih klonova i održavane na MS medijumu bez fitohormona. Primećeno je da dodavanje adenin sulfata dovodi do povećanja broja internodija. Jedan od pet klonova, označen kao PKB, koji je korišćen za transformaciju, bio je slobodan od virusa, prema ELIZA testu. Transformisani korenovi su dobijeni inokulacijom sa *Agrobacterium rhizogenes* sojem A4M70GUS. Korenovi su se pojavili na 90% eksplantata. Transformacija je potvrđena testom za određivanje aktivnosti β-glukuronidaze.