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Transformation of a recalcitrant potato cultivar by an *Agrobacterium* binary vector

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SUMMARY

The CzechoSlovak potato cultivar Lipa was recalcitrant to transformation using established procedures. A modified leaf disc procedure was developed, which allowed the transformation of Lipa with the *Agrobacterium* Ti plasmid-based binary vector pARC8. After transformation, leaf explants were incubated on callus-inducing selection medium containing 50 mg/l kanamycin for 6 weeks, before selective conditions for shoot induction were applied. Shoot induction was continued for up to 5 months. Transgenic plants were obtained from shoots on selective root-induction medium. Southern-blot analysis confirmed the presence of T-DNA from the vector pARC8 in 1–2 copies per tetraploid genome in all rooted transgenic clones.

Key-words: Agrobacterium tumefaciens, binary vector, plant transformation, potato (*Solanum tuberosum* L.).

INTRODUCTION

In recent years various methods have been developed for the introduction of single genes into the plant genome (for review, see Kung & Arntzen 1989). The Ti-plasmid of *A. tumefaciens* has been shown to be a convenient system for in-vitro transformation of dicotyledonous crops, such as tomato and potato, from which transformed cells can be regenerated to whole plants. Substantial differences are observed however, in the efficiency of transformation between species and between cultivars within one species. These differences in transformation efficiency are probably related to the relative ease of cell regeneration after DNA transfer.

In the present study, we have focused on the transformation and regeneration of the CzechoSlovak *Solanum tuberosum* cultivar 'Lipa'. This tetraploid potato cultivar originates from crossings between the cultivars Ker-Pondy and Tatranka carried out in 1973 at the Potato Research and Breeding Institute in Velka Lomnica, CSFR. Lipa has been selected as one of the cultivars that may become agronomically important in CzechoSlovakia because of its consumptive qualities and field performance (Smalik & Hrobak 1974). The cultivar is, however, relatively sensitive to potato leaf-roll virus and nematodes, and therefore it was not included in the CzechoSlovak list of licensed potato cultivars. Because of the favourable characteristics of this tetraploid cultivar, the application of a molecular breeding strategy that tackles specifically the few undesirable traits may prove to be faster in improving this cultivar than applying classical breeding programs.

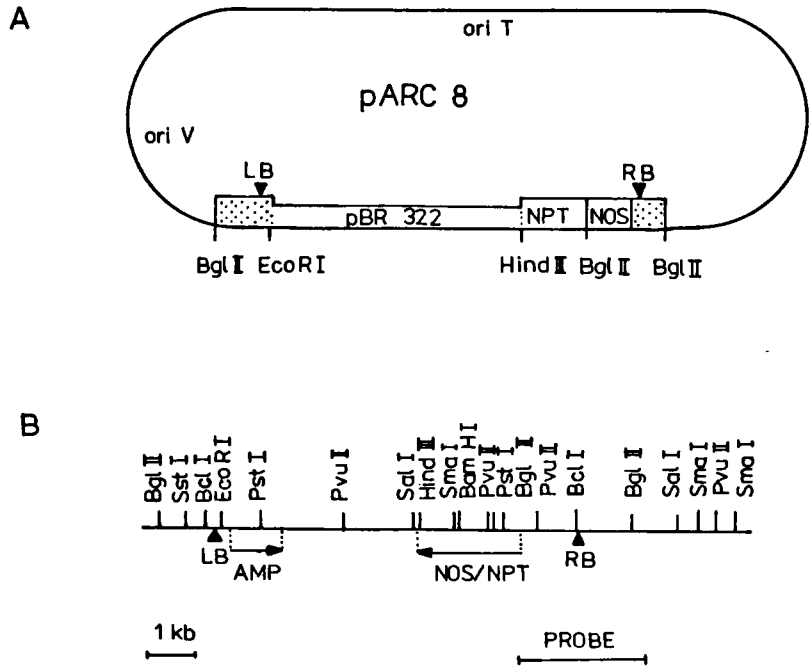

Fig. 1. Schematic drawing of the binary vector pARC8 (a) and restriction map of the T-DNA region from pARC8 (b). The black triangles indicate the left (LB) and right (RB) border of the T-DNA. In (b), the fusion between the promoter of the nopaline-synthase (NOS) gene to the neomycin phosphotransferase (NPT) coding region is indicated by an arrow. The β-lactamase gene for bacterial ampicillin-resistance is indicated by AMP and an arrow. The BglII fragment used as probe is indicated by a black bar.

Several procedures for obtaining transgenic potato plants have been published. These procedures differ in explant used, overall efficiency, and time required for regeneration. The transformation of potato callus by cocultivation with *Agrobacterium* required a long time of regeneration (Shahin & Simpson 1986; Ooms *et al.* 1987). Modifications of the leaf disc method, as originally described by Horsch *et al.* (1985), yielded transgenic potato plants in about 7–20 weeks, depending upon, among other things, the genotype used (An *et al.* 1986, De Block, 1988, Knapp *et al.* 1988; Tavazza *et al.* 1988). A more efficient method published was the regeneration in 4–6 weeks of transformed potato shoots from *Agrobacterium*-infected tuber discs (Sheerman & Bevan 1988; Stiekema *et al.* 1988), while the selection of a transformation-prone genotype (Wenzler *et al.* 1989), or the introduction of an adventitious shoot regeneration-protocol (Visser *et al.* 1989) also enhanced transformation efficiencies. As the established leaf and tuber disc transformation methods proved unsuccessful for cultivar Lipa, we focused on developing a transformation procedure starting from in-vitro grown leaf material using the binary vector pARC8 (Simpson *et al.* 1986). This vector has been shown to transfer its T-DNA from both *A. rhizogenes* (Simpson *et al.* 1986) and *A. tumefaciens* (McKnight *et al.* 1987), to plant cells including potato (Shahin & Simpson 1986).

MATERIALS AND METHODS

Plasmids and Bacteria

The binary plasmid pARC8 is a 28 kb plasmid, including a T-DNA of about 7 kb (Fig. 1a) (Simpson *et al.* 1986). The T-DNA houses the promoter of the nopaline-synthase gene

fused to the gene for neomycin phosphotransferase, thereby conferring resistance to the antibiotic kanamycin, in addition to the bacterial gene for resistance to ampicillin from the bacterial vector pBR322 (Fig. 1b). *Escherichia coli* HB101(pARC8) was used as donor strain for mobilizing the binary plasmid pARC8 to *Agrobacterium tumefaciens* LBA4404 (Hoekema *et al.* 1983) in a triparental mating using *E. coli* MM294/pRK2013 (Ditta *et al.* 1980) as helper strain. Transconjugants were selected at 30°C on minimal A medium (Miller 1972) containing 20 mg l^{-1} rifampicin and 5 mg l^{-1} tetracycline. For plant transformation, *A. tumefaciens* LBA4404(pARC8) was grown for 24 h in minimal A medium supplemented with 5 mg l^{-1} tetracycline, and diluted to a density of $1 \cdot 10^8$ cells ml^{-1} in MS medium.

Chemicals and enzymes

Plant growth hormones and vitamins were purchased from Serva, agar was from Difco Laboratories. A nick-translation kit was obtained from Amersham, and restriction endonucleases were obtained from USOL, CSFR.

Potato transformation and regeneration

Discs of about 0·5 cm in diameter were cut from the leaves of 4-week-old potato cultivar Lipa plantlets grown *in vitro* using a cork borer and sterile conditions. Discs were submerged in a diluted *A. tumefaciens* LBA4404(pARC8) culture for 10 min after which the discs were blotted dry. The discs were placed on callus-inducing agar plates, containing MS salts (Murashige & Skoog, 1962), 3% sucrose, 0·8% agar, 5 mg l^{-1} NAA and 1 mg l^{-1} BAP and were incubated at 25°C, 3500 lux and a regime of 16 h light and 8 h darkness. After 2 days of incubation, discs were transferred to agar plates containing the same callus-inducing medium containing 500 mg l^{-1} Claforan and 50 mg l^{-1} kanamycin. Discs were transferred to fresh medium every 3 weeks. From 6 weeks on, callus that developed at the cutting edge of the leaf discs was isolated and incubated on shoot-inducing medium containing MS salts, 3% sucrose, 0·8% agar, 0·01 mg l^{-1} NAA, 0·01 mg l^{-1} GA_3, 1 mg l^{-1} zeatin and 50 mg l^{-1} kanamycin. Calli were also transferred to fresh medium every 3 weeks. Shoots reaching a size of 1 cm were transferred to root-inducing medium (MS salts, 2% sucrose, 0·5% agar, 0·1 mg l^{-1} IAA and 50 mg l^{-1} kanamycin). Rooted plantlets were transferred to soil and maintained in the greenhouse. For in-vitro tuberization, stem cuttings were incubated in the dark on MS medium containing 8% sucrose and 2·5 mg l^{-1} BAP as described by Stiekema *et al.* (1988).

DNA manipulations

Plant DNA was extracted from 0·5 g in-vitro grown leaf material as described by Deblaere *et al.* (1987). Restriction enzyme digestions, agarose gel electrophoresis, Southern blotting, nick translation and hybridizations were performed according to established procedures and protocols (South 1975; Maniatis *et al.* 1982). Autoradiography was carried out at room temperature for 72 h using Medix Rapid film (Fotochema, CSFR). The probe used for hybridization was a 2·6 kb BglII fragment from pARC8, carrying the NOS promoter, the right border of the T-DNA and a part of vector DNA that is not transferred to the plant (Fig. 1).

RESULTS

In preliminary experiments the required level of kanamycin selection was determined. Callus induction on untransformed discs was substantially suppressed at a kanamycin

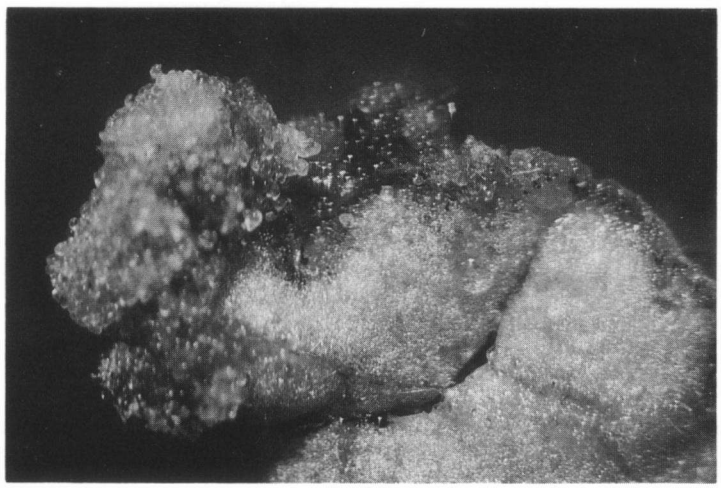

Fig. 2. Callus development on the cutting-edge of leaf discs from potato cultivar Lipa grown on kanamycin containing callus-inducing medium, 6 weeks after the discs had been immerged in a suspension of *A. tumefaciens* LBA4404(pARC8).

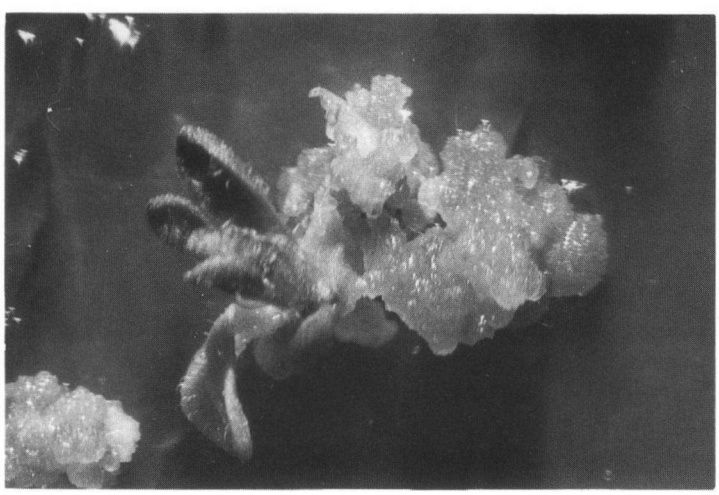

Fig. 3. Shoot development from callus incubated for 4 months on kanamycin containing shoot-inducing medium.

concentration of 25 mg l^{-1}, while at a kanamycin concentration of 50 mg l^{-1} no callus was formed at all. Therefore, selection for transformation was based on the ability to form callus and shoots on medium with 50 mg l^{-1} kanamycin. In a typical experiment, approximately half the number of discs formed callus on the kanamycin containing callus-inducing medium, some discs forming considerable amounts of callus (Fig. 2). The kanamycin resistant calli had to be cultivated 4–5 months on shoot-inducing medium before shoot formation was apparent (Fig. 3). The majority of shoots formed subsequently also rooted on root-inducing medium containing 50 mg l^{-1} kanamycin. All plants that rooted on 50 mg l^{-1} kanamycin also rooted on 100 mg l^{-1} kanamycin.

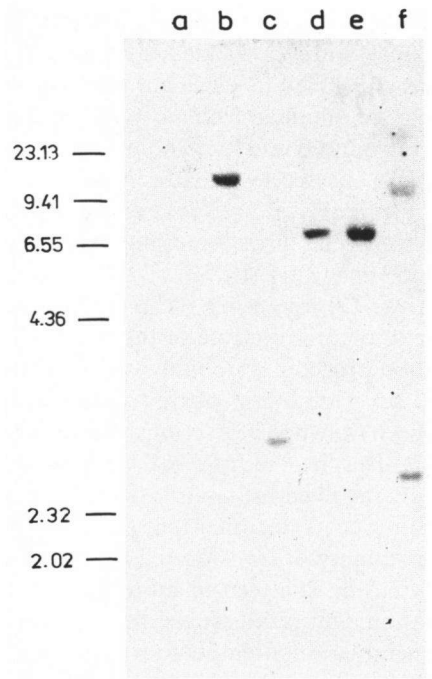

Fig. 4. Autoradiograph of a southern blot containing 10 μg of BglII-digested genomic potato DNA from the untransformed-cultivar Lipa (lane a) and five independent transformants (lanes b–f), hybridized with the ^{32}P-labeled BglII fragment from pARC8 indicated in Figure 1. The fragments of lambda DNA digested with HindIII were used as size markers.

Five transgenic potato plants were obtained from 70 leaf discs. After transfer to soil and propagation in the greenhouse, the transgenic regenerants were compared with the untransformed Lipa parent. Regenerated transgenic plants did not show significant changes from the untransformed parent with respect to the shape of leaves, vigour, tuber setting or tuber shape. Also no difference was observed in in-vitro tuberization between transgenic and untransformed plants.

To prove the transgenic nature of the kanamycin resistant plants at the molecular level, the stable integration of the T-DNA was verified by southern blotting and hybridization. The result of the hybridization analysis is shown in Figure 4. It can be concluded that in all kanamycin resistant plants (lane b–f) T-DNA sequences could be demonstrated, while such sequences were not detectable in DNA from the untransformed parent (Fig. 4, lane a). Four out of five plants analysed (lanes b–e) showed only one copy of the T-DNA, whereas the other plant (lane f) appeared to contain two copies of the T-DNA per tetraploid genome.

DISCUSSION

In our laboratory, the potato cultivar Lipa proved very recalcitrant to transformation. Neither the leaf disc method as described by De Block (1988) and Knapp *et al.* (1988), nor the tuber disc method as described by Stiekema *et al.* (1988) yielded any shoots, while the relatively easy-to-transform potato cultivar Desiree could be transformed using either

method, with efficiencies that were comparable to the published efficiencies. The procedure described in this paper however, yielded transgenic Lipa plants in 6–7 months. The procedure is time-consuming, but its efficiency is reasonable, as approximately five transgenic potato plants can be obtained from only 70 leaf discs. The number of one to two T-DNA copies stably introduced into the genome of Lipa does not differ from results published by others for either the species potato or the vector pARC8 (Simpson *et al.* 1986), although more detailed analyses are required for estimating the precise number of T-DNA inserts in our transgenic lines, especially with respect to the occurrence of (in)direct repeats and/or incomplete inserts.

Transformation of cultivar Desiree using the method described here took only 10 weeks, which supports the recalcitrant nature of the cultivar Lipa. The most important feature of the transformation procedure probably is the prolonged phase (six weeks) of callus induction, followed by a long-term phase (several months) of shoot induction. Somaclonal variation has been shown to be a serious problem in regeneration from potato callus (Sree Ramulu 1987). Despite the long callus-phase, somaclonal variation does not seem to be substantially involved in this transformation and regeneration scheme. Possibly the transformation event and concurrent selective conditions contribute to or select for a higher genetic stability of transformed callus. However, larger numbers of transgenic Lipa plants should be analysed in more detail to assess the occurrence of potential somaclonal variation. Our results suggest that the introduction of a prolonged callus phase may be of general use in attempts to transform and regenerate recalcitrant cultivars or species.

The procedure we have developed will enable the transfer to potato cultivar Lipa of genes that specify agronomically important properties. The plants then obtained are likely to be useful in agricultural practice, either directly or as source material for subsequent breeding programs, provided regulations for the introduction of genetically modified potato cultivars into the environment are established in our country.

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