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Selection of somatic hybrids between diploid clones of potato (*Solanum tuberosum* L.) transformed by direct gene transfer

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Summary. Five diploid potato clones have been transformed by electroporation of protoplasts with different selectable markers. The resulting diploid regenerated plants have been used in somatic hybridization. It has been shown that hybrid cell selection on the basis of antibiotic or herbicide resistances brought by the two parents of fusion is an efficient method for the recovery of tetraploid somatic hybrids.

Key words: *Solanum tuberosum* – Protoplast – Electroporation – Somatic hybrids

Introduction

Diploid clones of *Solanum tuberosum* L. have been used for crossing with diploid wild species. Introduction of resistance genes and increasing heterozygosity of the potato genus are the major goals of this breeding programme. However, tetraploid clones of potato are more vigorous and produce more tubers than diploid clones. Supplementary increase of heterozygosity during chromosome doubling for tetraploid creation is critical (Wenzel et al. 1979). One way to accomplish this is to fuse protoplasts from two different diploid clones and regenerate a tetraploid hybrid combining both parental genomes. Different approaches have been followed for protoplast fusion and recovery of somatic hybrids. Hybrid vigour in calli resulting from protoplast fusion of diploid protoplasts has been shown to have no relation to any real hybrid nature of the regenerated plants (Wenzel et al. 1979). After protoplast fusion and mass culture of the cells, certain particularly favourable types of hybrid cells have been recovered, such as interspecific hybrids between *Solanum tuberosum* and *Solanum brevidens* (Austin et al. 1985a; Ehlenfeldt and Helgeson 1987; Helgeson et al. 1986) or between two different *Solanum tuberosum* clones (Austin et al. 1985b; Deimling et al. 1988; Waara et al. 1989). Fused protoplasts could be selected by using a cell-sorter or by micromanipulation (Puite et al. 1986). In this case, and despite the high number of fusion products selected by cell-sorting, somatic hybrids were highly instable and chromosomes of one partner have been partially lost.

We propose an alternative procedure making use of introduced resistance genes. Chimaeric genes carrying coding sequences of proteins conferring antibiotic or herbicide resistance can be introduced into plants, and all their cells express this resistance (Paskowski et al. 1984). The use of such selectable markers at the cellular level could greatly simplify the recovery of somatic hybrids. For example, after fusion between protoplasts from a kanamycin-resistant diploid clone and protoplasts from a hygromycin-resistant diploid clone, colonies resistant to both antibiotics could be expected to be somatic hybrids. The high number of hybrid colonies which can be selected should permit the regeneration of several different plants, among which a true tetraploid somatic hybrid should be present. Foreign genes have been introduced into the potato genome by using *A. rhizogenes* (Ooms et al. 1986), but the regenerated plants displayed phenotypic abnormalities due to the expression of Ri T-DNA genes. Thus, these plants were not usable for agronomic purposes. Transformation using *Agrobacterium tumefaciens* (Ooms et al. 1987) is a suitable method for the introduction of cloned genes into the potato genome. But only a few clones have been transformed and a high proportion of regenerated plants exhibited somaclonal variation. Moreover, this transformation method is still restricted to tetraploid clones of potato. We describe here an alternative method for potato transformation using

direct gene transfer by electroporation. A procedure for protoplast culture and plant regeneration adapted to a wide range of diploid clones of potato has been previously described (Masson et al. 1987). This procedure seemed especially well adapted for low cell density culture and plant regeneration. Thus, even rare events such as transformed cells or hybrid cells could be recovered. Protoplasts from transformed plants have been used for fusion experiments, and cells have been selected for a combination of both parental resistances. The efficiency of cell selection for the recovery of tetraploid somatic hybrids was studied.

Materials and methods

Diploid clones of potato

The following clones, created in the INRA breeding station of Landerneau (France) have been used in these experiments. 45: *Solanum tuberosum* hybrid (50.12.4 H2); 90: *Solanum tuberosum* × *S. phureja* (Maris Page H1 × *S. phureja*); 71: *Solanum tuberosum* × *S. vernei* (50.12.4 H2 × *S. vernei*); 217: *Solanum tuberosum* × *S. chacoense* (Palma H4 × *S. chacoense*); 275: *Solanum tuberosum* × *S. stenotomum* (Keltia H9 × *S. stenotomum*).

Plasmids

The following plasmids have been used in transformation experiments. pABD1: (Paskowski et al. 1984) conferring kanamycin resistance to plants; pCel40: (Waldron et al. 1985) conferring hygromycin resistance to plants; and pGH1: (Haughn et al. 1988) conferring chlorsulfuron resistance to plants.

Protoplast isolation

Shoot tips *Solanum tuberosum* diploid clone 90 (Maris Page H1 × *Solanum phureja*) were grown for 3 weeks under the culture conditions described previously (Masson et al. 1987). Protoplasts were obtained by overnight digestion of sliced leaves in a medium containing 0.2% Cellulase R10, 0.2% Macerozyme, 2.9% glycine, 10^{-3} g/l naphthalene acetic acid, 10^{-3} g/l benzylaminopurine and 2×10^{-4} g/l 2,4 dichlorophenoxyacetic acid (pH of the preparation was adjusted to 5.8). Protoplasts were collected after filtration of the suspension through a 40 µ*M* steel mesh, by centrifugation at 100 g and washed once with a rinse medium containing 1.5% KCl, 0.2% $CaCl_2$ at pH 5.8.

Electroporation conditions

After isolation, protoplasts were rinsed in a pH 7.2 buffer – MKCL – (Guerche et al. 1987) containing 70 g/l mannitol, 0.372 g/l KCl and 0.042 g/l 3-(N-morpholino)propanesulphonic acid. Protoplasts were then resuspended at a density of 2×10^6/ml in MKCL buffer and kept at 4 °C. Ten micrograms of circular plasmid (purified on cesium chloride gradients) and 50 µg of sonicated calf thymus DNA were mixed with 1 ml of protoplast suspension. The mixture was kept at 4 °C for 10 min. A capacitor of 16 µF was charged at 250 V with a power supply. Samples were pulsed in a 1-cm^3 rectangular cuvette. The three electric pulses delivered were monitored with an oscilloscope (Masson et al. 1988).

Protoplast culture, selection of transformed cells and plant regeneration

After electroporation, 2×10^6 protoplast were plated into three dishes and cultivated as described in Masson et al. (1987). Medium C was supplemented with either 20 mg/l of paromomycin (Guerche et al. 1987), 20 mg/l hygromycin B or 16 µg/l chlorsulfuron for the selection of transformed cells. Transformed calli were transferred to regeneration medium D.

Drug resistance tests of transformed plants

Resistance tests were performed on shoot tips from transformed plants on the propagation medium P (Masson et al., 1987) supplemented with kanamycin, hygromycin B or chlorsulfuron. Drugs were added to the propagation medium after autoclaving. Resistance tests were also performed on protoplast-derived colonies from the clone 90, 90HR (hygromycin resistant), 90KR (kanamycin resistant) and Hybrid (90HR + 90KR) plants. Protoplasts were cultivated following the normal procedure. Colonies were pelleted by centrifugation of 15-day-old colony suspensions and plated at the same density (3×10^3 colonies/ml) for assays in C medium supplemented either with paromomycin, hygromycin or a combination of both antibiotics.

Protoplast fusion, selection of hybrid cells and plant regeneration

After isolation, protoplasts from KR, HR and CSR plants were resuspended at 2×10^6 protoplasts/ml in the rinse medium. Parental protoplasts were mixed in 1:1 proportion and fused by mixing 300 µl of suspension with 300 µl of 30% PEG 6000, 4% $CaCl_2 \cdot 2H_2O$, pH 6.5–7 in petri dishes. After 20 min, droplets were gently diluted with 10 ml of medium A. After 15 days of culture, hybrid colonies were selected in medium C containing 20 mg/l paromomycin and 20 mg/l hygromycin B, 20 mg/l paromomycin and 18 µg/l chlorsulfuron or 20 mg/l hygromycin and 18 µg/l chlorsulfuron. Antibiotic-resistant calli were transferred to regeneration medium D.

Molecular analysis of transgenic plant genomes

Plant DNA was extracted from 1 g of fresh green leaves of wild type, KR, and HR plants by the method of Dellaporta (Dellaporta et al. 1983), followed by centrifugation in a CsCl ethidium bromide gradient. For Southern blot analyses, 2 µg of plant DNA were restricted with enzymes (Bethesda Research Laboratories) according to the manufacturer's specifications. Fragments were separated by electrophoresis in 0.7% horizontal agarose gels and transferred to Hybond-N membranes following the Southern procedure (Southern 1975). Detections of APHIII' and APHIV coding sequences were performed with an EcoR5 fragment of pABD1 and a BamH1 fragment of pCel40, respectively. DNA probes were labelled with ^{32}P dCTP by random priming (Feinberg and Vogelstein 1983).

Cytological analysis

Root tips or buds from plants grown in the greenhouse were pretreated in bromonaphthalene (2% w/v in distilled water) and fixed in Carnoy's solution (95% ethyl alcohol, acetic acid and chloroform 6:1:3). Root tips were then subjected to hydrolysis in 2*N* HCl at 60 °C for 10 min followed by treatment with pectinase for 1 h. Staining was performed with Schiff's solution. Squash preparations were made in acetocarmine before chromosome counting.

Results

Regeneration of diploid-transformed potato clones carrying different selectable markers

Plant transformation with plasmids pABD1, pCel40 and pGH1. The standard procedure described in 'Material and methods' was used with protoplasts of diploid clones, nos. 45, 90, 71, 217, 275. Three different marker genes were used conferring kanamycin resistance (pABD1), hygromycin resistance (pCel 40) and chlorsulfuron resistance (pGH1). Keeping protoplast suspensions in the dark for 15 days was critical for obtaining fast-growing colonies and recovering transformed colonies. After dilution of colony supensions with C medium and addition of selective agents, petri dishes were transferred to the light. Resistant colonies began to appear 15 days after the start of selection (Fig. 1). The medium C used in selection steps seemed to be particularly well adapted to low cell density culture (single colonies were able to grow in 10 ml of medium C). Fifteen days later, calli were transferred to regeneration medium D either supplemented with the drug or not. All the regenerated plants were transferred to propagation medium supplemented with drugs for confirmation of their resistance. Transformed plants were able to grow on propagation medium supplemented with 20 mg/l hygromycin or 20 mg/l paromomycin or with 18 µg/l chlorsulfuron, whether they were obtained on a regeneration medium with or without the drug. This demonstrated that selection conditions for transformed colonies were efficient and that it was not necessary to maintain selection pressure through the regeneration phase. Analysis of resistance levels of transgenic plants showed that resistance to hygromycin concentrations from 50 to 200 mg/l were found for plants transformed with pCel40, as well as resistance to kanamycin concentrations from 50 to 200 mg/l for plants transformed with pABD1 and resistance to chlorsulfuron concentrations up to 36 µg/l for plants transformed with pGH1. Transgenic diploid plants have been obtained: one kanamycin resistant (45KR) for the clone 45, one kanamycin resistant (90KR) and one hygromycin resistant (90HR) for the clone 90, one chlorsulfuron resistant (71CSR) for the clone 71 and one kanamycin resistant (217KR) for the clone 217. Plants are under regeneration from hygromycin-resistant calli from clones 217 and 275.

Molecular analysis of DNA from transformed plants of clone 90. Integration of plasmids were analysed in 90HR and 90KR. Southern experiments indicated that pABD1 plasmid sequences were integrated in the genome of the KR plant, as well as pCel40 plasmid sequences in the genome of the HR plant (Fig. 3). A 1.1-kb band was detected in KR plant DNA digested with EcoR5 (Fig. 3a, 2), corresponding to the APHIII' coding sequence. An additional 3.5-kb fragment was found, probably resulting from recombinations between plasmid and plant DNA. A 5.4-kb fragment was detected in KR DNA digested with SmaI (Fig. 3a, 3), an enzyme which linearises pABD1. This demonstrates that at least one intact copy of pABD1 is flanked by modified plasmid DNA sequences. More than two copies of the plasmid have been integrated (Fig. 3a, 4 and 5). These conclusions were also confirmed by the higher intensity of the 1.1-kb

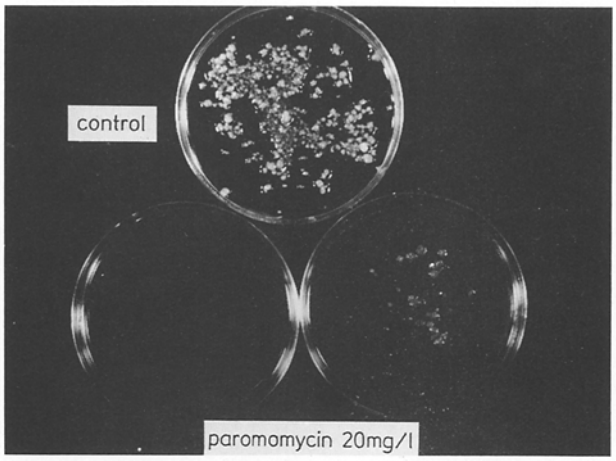

Fig. 1. Diploid potato protoplasts were electroporated in the presence of pABD1. Colonies were selected on C medium supplemented with 20 mg/l paromomycin. *Upper dish:* Control colonies from non-electroporated protoplasts of the wild-type clone 90. *Lower left:* same sample as above but the C medium was supplemented with 20 mg/l paromomycin. *Lower right:* transformed colonies derived from electroporated protoplasts (250 V, 16 µF capacitor) selected in C medium supplemented with 20 mg/l paromomycin

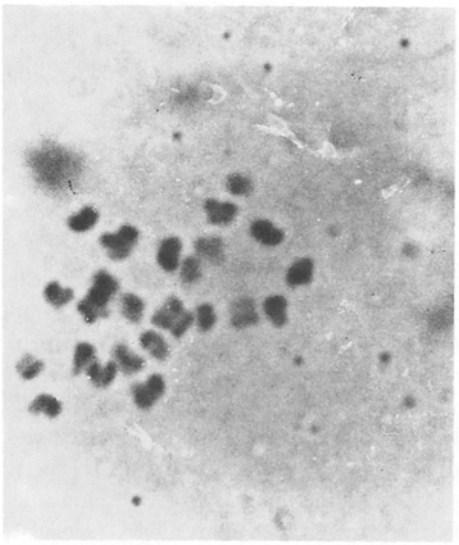

Fig. 2. Chromosomes of a KR plant shoot tip cell in metaphase

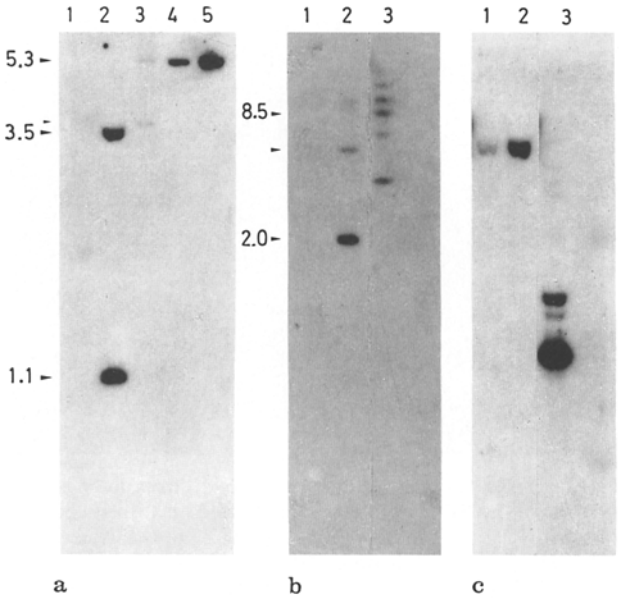

Fig. 3a–c. Molecular analysis of DNA from transformed plants. **a** *1* – EcoRV digestion of WT 90 DNA; *2* – EcoRV digestion of KR plant DNA; *3* – Sma1 digestion of KR plant DNA; *4* – reconstruction experiment with one plasmid copy/one plant genome; *5* – five plasmid copies/one plant genome; **b** *1* – BamH1 digestion of WT 90 DNA; *2* – BamH1 digestion of HR plant DNA; *3* – SST1 digestion of HR plant DNA; **c** *1* – Reconstruction experiment with one plasmid copy/one plant genome; *2* – five plasmid copies/one plant genome; *3* – BamH1 digestion of HR plant DNA

fragment as compared to the intensity of the additional 3.5-kb fragment in plant DNA digested with EcoR5 (Fig. 3a, 2). In BamH1 digests of HR plant DNA, a 2-kb fragment was detected (Fig. 3b, 2), corresponding to the coding sequence of the APHIV gene. A larger and additional fragment was also detected. An 8.5-kb fragment was found in an Sst1 (an enzyme which linearises pCel40) digest of HR plant DNA (Fig. 3b, 3), corresponding to the size of pCel40. Numerous other fragments were present. It seems that several copies of pCel40 have been integrated, one of them being flanked by recombinations between plasmid sequences and plant DNA (see also Fig. 3c). Approximately five copies of the plasmid were found per plant genome (Fig. 3c).

Genetic conformity of transformed plants. Transgenic plants were grown in soil, in the greenhouse and were compared to the controls with regard to leaf shape, flowering and tubers. Ploidy level of the plants was estimated by counting the number of chloroplasts present in their stomatal guard cells. Normal ploidy level of selected plants was confirmed by chromosome countings on root tips (Fig. 2). A proportion of 90% of the transformed plants regenerated were tetraploid but transgenic diploid plants were recovered for four different diploid clones (45, 90, 71 and 217). This phenomenon frequently occurs in potato cell culture (see Discussion).

Efficiency of selectable markers in somatic hybrid recovery

Protoplast fusion and selection of somatic hybrid cells. Three combinations have been performed in protoplast fusions between HR and KR clones (90HR + 90KR and 90HR + 217KR), between HR and CSR clones (90HR + 71CSR) and between KR and CSR clones (217KR + 71CSR). After protoplast fusion, hybrid colonies were selected in medium C supplemented with 20 mg/l hygromycin and 20 mg/l paromomycin, or with 20 mg/l hygromycin and 18 µg/l chlorsulfuron or with 20 mg/l paromomycin and 18 µg/l chlorsulfuron in the three combinations, respectively. The percentage of hybrid calli was estimated before transfer to regeneration medium and was compared to the number of colonies recovered in unselected fusion samples. The frequency of hybrids was estimated at 10^{-3} for the fusion between 90KR and 90HR and to 10^{-2} for the fusion between 90HR and 71CSR. Plants have been regenerated for the fusion between 90KR and 90HR. Selected calli are under regeneration for the fusions between 90HR and 71CSR, between 71CSR and 217KR and between 90HR and 217KR.

Validity of cell selection for drug resistance in somatic hybrid recovery. It has been described that protoplast fusion between 2 × clones of potato can result in genetic combinations in which the regeneration of somatic hybrids was either favoured or inhibited (Austin et al. 1985b). As described here, protoplasts from clone 90, 90HR, 90KR as well as calli from spontaneously doubled clone 90 had the same regeneration ability. Thus, by using the intraclone protoplast fusion between 90HR and 90KR, we should be able to avoid any particularity in cell growth. This fusion was studied for estimating the value of drug resistances in somatic hybrid cell selection. Plants have been regenerated from three doubly resistant calli. All were resistant to both antibiotics, but chromosome counts showed that four plants regenerated from one callus had different chromosome numbers (53, 56, 65 and 68). Two tetraploid plants (48 chromosomes) were recovered from the two other doubly resistant calli. One tetraploid doubly resistant plant (Hybrid) was studied. Shoot tips of this Hybrid plant were transferred to propagation medium D supplemented with a combination of both antibiotics. Plant growth was normal, whereas parental plants were killed on this medium. This plant displayed the normal phenotype of a tetraploid potato, with regard to leaf shape, flowers and tubers. The tetraploid plant was particularly studied to see whether it was the result of chimaera or of a true somatic hybrid. Protoplasts were isolated from WT90, 90HR, 90KR and

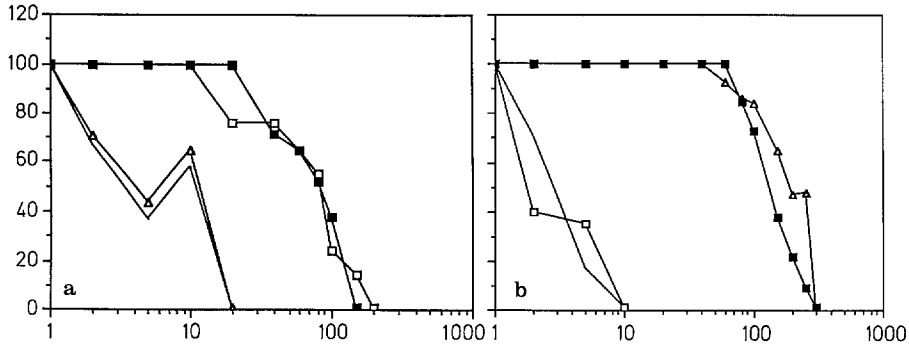

Fig. 4 a and b. Protoplast-derived colonies from wild-type, HR, KR and hybrid plants were diluted in C medium supplemented with various concentrations of paromomycin or hygromycin. Percentages of growing colonies were expressed relative to the number of growing colonies of the same clone in control medium. The 100% value represents approximately 3×10^3 colonies/ml. Percentages of growing colonies are represented on the *vertical axis* and antibiotic concentrations (mg/l) on the *horizontal axis*. Wild type: —, KR clone: □, Hr clone: △, Hybrid clone: ■. **a** Test of colony resistance to paromomycin. **b** Test of colony resistance to hygromycin

Table 1. Resistance test of protoplast-derived colonies of wild-type, KR, HR, a 1:1 mixture of HR and KR and colonies of the somatic hybrid. Protoplast derived colonies from wild-type, HR, KR and hybrid plants were plated in C medium (control) or C medium supplemented with hygromycin 20 mg/l (A), with paromomycin 20 mg/l (B) or with both antibiotics (C). Percentages of growing colonies were expressed relative to the number of growing colonies of the same clone in control medium. The 100% value represents approximately 3×10^3 colonies/ml

Cell types	Control	Culture media		
		A	B	C
WT	100	0	0	0
KR	100	0	107	0
HR	100	100	0	0
HR+KR	100	52	37	0
Hybrid	100	96	98	103

Hybrid plant, and colonies were submitted to various concentrations of hygromycin and/or paromomycin in medium C. Counts were performed as described in 'Material and methods'.

Colonies from 90KR were 20-fold more resistant to paromomycin than control colonies and colonies from 90HR were 30-fold more resistant to hygromycin than control colonies. Colonies from the Hybrid plant displayed resistance to both antibiotics with resistance levels comparable to those of the parents (Fig. 4). Protoplast-derived colonies from WT 90, KR, HR and Hybrid plants were plated at a density of 3×10^3 cells/ml in medium C, without antibiotic or supplemented either with 20 mg/l paromomycin, 20 mg/l hygromycin, or both. Colonies from all the clones were able to grow on control medium C (Table 1). Colonies from the KR plant were able to grow on C medium supplemented with paromomycin. Colonies from the HR plant were able to grow on C medium supplemented with hygromycin. In a 1:1 mixture of HR and KR colonies, only half of the colonies were able to grow on C medium supplemented with hygromycin or kanamycin, and no cell growth was found on medium supplemented with both antibiotics. These experiments confirmed the inability of the cells to detoxify the medium. In contrast, colonies from the Hybrid plant were able to grow on C medium supplemented with the combination of both antibiotics. These results confirmed that tetraploid cells derived from the Hybrid plant contained a combination of the genes of HR and KR parents. Thus, the Hybrid plant obtained through this procedure was shown to be not a chimera but a true somatic hybrid.

Discussion

We report here the first successful experiments in stable transformation of diploid potato by direct gene transfer. This technique has given transformation frequencies comparable to those obtained for direct gene transfer by electroporation in tobacco (Guerche et al. 1986). Transformation frequencies reached 0.5%–2%. Integration of the plasmids in plant genomes has been confirmed for two clones and seemed to be complex. Despite a high proportion of tetraploid plants resulting from spontaneous chromosome doubling, numerous phenotypically normal transformed plants can be regenerated 3–4 months after protoplast electroporation. Diploid transgenic plants have been obtained for four different diploid clones, and plants are under regeneration from hygromycin-resistant calli from two other diploid clones. Electroporation can thus be considered as a genotype-independent transformation method for diploid potatoes

as well as for tetraploid varieties (data not shown). Moreover, for potato clones which should be subjected to somatic hybridization experiments, this transformation technique can be considered as an alternative procedure of the transformation method using *Agrobacterium tumefaciens* described by De Block et al. (1988). There is some advantage in the sense that electroporation opens prospects in transient expression experiments in potato. Generally, the application of somatic hybridization to obtaining potato tetraploid clones suffers from a lack of reliable selection methods for early detection of somatic hybrids. The use of selectable markers previously introduced into diploid potato clones allowed us to obtain tetraploid somatic hybrids by selection of hybrid colonies directly after protoplast fusion. It appeared that a single marker was sufficient to maintain each diploid genome in the final tetraploid somatic hybrid. In this selection process, antibiotic resistance genes could be substituted by herbicide resistance genes which could be more useful for agronomic purposes. This method seems to be suitable for obtaining tetraploid somatic hybrids between combinations of a wide range of different diploid potato clones, without manipulation of a large number of regenerated plants. Electrical or chemical protoplast fusion methods are still relatively delicate. Hybrid selection could give rapid answers concerning the efficiency of these experiments. 1–2 months after protoplast fusion, which also avoids the need to manipulate large populations of regenerated plants.

Somaclonal variation frequently occurs in plants regenerated from potato protoplasts (Austin and Cassels 1983; Ramulu et al. 1983). Under our conditions, 90% of the transformed plants regenerated from diploid protoplasts were tetraploid and four hybrid plants regenerated from one selected callus were mixoploid. This chromosome instability significantly complicates the application of transformation or somatic hybridization to potato. Ramulu et al. (1989) showed that during the culture process, abnormal cell divisions could produce tetraploid as well as aneuploid or polyploid cells from diploid protoplasts. This low proportion of diploid-regenerated plants could be partially overcome by the use of a protoplast culture method leading to high regeneration rates and the manipulation of numerous different transformed or hybrid cells obtained through a selection procedure. Ramulu et al. (1989) showed that, just after isolation, protoplasts from diploid plants had the normal nuclear DNA content. This result is critical for the application of protoplast fusion in potato.

Several other transformed interspecific hybrid diploid clones are being developed for use in a somatic hybridization scheme. Somatic hybrids resulting from protoplast fusion and cell selection are under work and their agronomic value will be compared to that of tetraploid cultivars currently used in agriculture.

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