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Production and identification of somatic hybrids between *Solanum tuberosum* and *S. papita* by using the *rolC* gene as a morphological selectable marker

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Abstract A successful hybridization of a diploid clone of *Solanum tuberosum* with a *rolC*-transgenic, diploid *S. papita* clone is reported. By using leaf explants of this *S. papita* clone, which after transformation expressed kanamycin resistance, intact protoplasts were obtained, but these protoplasts did not develop to microcalli or regenerate to mature plants. However, protoplasts of the *S. tuberosum* clone showed a high capacity to regenerate plants from isolated protoplasts. On a medium containing kanamycin only calli regenerated to plants, which revealed a *rolC* phenotype (reduced apical dominance with a large number of adventitious shoots and a pale green color of leaves) and later on turned out to be true hybrids. Self fusions of *S. papita* never developed to microcalli and those of *S. tuberosum* ceased to develop on the kanamycin-containing medium. Identification of somatic hybrids was done by RFLP and RAPD analysis. In the greenhouse, out of four selected hybrids only FK3.1 was successfully crossed with two standard *S. tuberosum* varieties (Datura, Desirée). Out of all the seeds germinated, only *rolC*-negative F^1 seedlings were further characterized. Within the seedling population obvious differences were evident in respect of the *S. papita* and *S. tuberosum* characteristics.

Key words Marker genes · Protoplast fusion · *RolC* · Somatic hybrid · Transgenic potato

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Introduction

An important factor for the successful breeding of new varieties is the availability of a broad genetic spectrum for inclusion into breeding programs. Wild relatives are crossed with cultivars for this general reason, but also to introduce specific genes or traits into breeding lines. However, when sexual incompatibility, or wide genetic distances, limit the sexual introduction of the genetic resources from wild relatives into crop plants, somatic hybridization can be used to combine complete or partial genomes of sexually incompatible species (Melchers et al. 1978; Ehlenfeldt and Helgeson 1987). This technique involves the isolation and fusion of intact protoplasts, and the regeneration of mature plants from the fused cells. A first selection in vitro of putative somatic hybrids is possible when these genotypes carry selectable markers (Komari et al. 1989; Masson et al. 1989).

The identification of somatic hybrids can be achieved by one or more of the following methods: (1) characterization of an intermediate morphology (Schieder 1978), (2) the use of isozyme markers (Schieder 1978; Gleba et al. 1984; Möllers and Wenzel 1991), restriction fragment length polymorphism (RFLP; Williams et al. 1990 a), and species-specific DNA probes (Pehu et al. 1990). In potato, the technique of somatic hybridization is well established (Möllers and Wenzel 1991). In most of the cases reported, protoplasts of diploid clones of the tuber-bearing species *Solanum tuberosum* were fused with protoplasts from a number of different tuber and non-tuber-bearing *Solanum* species, either to combine parthenogenetic, androgenetic and protoplast fusion techniques in a single breeding scheme (Wenzel et al. 1979), or to introduce specific traits to certain breeding strains (Ferreira and Zelcer 1989).

For *S. papita*, a tuber-bearing species growing naturally in Mexico and Latin America (Hawkes 1990) and sexually only poorly compatible with *S. tuberosum* (Gorea 1970; Woodwards and Jackson 1985), no successful somatic hybridization with *S. tuberosum* has been reported so far. However, besides other useful characters, heat and drought

resistance has been described for *S. papita* (Hanneman and Bamberg 1986).

In the present paper we report the successful hybridization of a diploid clone of *S. tuberosum* with a *rolC*-transgenic, diploid *S. papita* clone. The *rolC* gene is one out of four genes located on the TL-DNA of the Ri plasmid of *Agrobacterium rhizogenes*, and the gene is involved in the pathogenesis of the hairy root disease (White et al. 1985; Spena et al. 1987). When the *rolC* gene is expressed alone in transgenic *S. tuberosum*, under the transcriptional control of the 35*s* promotor of the cauliflower-mosaic-virus (Fladung 1990; Fladung and Ballvora 1992), or in *S. papita* (D'Ambrosio, unpublished), characteristic phenotypic alterations, such as reduced apical dominance with a large number of secondary-shoots, pale green colour of leaves and altered yield parameters (more, but smaller tubers), were noted. The characterization of somatic hybrids was achieved by the use of RAPD markers (Williams et al. 1990b).

Materials and methods

Plant material and growth conditions

S. papita was transformed with the plasmid pPCV002-CaMVC (Spena et al. 1987), which carries the *rolC* gene from *A. rhizogenes* under the control of the cauliflower mosaic virus 35*s* RNA promotor (Pietrzak et al. 1986), and with the NPT-II gene as a selection marker (kanamycin resistance) for transformation. Regeneration yielded the clone *S.p.*6TE (D'Ambrosio, unpublished). The binary vector pPCV002 (without the *rolC* gene; Koncz and Schell 1986) was used in transformation to obtain the clones *S.p.*7TA and *S.p.*7TA1 (D'Ambrosio, unpublished). The second fusion parent was the kanamycin-sensitive diploid *S. tuberosum* clone H84.426/1 (internal collection of the MPI Züchtungsforschung, Köln). This clone exhibited a high capacity to regenerate from protoplasts. Aseptic shoot cultures were grown in a culture room at 22°C in day/night rhythms of 16/8 h. Hybrids and parental plants were transferred, after rooting from in vitro shoot cultures, to the greenhouse.

Isolation, fusion and regeneration of protoplasts

Protoplasts were obtained after enzymatic digestion of 2 g of leaf material from 4-week old axenic plant material (Knapp 1989). Isolated protoplasts were fused in an electric field using a Zimmermann Cell FusionTM Instrument (GCA Corporation, Chicago, USA; voltage of alignment: 12 V; duration and voltage of one pulse: 25 µs and 150 V) in solution of 0.5 ml in a fusion chamber (Krüss, Hamburg, Germany) according to Möllers (1990). The fusion aggregates were sub-cultivated for 7–21 days in a liquid medium in a concentration of 10^4 cells/ml to obtain microcalli, which were further sub-cultivated in regeneration medium (Knapp 1989). Selection of hybrids were done by transferring 3/4 of the calli onto regeneration medium containing 100 mg/l of kanamycin sulphate, or by RAPD analysis after sub-cultivating 1/4 of the calli on regeneration medium without kanamycin.

DNA-, RFLP- and RAPD-analysis

Isolation of DNA and determination of the copy number of the *rolC* gene in *S.p.*6TE was established by Southern experiments as described in Fladung and Ballvora (1992). RFLP analysis of putative hybrids were done according to Meyer (1991). For RAPD analysis, DNA was isolated as described by Edwards et al. (1991). Primers were obtained from Operon Technologies (Alameda, Calif., USA). The PCR reactions were done according to Williams et al. (1990b). Bands were visualized after electrophoresis on agarose gels and photographed under UV light (Maniatis et al. 1982).

Determination of chromosome number and pollen vitality

The chromosome number was determined by microscopical analysis of shoot tips soaked for 4–5 h in 0.03% hydroxyquinoline solution and fixed for 24 h in Carnoy solution. Pollen vitality was estimated by staining of pollen in lactophenol acid fuchsin solution for 10 min (Meyer 1991).

Results

Characterization of parental clones

Protoplast isolation of both kanamycin-resistant *S. papita* clones 7TA and 7TA1 (without the *rolC* gene) failed in all experiments. When using leaf explants of the *rolC*-transgenic *S.p.*6TE, intact protoplasts were obtained. However, these protoplasts did not divide or develop into microcalli. Therefore, *S.p.*6TE has been selected as a fusion partner with protoplasts of the *S. tuberosum* clone H84.426/1. The ploidy levels of the *S. papita* and *S. tuberosum* clones were found to be 2n=2x=24 (Table 1). The *rolC*-transgenic *S.p.*6TE shows a bushy phenotype with reduced apical dominance and a high number of short shoots (Fig. 1 A). During the whole season, plants of this clone never formed flowers. Leaves were much smaller compared to controls but had a normal shape (Fig. 1 B). In Southern experiments only one copy of the *rolC* gene was found in the genome of *S.p.*6TE (Fig. 2 A). For RAPD analysis various primers were screened in PCR reactions to differentiate both *Solanum* fusion partners. The fourth primer out of ten screened turned out to be an appropriate marker to identify specific parental bands (Fig. 2 B) which would be combined in putative hybrids.

Regeneration of plants from fused protoplasts

Under non-selective conditions, protoplasts from various fusion experiments developed to microcalli when both fusion partners (*S.p.*6TE and H84.426/1) were combined or when self-fusions of H84.426/1 were carried out. Self-fusions of the other parental line *S.p.*6TE did not result in microcalli. On selection medium containing 150 mg/l of kanamycin sulphate only calli regenerated to complete plants, which later on were identified as true hybrids, whereas none of the calli which originated from the self-fusion experiments of H84.426/1 developed any further. Under these conditions, a total of eight putative hybrids were obtained. On regeneration medium without selection pressure 15 putative hybrids were obtained.

Table 1 Comparison of morphological characteristics, chromosome numbers and RAPD profiles of parental *S. tuberosum* and *S. papita* clones, and somatic hybrids

Clone number	*RolC* phenotype[a]	Chromosome number	RAPD profile[b]	Leaf shape[c]	Pigmentation[d]	Petal color[d]		Total chlorophyll[e]
						Front	Back	
Parental								
S.p. 6TE	++	24	P	a	+++	/	/	0.41
H84.426/1	–	24	T	b	–	–	–	0.50
*S.p.*7TA	–	24	P	c	+++	+	++	0.59
Somatic hybrids								
FK15.1	+	48	H	b	+++	++	+++	0.31
FK27.1	+	48	H	b	+++	++	+++	0.28
FK3.1	+	48	H	c	+++	++	+++	n.d.[f]
FK17.1	+	48	H	c	+++	++	+++	0.40
FK26.1	+	72	H	a	+++	/	/	0.38

[a] ++ = strong; + = intermediate; – = wild-type (see also Fig. 1)
[b] T = *S. tuberosum* pattern; P = *S. papita*; H = hybrid pattern (see also Fig. 2)
[c] a = very small leaves; b = more like *S. tuberosum*; c = more like *S. papita* (see also Fig. 1)
[d] +++ = very strong; ++ = strong; + = weak; – = none; / = no flower produced
[e] $g*m^{-2}$ leaf area
[f] n.d. = not determined

Characterization of somatic hybrids

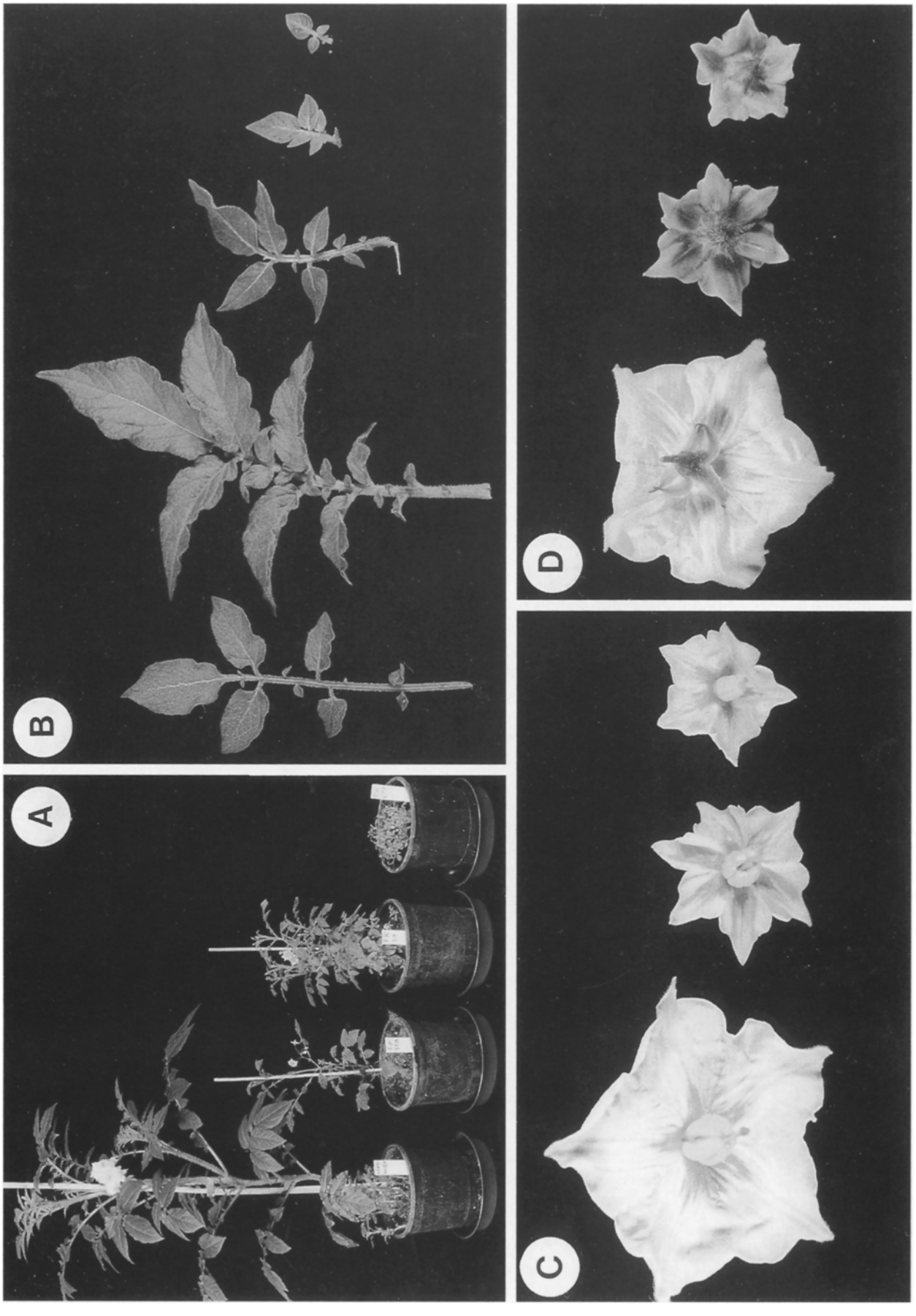
Phenotypically, the regenerants obtained either showed a phenotype similar to *rolC*-transgenic plants, with more and smaller leaves and short internodes, or else a high similarity to the regenerants of the clone H84.426/1. Regenerants of this latter group were only observed on regeneration medium without the selection marker kanamycin. RFLP and RAPD analysis of the regenerants (Fig. 2 B, C) revealed 15 true hybrids originating from selective (8) and non-selective (7) conditions, respectively.

After transfer to the greenhouse the somatic hybrids could be divided into two groups with respect to their phenotypes: one group displayed the phenotype of the *rolC*-transgenic clone *S.p.*6TE, although they were characterized as hybrids, and the second group showed a considerably weaker influence of the *rolC* gene on the plant phenotype (Fig. 1 A). Like the parental clone, flower formation was never observed in *S.p.*6TE hybrids from the first group. Within the second group, hybrids were distinguished into two groups in respect of leaf color and shape, and flower pigmentation (Fig. 1 C, D): one group resembled more the phenotype of the *S. papita* parent, whereas the other hybrids exhibited the phenotype of the *S. tuberosum* parent (Table 1). Anthocyanidin pigmentation of the leaves was very strong in all hybrids (Table 1). Total chlorophyll content in mature leaves was reduced in all hybrids compared to the non-*rolC S. papita* and *S. tuberosum* clones, but was similar to the *rolC*-transgenic *S. papita* clone 6TE (Table 1).

The ploidy level of the somatic hybrids was determined by counting the chromosomes in cells of shoot tips. All hybrids displaying the weaker *rolC* phenotype were tetraploid, whereas one hybrid from the bushy group was hexaploid (Table 1). The determination of pollen vitality from the parental lines and the hybrids demonstrated male sterility in all hybrids showing the weaker *rolC* phenotype, whereas the non-*rolC S. papita* and H84.426/1 revealed high pollen fertility. The *rolC S.p.*6TE plant did not form flowers.

Characterization of F_1 plants

Out of four tetraploid hybrids only FK 3.1 was successfully crossed with the two standard *S. tuberosum* varieties 'Datura' and 'Desirée'. The clones FK15.1, FK17.1 and FK27.1 produced seeds only after pollination with 'Desirée'. From each of the two backcross combinations, (FK3.1 × Datura) and (FK3.1 × Desirée), 100 seeds were placed on MS medium supplemented with 0.1 mg/l of gibberellic acid for germination. From the cross FK3.1 with 'Datura', 25 plants developed; the second combination (FK3.1 × Desirée) yielded 42 plants. Characterization of the *rolC* phenotype of the F_1 plants was performed visually based on the typical features of shortened internodes and smaller leaves. The total content chlorophyll in mature leaves was always lower in *rolC*-transgenic backcross plants. Values ranged from 0.31 to 0.43 and from 0.53 to 0.63 mg of total chlorophyll$*m^{-2}$ for F_1 plants with and without the *rolC* phenotype, respectively. From the 25 and 42 germinated seeds, 12 and 13 *rolC*-negative F_1 plants, respectively, were selected: this compares to a segregation ratio equal to 1:1 and 1:2. The chromosome number in all selected F_1 plants without a *rolC* phenotype was 48 indicating a tetraploid genetic structure (Table 2). Only one plant (2014/6) was triploid with 36 chromosomes. Within the seedling population there were marked differences


A
B
C
D

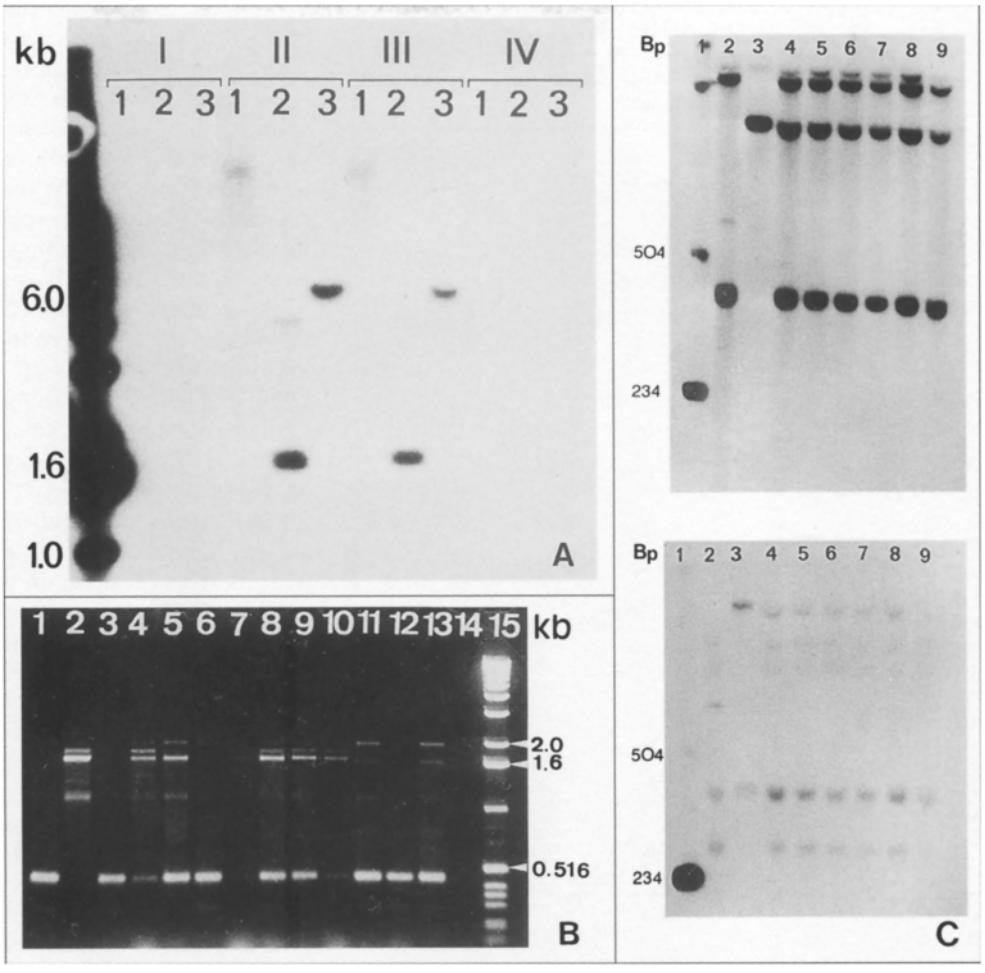


Fig. 2 Southern-blot analysis (**A**), RAPD profile (**B**) and RFLP-pattern (**C**) of DNA extracted from potato leaves of parental clones and somatic hybrids. (**A**) Ten milligrams of restriction enzyme-digested DNA (*1=Eco*RI, *2=Eco*RI/*Hin*dIII, *3=Hin*dIII) was loaded in each slot, blotted and probed against 0.1 mg of a *rolC* insert. The double digest showed the isolated insert of about 1.6 kb. I: H84.426/1; II: *S.p.*6TE; III: FK3.1.; IV: *S.p.*7TA. (**B**) After the PCR reactions ten µl of the incubation volumes were loaded on 1.4% agarose gels. The primer used was G4 from Operon (Alameda, USA). *Lane 1*: *S. tuberosum* H84.426/1; *2: S.p.*6TE; *3:* regenerant of H84.426/1; *4:* FK3.1; *5–13:* various regenerants after fusion; *14:* control without DNA; *15:* molecular weight marker. (**C**) Ten milligrams of *Hin*dIII-digested DNA was loaded in each slot, blotted and probed against the marker GP 27 (upper) and GP 79 (lower). *Lane 1*: molecular weight marker; *2*: *S.p.*6TE; *3*: H84.426/1; *4:* FK3.1; *5–9:* somatic hybrids, showing a combined banding pattern, 5–7 are different regenerants of the same callus

between the shape of the last pinnates and the pigmentation level of the leaves (Table 2). With regard to the level of pigmentation of the leaves (Table 2), in *rolC*-negative F_1 plants in the cross (FK3.1 × Datura), only one, and in the cross (FK3.1 × Desirée) no, plant showed absence of pigmentation. Most plants revealed intermediate or strong pigmentation.

Discussion

The two kanamycin-resistant transformants of the same clone of *S. papita* used in this study turned out to be different in respect of their ability to form intact protoplasts using a standard isolation protocol developed for leaves of *S. tuberosum* clones. The only genetical difference between these two clones was the additional insertion of the *rolC* gene of *A. rhizogenes* into the genome of the *S.p.* clone 6TE (Fig. 2 A). While with this *S. papita* 6TE clone it was possible to produce protoplasts from leaves at appropriate efficiencies, intact protoplasts have never been formed with the *S. papita* transformant carrying only the gene for kanamycin resistance. This is the first occasion on which an influence of the *rolC* gene on the isolation of protoplasts has been described.

The *rolC* gene encodes for a cytokinin-β-glucosidase, which is able to release free active cytokinins from inactive precursors (Estruch et al. 1991). In transgenic plants of *S. tuberosum*, *rolC* expression leads to up to a four-fold increase in the content of isopentenyladenine, dihydro-zeatin riboside and trans-zeatin riboside-type cytokinins (Schmülling et al. 1993). Furthermore, it was shown in the same study, as well as in Nilsson et al. (1993), that the dwarfism of *rolC* transgenic tobacco and potato plants is possibly linked to a reduction of gibberellic acid A_1 concentration. In addition to the alteration of hormonal levels of transgenic aspen plants carrying the same *35s-rolC* construct, the abscisic acid content of leaves is also changed (Fladung et al. unpublished results). Walden et al. (1993) found that *rol* genes alter the hormonal requirements for protoplast growth.

Fig. 1 Plant phenotype (**A**), leaf shape (**B**), and pigmentation of the front (**C**) and the back (**D**) of the petals of *S. tuberosum* and *S. papita* clones. Left to right: (**A**) *S. tuberosum* H84.426/1, *S.p.*7TA, hybrid FK 3.1, *S.p.* 6TE. (**B**) *S.p.*7TA, *S. tuberosum* H84.426/1, hybrids FK 17.1 and FK 15.1, *S.p.*6TE. (**C**), (**D**) *S. tuberosum* H84.426/1, hybrid FK 3.1, *S.p.*7TA; *S.p.* 6TE never formed flowers

Table 2 Chromosome numbers and pigmentation of leaves of F_1 seedlings from 'FK3.1 × Datura' and 'FK3.1. × Desirée'

Clone number	Chromosome number	Pigmentation[b]
FK3.1 × Datura		
2014/2	48	+
2014/3	n.d.[a]	+
2014/4	48	+++
2014/5	48	++
2014/6	36	++
2014/7	48	+
2014/9	48	+++
2014/12	48	–
2014/13	48	+
2014/14	48	+++
2014/23	n.d.	++
2014/25	n.d.	+++
FK3.1 × Désirée		
2015/2	48	++
2015/7	48	++
2015/9	n.d.	+++
2015/10	48	+++
2015/11	48	+
2015/12	48	+++
2015/13	48	++
2015/16	48	+
2015/17	48	++
2015/18	48	+++
2015/21	48	+++
2015/25	48	++
2015/27	48	++

[a] n.d. = not determined
[b] +++ = very strong; ++ = strong; + = weak; – = none

Therefore, an altered hormonal balance leading, for example, to altered osmotic conditions in cells is a possible explanation for the different responses of the *rolC*- and non-*rolC*-transgenic *S. papita* to the isolation of protoplasts.

Isolated protoplasts of the kanamycin-resistant and *rolC* transgenic clone *S.p.*6TE, however, were not capable of forming microcalli. The opposite was shown for the *S. tuberosum* clone H84.426/1. Protoplasts of this clone showed a high frequency of regeneration to microcalli and mature plants which were sensitive to kanamycin treatment.

After fusing protoplasts of the two clones *S.p.*6TE and H84,426/1 it was possible to regenerate plants on kanamycin-containing media, while no growth was observed from protoplasts of each parental clone cultivated alone under selective conditions. It can be concluded that a complementation of the two traits 'ability to regenerate' and 'kanamycin resistance' took place after protoplasts of the two different genotypes were fused. A similar observation was reported by Schieder (1978). He described the fusion of a chlorophyll-deficient mutant of *Datura innoxia* that had the ability to regenerate with wild-type protoplasts of the two species *D. stramonium* and *D. discolor*, neither of which could themselves regenerate. In both combinations green calli, and subsequently also green plants, were obtained. It could be shown that these plants were hybrids. Wijbrandi et al. (1988) fused kanamycin-resistant protoplasts of *Lycopersicon esculentum* that did not regenerate with protoplasts of the clone *Lycopersicon peruvianum* that had a high regeneration capacity. After regeneration on kanamycin they obtained somatic hybrids; they described 'regeneration ability' as a dominant and selectable marker for somatic fusions. Furthermore, kanamycin resistance was successfully applied in several other experiments involving selection for somatic hybrids (Brunhold et al. 1987; Komari et al 1989; Masson et al 1989).

All regenerants isolated under selective conditions in the present study showed the reduced internodes and small leaves typical of the *rolC* phenotype in potato (Fladung 1990; Fladung and Ballvora 1992). Regeneration of somatic hybrids without kanamycin selection resulted in regenerants with and without the *rolC* phenotype. Regenerants of this group without the *rolC* phenotype proved to be regenerants of the *S. tuberosum* parent, whereas regenerants with the *rolC* phenotype turned out to be true somatic hybrids. This observation shows that the *rolC* gene can be used as a positive morphological selection marker at a very early stage of plant regeneration and development. The characterization of true somatic hybrids at early stages of development is important because discarding non-hybrid plants saves both labour and space.

Somatic hybrids that were able to produce flowers in the greenhouse showed a phenotype similar to the *35s-rolC*-transgenic *S. tuberosum* plants obtained from transformation experiments (Fladung 1990; Fladung and Ballvora 1992). Like those plants they were male sterile, but female fertile, and produced tubers that were rather long and small, and had deep eyes.

Evaluation of the offspring of a backcross between one somatic hybrid and two standard potato varieties showed that it was possible to differentiate phenotypically between *rolC*-positive and *rolC*-negative F_1 plants. This phenotypic impression was supported by measurements of the content of total chlorophyll in mature leaves, which was always decreased in *rolC* transgenic plants (Fladung 1990; Fladung and Gieffers 1993). Therefore, it was possible to use *rolC* as a marker gene a second time and to select F_1 seedlings without a *rolC* phenotype in order to obtain those plants that combine genes of *S. papita* and *S. tuberosum* without the influence of the *rolC* gene. The existence of fragments of the T-DNA in those plants cannot, however, be excluded.

The identification of somatic hybrids at the DNA level can be carried out using the polymerase chain reaction (PCR) with 10-mer primers to generate random amplified polymorphic DNA (RAPD, Welsh et al. 1990; Williams et al. 1990 b). One primer out of the ten commercially available primers tested showed different banding profiles for the two parental *S. tuberosum* and *S. papita* clones, while somatic hybrids displayed a combination of the parental patterns. The hybrid character of those plants could also be confirmed through RFLP analysis (Fig. 2 B, C). This result is in agreement with a similar study identifying somatic hybrids between *S. tuberosum* and *S. brevidens* (Xu et al. 1993). The amplification products of the PCR reac-

tion were visualized by agarose-gel electrophoresis and ethidium-bromide staining. Compared to RFLP the screen with RAPD can be done at a much earlier stage of plant development because much less DNA is needed for an assay. Furthermore, the DNA used in the RAPD assay is quickly isolated and does not require long cleaning steps. The RAPD technology proved to be a useful screening system and a single RAPD marker is sufficient to establish a quick preliminary screen for putative somatic hybrids. However, for the analysis of the F_1 seedlings obtained from crosses between hybrids and standard potato varieties, a set of different primers should be used to detect parts of the *S. papita* genome in the F_1 seedling plants, because the DNA fragments amplified with one primer most probably do not represent the whole genome.

In the present study the successful somatic hybridization of the cultivated *S. tuberosum* and the wild species *S. papita* has been reported. As a result of the fusion, tetraploid plants were obtained that were female fertile and could be backcrossed with two standard *S. tuberosum* varieties yielding tetraploid F_1 plants. Only two attempts to sexually combine the two species *S. tuberosum* and *S. papita* have been described so far (Gorea 1970; Woodwards and Jackson 1985) while no somatic combination has yet been reported. While Gorea (1970) described triploid F_1 plants that were male sterile (female fertility was not tested), Woodwards and Jackson (1985) only mention seeds coming from this combination, but no hybrid plant.

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