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Somatic hybridization of sexually incompatible top-fruit tree rootstocks, wild pear (*Pyrus communis* var. *pyraster* L.) and Colt cherry (*Prunus avium* × *pseudocerasus*)

S. J. Ochatt, E. M. Patat-Ochatt, E. L. Rech, M. R. Davey and J. B. Power

Plant Genetic Manipulation Group, Department of Botany, University of Nottingham, Nottingham NG7 2RD, UK

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Summary. Mesophyll protoplasts of wild pear (*Pyrus communis* var. *pyraster* L., Pomoideae) were chemically fused with cell suspension protoplasts of cherry rootstock Colt (*Prunus avium* × *pseudocerasus*, Prunoideae), following an electroporation treatment of the separate parental protoplast systems. Fusion-treated protoplasts were cultured, on modified K8P medium, where it had been previously established that neither parental protoplasts were capable of division. Somatic hybrid calli were recovered and, following caulogenesis on MS medium with zeatin and after rooting of regenerated shoots, complete trees were obtained and grown in vivo. Hybridity of these trees was confirmed based on morphological characters, chromosome complement and isozyme analysis. Two separate cloned lines of this intersubfamilial rootstock somatic hybrid (wild pear (+) Colt cherry) were produced. This is the first report of the production of somatic hybrid plants of two woody species, of agronomic value, within the order Rosales.

Key words: Somatic hybrids – Fruit tree rootstocks – *Pyrus* (wild pear) – *Prunus* (Colt cherry) – Plant breeding

Introduction

The fusion of protoplasts by chemical or electrofusion methods has led to the production of several somatic hybrids, but rarely have these involved woody (tree) species (Power and Davey 1988). This has largely been due to the lack of reproducible protoplast-to-tree systems, thus, for fruit tree species in particular, making the production of somatic hybrids seem a remote prospect. The extension, however, of successful protoplast fusion techniques to the fruit trees would, nevertheless, have a major impact on their propagation and breeding and on fruit production in general (James 1987; Ochatt et al. 1988a). In this context, there are many possible targets for somatic hybridization in top-fruit tree species, including both rootstock/scion hybridization and, as described here, the production of new hybrid rootstocks.

Recently, protocols for the efficient regeneration of plants from fruit tree protoplasts have been described for several genotypes (Kobayashi et al. 1983; Ochatt and Caso 1986; Ochatt and Power 1988a, b; Ochatt et al. 1987, 1988b; Patat-Ochatt et al. 1988; Vardi et al. 1982) with the production of somatic hybrids within the family Rutaceae (Grosser et al. 1988a, b; Kobayashi et al. 1988; Ohgawara et al. 1985) and the establishment of heterokaryon division for species of Carica, a tropical fruit tree genus (Jordan et al. 1986). There is one report on protoplast fusion involving a temperate fruit tree species, where cell suspension protoplasts of apple were fused with mesophyll protoplasts of hop. Heterokaryons, however, failed to divide (James et al. 1986). On the other hand, the electroporation of protoplasts has been shown to improve the efficacy of regeneration for several species, including Colt cherry (Ochatt et al. 1988b).

Against this background, experiments were undertaken aimed at the production of novel temperate fruit tree somatic hybrids of two sexually incompatible rootstock genotypes: the cherry rootstock, Colt (*Prunus avium* × *pseudocerasus*) and the pear rootstock, wild pear (*Pyrus communis* var. *pyraster* L.), which are members of the subfamilies Prunoideae and Pomoideae, respectively, within the Rosaceae.

Materials and methods

Isolation of pear and Colt cherry protoplasts

Protoplasts of both parents were isolated based on methodologies previously described. Thus, for the wild pear rootstock,

the largest expanded leaves of in vitro-cultured shoots were incubated, for 8 h, in an enzyme mixture which contained 0.1% (w/v) Macerozyme R-10, 0.5% (w/v) Cellulase Onozuka R-10 and 0.1% (w/v) Driselase, in 10% strength MS (Murashige and Skoog 1962) salts, lacking NH_4NO_3, but with 0.35 *M* sucrose as osmoticum, pH 5.6 (Ochatt and Caso 1986). Cell suspensions of Colt cherry, taken 21 days after subculture, were digested overnight (16 h) in an enzyme solution which consisted of 0.03% (w/v) Macerozyme R-10, 2.0% (w/v) Meicelase and 2.0% (w/v) Rhozyme HP-150, in CPW salts (Power et al. 1984) with 0.7 *M* mannitol as osmoticum, pH 5.6 (Ochatt et al. 1987). All subsequent procedures were as previously described.

Protoplast fusion procedures

Protoplasts of wild pear and Colt cherry were maintained for 1 h in their respective osmotica, as used in the enzyme mixtures, after which they were either fused directly with polyethylene glycol, using the PEG/high pH method (Power et al. 1984), or electroporated separately (Rech et al. 1987) prior to their mixing and subsequent fusion.

For direct fusion, the density of both protoplast populations was adjusted to 4×10^5 protoplasts/ml with appropriate culture medium (Table 1) and the protoplasts were mixed (1:1), fused and washed twice by resuspension and centrifugation ($100 \times g$; 5 min) with CPW salts (Power et al. 1984) supplemented with 0.6 *M* mannitol and 0.74% (w/v) $CaCl_2 \cdot 2H_2O$.

Alternatively, for electroporation of the parental protoplasts prior to their fusion, *Pyrus* protoplasts were adjusted to a density of 1×10^6/ml and *Prunus* protoplasts to 4×10^6/ml, in their respective culture media (Table 1), whereupon they were electroporated as separate populations at 250 V/cm, by discharging a 30 nF capacitor, for three successive 87 µs pulses at 10 s intervals (room temperature) as described previously (Rech et al. 1987). Following electroporation, the density of both protoplast preparations was returned to that as used for fusion (with their respective culture media, Table 1), and the protoplasts were mixed and fused as described earlier.

Culture of heterokaryons and proliferation of somatic hybrid microcalli

Fusion-treated protoplasts (with or without an electroporation pretreatment) and viability controls were adjusted to a density of 1×10^5/ml in semisolid (0.625% (w/v) Seaplaque agarose) modified K8P medium (Kao and Michayluk 1975) (Table 1). Protoplasts were cultured as 100 µl droplets (10 droplets per each 5 cm in diameter disposable plastic petri dish) submerged in liquid medium (4.0 ml) of the same composition. Dishes were sealed with Nescofilm and kept at 25 °C, in the dark. A step-wise reduction of the osmotic pressure of the culture medium was initiated after 21 days, when the heterokaryons had regenerated a cell wall and divided at least once, This was performed by replacing the liquid layer with fresh medium of the same composition but with the osmotic pressure reduced to 75% of its previous level. Protoplasts of both parental species (electroporated or not) were unable to divide in their own right or as 1:1 mixtures in the modified K8P medium as used for the culture of the fusion-treated protoplasts, thereby providing a selection strategy for the preferential recovery of somatic hybrid colonies/calli.

The putative somatic hybrid microcalli (see 'Results') were individually transferred (after 120 days of culture) to 5.0 ml of MS medium with 2.0 mg/l NAA (1-naphthalene acetic acid), 0.25 mg/l BAP (6-benzylaminopurine) and 8% (w/v) agar, pH 5.7, for further proliferation. Cultures were subsequently kept at 25 °C, with a constant illumination (1000 lx, cool white fluorescent tubes), and subcultured onto the same medium every 2–3 weeks.

Table 1. Protoplast washing/culture media and growth conditions used for the production of wild pear (+) Colt cherry somatic hybrid rootstocks. Figures in brackets mg/l except where otherwise stated

Medium/washing solution	Wild pear (leaf protoplasts)[a]	Colt cherry (cell suspension protoplasts)[b]	Wild pear (+) Colt cherry (protoplast selection medium)
(Basal component)	MS salts[3] (10% strength); NH_4NO_3-free	MS salts[c]	K8P medium[d]
(Organics)	Complex organic mixture[a]; caseine hydrolysate (50)	As in MS medium[c]	Thiamine-HCl (1.0); coconut milk (1.0%); casaminoacids (125.0)
(Growth regulators)	NAA (1.0); BAP (0.4)	NAA (1.0); BAP (0.25); Z (0.5)	2,4-D (0.1); Z (0.2)
(Main osmotica)	Sucrose (0.18 *M*); xylitol (0.025 *M*); mannitol (0.025 *M*); sorbitol (0.025 *M*); inositol (0.025 *M*)	Sucrose (0.05 *M*); mannitol (0.5 *M*)	Sucrose (0.06 *M*); glucose (0.55 *M*)
Mode of culture	Agarose (1.0%, w/v) as semisolid layers; pH 5.6	Agarose (0.625%, w/v) as semisolid layers; pH 5.8	Agarose (0.625%, w/v) as 100 µl droplets submerged in liquid medium; pH 5.8
Growth conditions			
(Temperature)	25±1 °C	25±2 °C	25±2 °C
(Irradiance)	2,000 lx, 16 h day length	Dark	Dark

[a] Ochatt and Caso (1986)
[b] Ochatt et al. (1987)
[c] Murashige and Skoog (1962)
[d] Kao and Michayluk (1975)

Regeneration of rootstock somatic hybrid plants

After 20 weeks, calli portions (200 mg fresh weight), which were recovered only from the electroporated/fusion-treated preparations, were transferred to a regeneration medium which consisted of MS salts and organics medium with 1.0 mg/l Z (zeatin) alone, whereupon shoot buds were differentiated within 4 weeks. These were detached from the callus and transferred, for shoot multiplication and internode elongation, to MS medium with 0.05 mg/l IBA (4-indole-3yl-butyric acid), 0.75 mg/l BAP and 0.05 mg/l GA_3 (gibberellic acid). Elongated shoots (3.0 cm in length) were rooted, after being individually transferred to half-strength MS medium with 3.0 mg/l IBA, for 3 weeks for root initiation and then to half-strength, hormone-free MS medium, for a further 4 weeks for root proliferation. All cultures, for both shoot multiplication and rooting, were kept at 25 °C with constant illumination as described earlier.

The regenerated, putative somatic hybrid plants were subsequently transferred individually to pots (9 cm in diameter) which contained Professional Levington M3 soil-less compost (Fisons, UK), with each pot being enclosed after watering in a polyethylene bag. Plants were maintained under glasshouse conditions, with a temperature of 23 ± 5 °C, and a 16 h light photoperiod (10,000 lx, cool white fluorescent tubes). Bags were progressively opened over a 3-week period and by 4 weeks, ex vitro transfer and acclimatization was completed. Plants of both parental rootstocks, taken from axenic shoot cultures of protoplast origin, were maintained alongside the regenerants for comparative purposes.

Confirmation of hybridity of the regenerated trees

Two months after ex vitro transfer, the putative somatic hybrid trees were transferred to larger pots (12.5 cm in diameter), and kept outdoors.

Hybridity of the selected regenerants was confirmed, through a comparative analysis with protoplast-derived trees of both parents of the same age, by means of somatic chromosome number determination, morphology, leaf isozyme profiles of each parent, a physical mixture of extracts and the cloned individuals of hybrid origin. A comparison of floral morphologies was not possible since such trees would not be expected to flower for at least 2–3 years.

For chromosome counts, root apices (0.5 cm long) were taken from the parental and somatic hybrid regenerants, at the time of repotting, and were incubated (30 min, 28 °C) in an aqueous solution of 1.0% (w/v) Pectinase (Sigma), pH 5.8, so as to soften the root tissues. Root tips were kept in water for 16 h (4 °C) and then pre-treated with a saturated aqueous solution of alpha-bromo naphthalene for a further 4 h (4 °C). Tips were fixed in ethanol : acetic acid (3:1) overnight (16 h), and stained with leuco-basic fuchsin in the dark, for 2–3 h, at 25 °C.

For electrophoresis of isozymes, leaf tissues were extracted as described by Weeden and Lamb (1987) and 50 µl samples were loaded onto 12% polyacrylamide gels. These were run for 8 h, at 4 °C and 100 V. Gels were stained for esterase, phosphoglucomutase and malate dehydrogenase following standard procedures (Vallejos 1983).

Results

When the parental protoplast populations were fused without an electroporation pretreatment, this gave a heterokaryon frequency of 1.5% (equivalent to 1,200 heterokaryons/ml). Such heterokaryons regenerated a cell wall by day 28, but invariably ceased mitotic division by day 40.

Conversely, protoplasts electroporated prior to their fusion typically gave a population of 6.9% heterokaryons (equivalent to 5,520 heterokaryons/ml, Fig. 1 a). After 28 days in modified K8P medium (Table 1), these heterokaryons had regenerated a cell wall and entered division. The heterokaryons had a plating efficiency of 8.2% (the percentage of heterokaryons entering sustained division), but by day 40 the putative hybrid cell colonies, which were composed of 4–10 cells, reflected a heterokaryon plating efficiency of 0.95% (equivalent to 53 potential hybrid colonies/ml medium). Unfused parental protoplasts (in both the control and the fusion-treated plates) and physical mixtures (1 : 1) of untreated protoplasts did not undergo cell wall regeneration or division in the modified K8P medium. This situation also held for unfused protoplasts that had been electroporated. By day 40, all unfused protoplasts (and by implication homokaryons of both parents) were dead. In addition, heterokaryons were unable to divide when plated in either of the media previously used for successful protoplast culture of wild pear (Ochatt and Caso 1986) or Colt cherry (Ochatt et al. 1987).

Beyond the 10-cell stage, many of the colonies in the fusion plates ceased to proliferate, so that by day 100, surviving microcalli (50-cell stage) reflected a heterokaryon plating efficiency of 0.36% (equivalent to 22 putative hybrid microcalli/ml of medium, Fig. 1 b). In all, the experiments were repeated eight times with a total of 3.2×10^6 parental protoplasts being subjected to a fusion treatment per experiment. By day 120, 7 putative hybrid calli were recovered, from one experiment only. These were transferred (see 'Materials and methods') for plant regeneration and micropropagation. After three successive bi-weekly subcultures on the callus proliferation medium, plant regeneration was attempted.

Of the 7 hybrid calli, 2 underwent differentiation to give three shoot buds each per callus line (Fig. 1 c). The regeneration capacity in these calli was irreversibly lost after 30 days on regeneration medium.

The regenerated shoot buds, detached and transferred for multiplication, grew slowly with extensive phenolic oxidation associated with vitrification. This ultimately resulted in the death of three of the six original shoot buds. Of the three remaining shoot buds, fast-growing axenic shoot cultures were established for two separate clones (hereafter referred to as clones A and B), whilst slow-growing, non-rootable shoots, were obtained for a third (clone C). Subsequently, shoots of clone A proved to be easy to root (comparable to the Colt cherry parent) with an 80% success rate (Fig. 1 d); shoots of clone B were more difficult to root with a 30% success rate (similar to the wild pear parent). To date, over 30 plants of clone A and 14 plants of clone B have been

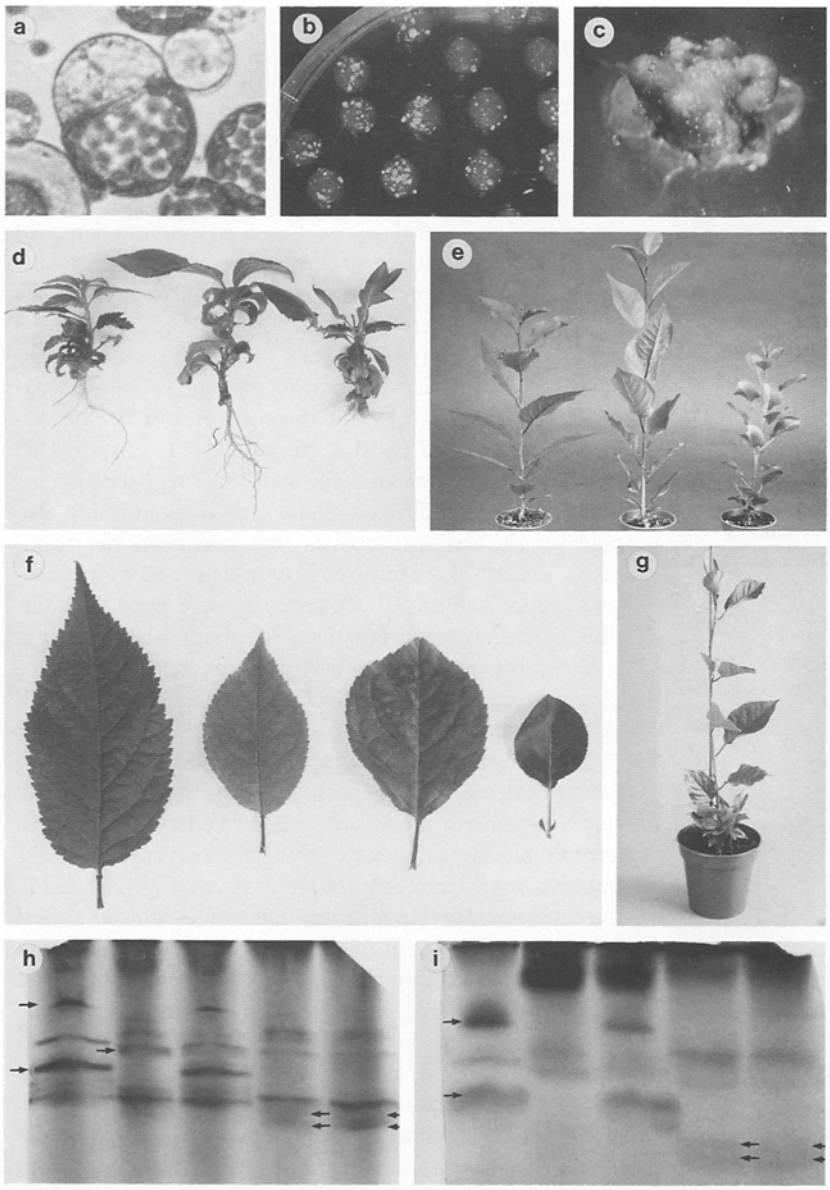

Fig. 1 a–i. Somatic hybridization of wild pear and Colt cherry rootstocks. **a** PEG-induced adhesion of a cell suspension protoplast of Colt cherry with a mesophyll protoplast of wild pear (×450). **b** Somatic hybrid microcalli developing in agarose beads (100 days) (×0.5). **c** Early stage shoot differentiation of somatic hybrid callus (180 days) (×2). **d** Rooted shoots of (left to right): Colt cherry rootstock, a somatic hybrid (Colt cherry [+] wild pear, clone A), wild pear rootstock. The somatic hybrid plant was 100 days old taken from the time of callus differentiation (×0.8). **e** Young trees, established ex vitro for 2 months of (left to right): Colt cherry rootstock, a somatic hybrid tree (clone B), wild pear rootstock (×0.1). **f** Leaf morphologies of (6-month-old) plants of (left to right): Colt cherry rootstock, a somatic hybrid (clone A), a somatic hybrid (clone B), wild pear rootstock (×0.5). **g** A suckering, aneuploid somatic hybrid (clone A) (2 months ex vitro) (×0.1). **h, i** Isoenzyme banding profiles, in vertical polyacrylamide gels, of esterases **h** and phosphoglucomutase **i**, *lane 1* – wild pear rootstock, *lane 2* – Colt cherry rootstock, *lane 3* – a physical mixture (1:1) of both parental rootstocks, *lane 4* – a somatic hybrid clone A, *lane 5* – a somatic hybrid clone B. The *arrows* (→) highlight the bands from each parental rootstock missing in the somatic hybrids, and the *arrows* (←) highlight the new (hybrid) bands present in the somatic hybrids

established as young trees (Fig. 1 e), some of which are now growing under field (open-air) conditions.

For the confirmation of hybridity based on morphological markers, the somatic hybrid trees were compared, 2 months after ex vitro transfer, with representative examples of both parents (of a similar age and background). However, most phenotypic differences were apparent after the in vitro rooting stage. All plants within either clone A or B were, with one exception, highly homogeneous in appearance, as were the respective parents, but there were consistent differences apparent between the two cloned populations. For most morphological characteristics, the hybrid plants were intermediate as compared to the wild pear and Colt cherry parents (Table 2, Fig. 1 f).

In addition, the somatic chromosome number showed that, in spite of minor differences in morphology, all somatic hybrid plants (clones A and B) had a complement of 58 chromosomes. This was equivalent to the summation of the chromosome numbers of wild pear ($2n = 2x = 34$) and Colt cherry ($2n = 3x = 24$). The hybrid plants were therefore designated ($2n = 2x + 3x = 58$). The one exception to this was for one plant, of clone A, which was aneuploid, with a complement of ($2n = 2x + 3x = 57$). This plant, although consistent in terms of leaf morphology with individuals of clone A, suckered prolifically from its roots (Fig. 1 g).

Isozyme banding patterns provided further confirmation of hybridity when leaf extracts from individual plants of the two somatic hybrid clones, A and B, were

Table 2. Vegetative characteristics of the somatic hybrid [wild pear (+) Colt cherry] and parent species. All assessments were made 2 months after ex vitro transfer on plants of comparable age and, where appropriate, origin

Phenotypic character	Colt cherry	Wild pear (+) Colt cherry		Wild pear
		Clone A	Clone B	
Mean height aerial part (cm)	20.0	50.0	50.0	35.0
Leaf shape	Lanceolate	Oval-lanceolate	Oblong-lanceolate	Oval-oblong
Fully expanded leaf index (length × width) (cm)	12.0 × 5.0	10.0 × 8.0	8.0 × 5.0	4.0 × 3.0
Leaf colour[a]	Green Group, 139A (normal green)	Green Group, 143A (pale green)	Green Group, 136B (dark green)	Green Group, 131A (very dark green)
Leaf margin[b]	Grossly double serrate	Single serrate	Grossly double crenate	Fine crenate
Leaf apex[b]	Acuminate	Acute	Obtuse	Blunt
Petiole: length (cm)	1.5	2.8	1.7	2.5
diameter (mm)	4.0	3.0	2.0	1.0
pigmentation (+/−)	+	+	+	−
Stipule: leaf[b]				
form	Absent	Filiform	Rudimentary	Filiform
margin	–	Fine, double serrate	Entire	Entire
length (cm)	–	0.5–1.0	0.5	2.0
Petiole glands: no.	4–6	1–3	1–3	1 or absent
form	Prominent, reniform	Prominent, reniform	Small, reniform	Small, flat
colour[a]	Greyed Purple Group 187A	Yellow Green Group 145A	Greyed Purple Group 183A	Green Group, 137D
Trichomes	Abundant, along secondary veins	Abundant, only on main vein	Sparse, only in vein ramifications	Absent

[a] Data referring to colour assessments of leaves and petiole glands based on Royal Horticultural Society Colour Chart, R.H.S. (London)/Flower Council of Holland (Leiden) (1988) with respect to colour group, hue, saturation and brightness
[b] See also Fig. 1 f. Data was an average of a minimum of 10 measurements per genotype

compared with extracts of the two parents individually or as physical mixtures. For esterases, plants of clones A and B had a similar banding profile, where some bands of both parents were retained whilst others were lost. As shown in Fig. 1 h, a new (hybrid) band was also evident. Similarly, for phosphoglucomutase, two novel bands were present in the hybrids, with only one, or in some cases two, bands being retained corresponding to both parent species (Fig. 1 i). Additionally, the banding patterns for phosphoglucomutase showed a difference between individuals at the clone level, which might correlate with the differences in morphological characters also observed between them. For malate dehydrogenase, the banding patterns, whilst confirming hybridity, showed no difference between clones A and B. Samples taken for isoenzyme analysis from the aneuploid individual (clone A) were consistently smeared, which made it impossible to identify individual profiles. This might imply an altered physiological status for this individual.

Discussion

Conventional plant breeding is based on parental selection, F_1 hybrid production followed by often lengthy selection and refinement in the resulting progeny. Most vegetatively propagated genotypes such as fruit tree rootstocks are likely to be improved with the introduction or modification of a few characteristics. In this context, however, since fruit trees (scions and stocks) are highly heterozygous outbreeding individuals with extended life cycles and with widespread asexual propagation modes (Childers 1976), genetic improvement is most likely to be achieved through somatic methods including somatic hybridization (James 1987; Ochatt et al. 1988 a). Superficially, a drawback of somatic hybridization (involving herbaceous species) is that hybrid plants are often found to be partially or totally sterile (Power and Davey 1988). Yet, in the context of rootstock improvement, somatic hybridization may be particularly appropriate since fertility considerations are largely irrelevant. Even if such somatic hybrids were to be introduced ultimately into a conventional breeding programme, a reduced fertility would not necessarily be of great concern, since fruit tree species are highly floriferous, multi-ovuled individuals where minimal seed set is required for fruit growth (Childers 1976; James 1987).

Several species within the family Rutaceae provided the first examples of woody species somatic hybrids (Grosser et al. 1988 a, b; Kobayashi et al. 1988; Ohgawara et al. 1985) and generally utilized protoplasts of embryogenic cell lines of one parent fused with leaf protoplasts or with callus protoplasts. The selection strategies relied on either a lack of protoplast division and/or

regeneration competence of one of the parents. In addition, somatic hybrid plants were always recovered via the embryogenic pathway.

In the experiments described here involving wild pear and Colt cherry, although both parental partners were capable of the reconversion of protoplasts to trees and always via organogenesis (Ochatt and Caso, 1986; Ochatt et al. 1987; 1988 b), the selection strategy for the recovery of their somatic hybrids was based on the use of a culture medium, previously shown to be suitable for successful protoplast culture of other rosaceous fruit tree species (Patat-Ochatt et al. 1988), but where neither of the parental protoplasts were capable of growth in their own right. Consequently, the ability to recover somatic hybrids was based solely on complementation to growth proficiency coupled with an anticipated heterosis effect.

Previous research had shown that electric pulses as applied to isolated Prunus and Pyrus protoplasts enhanced the division and regeneration capacity of protoplast-derived cells (Rech et al. 1987), coupled with increased DNA synthesis (Rech et al. 1988). It was felt that linking an electroporation treatment prior to protoplast fusion might, thereby, enhance the throughput of somatic hybrid cells from heterokaryons as proved to be the case in these experiments. A bonus of this approach was an enhanced level of heterokaryon formation, which was also likely to improve the probability of recovering hybrids. Electroporation treatment was also likely to be implicated in fostering plant regeneration from heterokaryon-derived calli, since the stimulatory effect of electroporation on the competence of plant regeneration had been previously reported for cell suspension protoplast-derived calli of Colt cherry (Ochatt et al. 1988 b).

Initial confirmation of hybridity for the wild pear (+) Colt cherry rootstock trees was provided by an intermediate phenotype. In this connection, particular relevance was attached to the presence or absence of stipules, since these are highly conspicuous for members of the Pomoidea but always absent in *Prunus* species (Childers 1976). All the hybrid rootstocks, some of which have been growing under field conditions for 1 year, retained an unaltered phenotype in relation to this and the other morphological markers.

Conclusive evidence of hybridity was provided by chromosomal and biochemical assessments. Isozyme analysis, which has been used for the confirmation of hybridity in other somatic hybrids (Grosser et al. 1988 a, b; Lo Schiavo et al. 1983; Power et al. 1976; Shepard et al. 1983) also provided proof that these plants were not chimaeras, since additional bands were present.

Preliminary assessments, in vitro, on graft compatibility of these wild pear (+) Colt cherry somatic hybrid rootstocks have shown that the hybrid trees are indeed graft-compatible with members of both the Pomoideae and the Prunoideae (unlike the wild pear or Colt cherry parent rootstocks). Although these observations have to be confirmed ultimately under field conditions, they strongly suggest that these somatic hybrid rootstocks might provide the basis of a universal rootstock for all Rosaceous fruit tree species. Equally relevant, though, is the fact that the production of these novel rootstocks has demonstrated that barriers to gene flow can be overcome for species that do not lend themselves to conventional breeding strategies and, this in turn, may provide plant material that will provide a basis for a better understanding of the fundamental physiological phenomena involved in scion/rootstock graft-compatibilities.

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