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Intergeneric somatic hybridization of sexually incompatible parents: *Citrus sinensis* and *Atalantia ceylanica*

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Abstract. Protoplast fusion using polyethylene glycol (PEG) was conducted to combine *Citrus sinensis* (L.) Osbeck cv. 'Hamlin' sweet orange protoplasts, isolated from nucellus-derived embryogenic callus with *Atalantia ceylanica* (Arn.) Oliv. leaf protoplasts. Five plants regenerated from independent fusion events following protoplast culture were identified as intergeneric allotetraploid somatic hybrids of 'Hamlin' sweet orange and *A. ceylanica*, and confirmed by isozyme analysis and chromosome number determination in root tip cells (2n=4x=36). Two different types of leaf morphology were observed among the hybrids (normal and narrow), although no differences in chromosome number nor isozyme banding patterns were observed. This is the first report of the production of hybrid plants between these sexually incompatible genera.

Introduction

Wild genera related to *Citrus* may be a source of useful genetic traits for rootstock improvement. However, sexual incompatibility has been a barrier to production of hybrid plants through sexual means. *Atalantia ceylanica*, a member of the Citrus Fruit Trees subtribal group, is sexually incompatible with *Citrus* and attempts to cross these two genera via sexual methods have not been successful (Iwamasa et al. 1988). *A. ceylanica* grows well in wet soil (Bitters et al. 1964), suggesting *Phytophthora* resistance; and it has survived temperatures of -5 to -7°C in California (T. Williams, 1992 pers. comm.). However, it is difficult to graft with *Citrus* (Bitters et al. 1977) and has not been used successfully as a *Citrus* rootstock. Somatic hybridization with *Citrus* offers a potential means to utilize the disease resistance and cold-hardiness of *Atalantia* in citrus rootstock development.

Somatic hybridization via protoplast fusion has been used successfully as an alternative breeding method to bypass the sexual incompatibility observed between *Citrus* and some of its wild relatives (Grosser and Gmitter 1990c). Several somatic hybrids between sexually incompatible parents have been obtained including: *Citrus sinensis* (L.) Osb. 'Hamlin' sweet orange + *Severinia disticha* (Blanco) Swing. (the Philippine box orange) (Grosser et al. 1988), *Citrus sinensis* 'Hamlin' sweet orange + *Severinia buxifolia* (Poir.) Tenore (Chinese box-orange) (Grosser et al. 1992b), *Citrus sinensis* 'Hamlin' sweet orange + *Citropsis gilletiana* (Swing.) M. Kell. (Gillet's cherry orange) (Grosser and Gmitter 1990b), and *Citrus reticulata* (Blanco) Cleopatra mandarin + *Citropsis gilletiana* (Grosser et al. 1990).

The present work describes the production of somatic hybrid plants of *C. sinensis* 'Hamlin' sweet orange and *A. ceylanica.*

Materials and methods

Protoplast isolation and fusion. Leaf protoplasts of A. ceylanica were isolated from young seedlings maintained in a growth chamber with a 16 h photoperiod of 300 μmol $\cdot s^{-1} \cdot m^{-2}$ light intensity at 26-30°C (Grosser and Chandler 1987). Protoplasts of 'Hamlin' sweet orange were isolated from friable nucellus-derived embryogenic callus cultures maintained on either EME or H+H solid media (Grosser and Gmitter 1990a). All protoplasts were purified by passage through a 45 μm stainless steel filter followed by centrifugation on a 25% sucrose-13% mannitol gradient prior to mixing (Tusa et al.

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1990; Grosser and Gmitter 1990a). Approximately equal volume of protoplasts from each parent were mixed and fused using the polyethylene glycol (PEG) method described by Grosser and Gmitter (1990a).

Protoplast culture and plant regeneration. Following fusion, protoplasts were cultured directly in 60 mm x 15 mm plastic petri dishes (Falcon, Lincoln Park, NJ) in a 1:1 (v:v) mixture of EMEP and BH3 protoplast culture media as described by Grosser and Gmitter (1990a). Recovered somatic embryos were germinated on either B+ embryo germination medium, DBA3 medium (Grosser and Gmitter 1990a), or BGN medium (Louzada et al. 1992) in Magenta boxes (Magenta Corp., Chicago, IL). Embryos that developed shoots were transferred to RMAN rooting medium (Grosser and Gmitter 1990a).

Cytology. The chromosome number of regenerated plants was determined in actively dividing root-tip cells using the modified hematoxylin staining method (Grosser and Gmitter 1990a; Gmitter et al. 1990).

Electrophoretic analysis of leaf isozymes. Isozyme banding patterns of crude leaf tissue extracts from A. ceylanica, C. sinensis 'Hamlin' sweet orange, and regenerated plants were performed on horizontal mixed starch (9.85%) and agarose (0.15%) gels with histidine-citrate buffer (pH 5.7) (Cardy et al. 1981). Electrophoresis was carried out for 3 h and 4°C at 60 mA constant current. The gel was sliced and stained for peroxidase (PER, E.C.1.11.1.7.), phosphoglucosemutase (PGM, E.C.2.7.5.1.), and phosphoglucose isomerase (PGI, E.C.5.3.1.9.) as described by Vallejos (1983).

Results and discussion

Following protoplast fusion and culture, many somatic embryos with normal morphology were produced, but they were unable to regenerate plants on either DBA3, B+, or BGN media in plastic petri dishes. From approximately 30 embryos cultured in 50 ml of B+ or BGN medium in Magenta Boxes (Magenta Corp., Chicago, IL), five shoots were regenerated. Root induction from regenerated shoots was slow, requiring three one-month passages on the RMAN rooting medium (Grosser and Gmitter 1990a). One shoot never produced roots. One plant had the expected leaf morphology intermediate to that of the parents (Fig. 1b). The remaining four plants were less vigorous and exhibited an unexpected narrow leaf morphology (Fig. 1c). The four rooted plants were successfully transferred to soil, and the one with intermediate leaf morphology is growing normally, while the three with narrow leaf morphology are exhibiting slow growth, with one not growing. Although the protoplast culture medium used (1:1-v:v- mixture of EMEP and BH3 protoplast culture media) allows nonfused 'Hamlin' protoplasts to grow and regenerate (Grosser et al. 1992a), no 'Hamlin' plants were regenerated. It is possible that the 'Hamlin' sweet orange callus line used to provide the protoplasts has lost its capacity for plant regeneration. No plants were regenerated from nonfused *Atalantia* leaf protoplasts.

Although two distinct types of leaf morphology were observed in the regenerated somatic hybrid plants (Fig. 1b, 1c and 2), there were no differences observed in chromosome number and isozyme banding patterns. The chromosome number (Fig. 3) revealed the amphidiploid number (2n=4x=36) as expected for the true hybrid (each parent being 2n=2x=18).

Banding patterns of 'Hamlin', *A. ceylanica*, and somatic hybrid leaf isozymes are shown in Fig. 4a-c. As with previous somatic hybrids of *Citrus*, the isozyme activity of the purported somatic hybrids revealed the additive expression of alleles unique to the donor parents, thereby confirming hybridity. This additivity can be most clearly seen in the PER and PGM zymograms (Fig. 4a, b). The PGI zymogram of the somatic hybrid shows an allelic dosage effect that results in the greatest staining intensity at the position SS homodimer, intermediate intensity at the position of the MS heterodimer, and the least intensity at the MM homodimer position.

There are several possible explanations for the differences observed in the morphology and growth rate between the two types of somatic hybrid plants recovered. These include: somaclonal variation (possibly growth regulator induced during embryo germination or root induction); genetic variability in the parental embryogenic callus line; or differential organelle inheritance. Kobayashi et al. (1991) reported differential inheritance in chloroplast genomes in somatic hybrid plants of navel orange (*C. sinensis*) and 'Murcott' tangor, but it was not associated with morphological variation. Populations of *Citrus* somatic hybrid plants from any specific parental combination are generally very uniform. We have produced *Citrus* somatic hybrid plants from more than 50 parental combinations, and morphological variation was observed on only one other occasion. Somatic hybrid plants from the fusion of sour orange (*C. aurantium*) with a Volkamer lemon zygotic seedling (*C. volkameriana*) exhibited two distinct non-aberrant morphological types, of which the cause has yet to be determined (Louzada et al. 1992).

The acclimation process for the four hybrid plants was much longer than normal, requiring high humidity for approximately 3 months. The more vigorous hybrid plant will be propagated for rootstock evaluation by grafting to vigorous citrus rootstocks to increase vegetative material, followed by the rooting of cuttings according to the procedure of Sabbah et al. (1991). Successful somatic hybridization of *Citrus* with *Atalantia* further increases the germplasm available for citrus cultivar improvement.

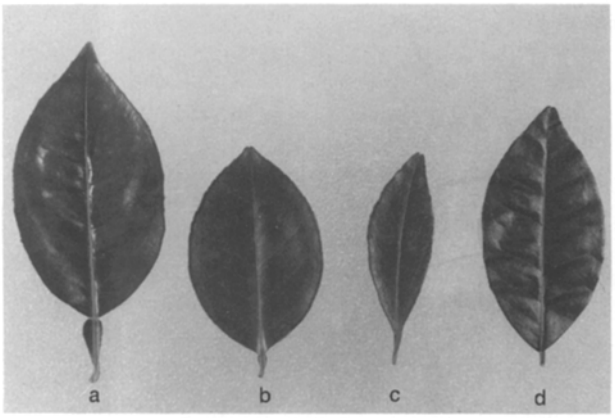

Fig. 1. Leaf morphology of 'Hamlin' sweet orange (1a), 'Hamlin' sweet orange + Atalantia ceylanica somatic hybrid type I (1b), 'Hamlin' sweet orange + Atalantia ceylanica somatic hybrid type II (1c), and Atalantia ceylanica (1d).

Fig. 2. 'Hamlin' sweet orange + Atalantia ceylanica somatic hybrid plants exhibiting the normal (left) and aberrant (right) leaf morphologies.

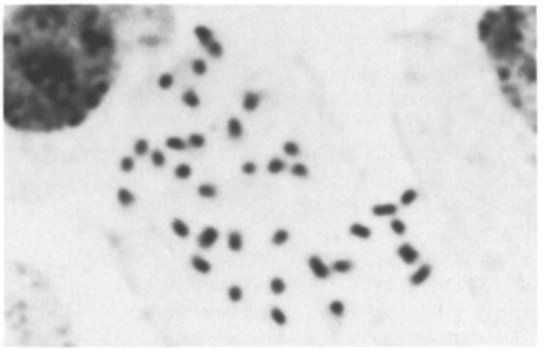

Fig. 3. Root tip squash prepared from 'Hamlin' sweet orange + Atalantia ceylanica somatic hybrid showing the allotetraploid chromosome number (magnification = 600X).

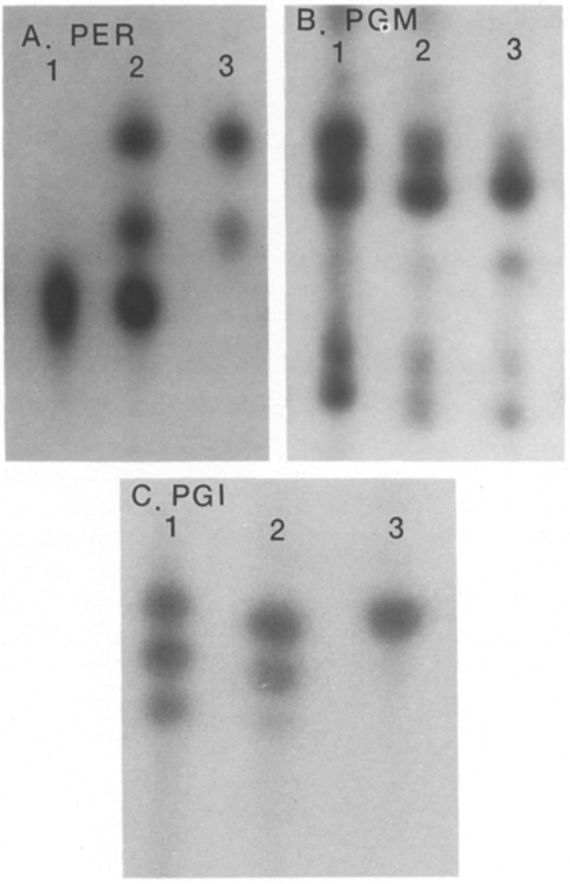

Fig. 4. Starch gel stained for (A) PER, (B) PGM, and (C) PGI activity. Designated genotypes are as follows: 'Hamlin' sweet orange, PER = FF (lane A1), PGM-1 = FS (lane B1), PGI = MS (lane C1); 'Hamlin' + A. ceylanica somatic hybrid, PER = FFXY (lane A2), PGM-1 = FFPS (lane B2), PGI = MSSS (lane C2); A. ceylanica, PER = XY (lane A3), PGM-1 = FP (lane B3), PGI = SS (lane C3).

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