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Intergeneric somatic hybrid plants from sexually incompatible woody species: *Citrus sinensis* and *Severinia disticha**

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Summary. The fusion of *Citrus sinensis* cv. Hamlin (sweet orange) protoplasts isolated from an embryogenic suspension culture with *Severinia disticha* (Philippine box orange) protoplasts isolated from epicotyl-derived callus with organogenic potential, resulted in the regeneration of allotetraploid somatic hybrid plants. Plant regeneration was a function of complementation, combining the capacity for somatic embryogenesis of *C. sinensis* with the organogenic ability of *S. disticha*. Confirmation of somatic hybrid identity was based on leaf morphology, chromosome number, and analyses of phosphoglucose mutase (PGM) and malate dehydrogenase (MDH) zymograms. Hybrid plants were multiplied organogenically and exhibited morphology intermediate to that of the parents. This is the first example of somatic hybrid plants produced between sexually incompatible woody genera.

Key words: Protoplast fusion – Plant regeneration – Rootstock – Cultivar improvement – Plant breeding

Introduction

Somatic hybridization has been touted as a means of bypassing barriers to sexual hybridization in order to access unavailable but desirable germplasm for crop improvement. However, successes in this area have been limited largely to the *Solanaceae* (Collins et al. 1984) and more recently the *Cruciferae* (Sundberg and Glimelius 1986) for various reasons, including a lack of efficient protoplast to plant regeneration system outside the *Solanaceae*, and the existence of somatic incompatibilities between distantly related species (Harms 1983). The elevated status of *Citrus* tissue and protoplast culture have made it an excellent candidate for efforts in somatic hybridization. Plant regeneration from protoplasts of several citrus types has been achieved via somatic embryogenesis (Vardi et al. 1982; Kobayashi et al. 1983). An intergeneric somatic hybrid produced between sexually compatible species *Citrus sinensis* L. Osbeck cv. Trovita and *Poncirus trifoliata* L. Raf. was reported by Ohgawara et al. (1985). Recently, we have produced a similar somatic hybrid between *C. sinensis* cv. Hamlin and *P. trifoliata* cv. Flying Dragon (Grosser et al. 1987) and have proposed that the system used to generate this somatic hybrid should serve as a model for the production of other intergeneric hybrids between *Citrus* and sexually incompatible species. The proposed model system allows for plant regeneration from only somatic hybrid tissue and takes advantage of distinct morphological markers unique to the respective parents for somatic hybrid identification. Using a modification of this model system, we have produced somatic hybrid plants between *S. disticha* (Blanco) Swing. (the Philippine box-orange) and *C. sinensis* cv. Hamlin (a sweet orange). This report deals with the production and verification of vigorous somatic hybrid plants generated between these sexually incompatible woody genera.

Both *S. disticha* and *C. sinensis* are members of the family *Rutaceae*, orange subfamily *Aurantioideae*, Tribe II *Citreae* and subtribe *Citrinae*, consisting of three subtribal groups, 13 genera and 65 species (Swingle and Reece 1967). *Citrus* is a member of the true citrus fruit tree subtribal group and *Severinia* belongs to the primitive citrus fruit tree subtribal group. *S. disticha* is

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graft compatible with *Citrus,* but there have been no sexual hybrids produced between these two distinct subtribal groups. The genus *Severinia* is a potential germplasm source for many traits desirable for citrus rootstock improvement including cold hardiness, salt and boron tolerance (Cooper 1961), and resistance to *Phythopthora* (Hutchison and Grimm 1973) and nematodes (Hutchison and O'Bannon 1972).

Materials and methods

Protoplast isolation

Fruit of *S. disticha* were obtained from the Florida Citrus Arboretum, Winter Haven, FL. Seeds were extracted and surface sterilized by immersion in 1N HCl for 30 s, followed by a 20 min immersion in 0.525% sodium hypochlorite with a drop of Liquinox detergent and rinsed twice in double distilled sterile H_2O. Treated seeds were germinated on a 1/10 strength Murashige and Tucker basal medium (MT, 1969) containing 25 g/l sucrose. Seedling epicotyls were dissected, sectioned and placed on a callus induction-maintenance medium (designated MTC), consisting of MT basal medium supplemented with 0.55 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.22 mg/l kinetin, 20 ml/l coconut H_2O (Gibco) and 50 g/l sucrose. The resulting callus cultures were subcultured monthly. For protoplast isolation, approximately 0.5 g of vigorous friable callus, six months post-initiation, was macerated gently into 2 ml BH3 protoplast culture medium (Grosser and Chandler 1987) in a 60×15 mm plastic Petri dish, to which 2.5 ml enzyme solution (Grosser and Chandler 1987) was added drop by drop to facilitate maceration and enzyme penetration. The Petri dishes were sealed with Nescofilm and incubated 2–4 h on a rotary shaker (50 rpm) at 28 °C. Resulting protoplasts were filtered through a 45 μm stainless steel filter, removed from the enzyme-medium mixture by centrifugation (10 min × 100 g) and resuspended in BH3 medium. Protoplasts of *C. sinensis* cv. Hamlin were isolated from an habituated suspension culture (1-year-old) initiated from 3-year-old ovule-derived callus, as described previously (Grosser et al. 1987).

Protoplasts fusion and culture

Approximately equal volumes of protoplasts from each parental source were mixed and fused using the polyethylene glycol (PEG) method of Menczel et al. (1981). Prior to fusion, pelleted mixed protoplasts were resuspended in a total volume of BH3 medium equal to 2× or 4× the volume of the original pellet, and 2 drops of mixed protoplasts were used per fusion Petri dish. A typical mixture provided enough protoplasts to perform 6–12 fusions at each density. BH3 medium was used for all volume adjustments and washe. Fusion solutions were eluted by five washes, each consisting of careful removal of medium with a pasteur pipette and replacement with 15 drops fresh medium. Protoplasts were cultured directly in the fusion Petri dishes in 8–20 drops of BH3 medium. Fusion plates were sealed with Nescofilm and stored in sealed plastic containers in the dark at 28 °C. The fusion cultures were supplemented with 10–12 drops of a 1:2 (v:v) mixture of BH3 medium and liquid MT basal medium containing 125 g/l sucrose and 0.5 g/l malt extract six weeks following fusion, 10–12 drops of liquid MT basal medium containing 100 g/l sucrose and 0.5 g/l malt extract eight weeks following fusion and 10–12 drops of liquid MT basal medium containing 50 g/l sucrose and 0.5 g/l malt extract every subsequent two weeks. Cultures were transferred to a low light environment (20 μmol $S^{-1}m^{-2}$) six weeks post-fusion. Larger colonies (1–2 mm in diameter) and somatic embryos developing directly in liquid medium were transferred to agar-solidified MT basal medium containing 0.5 g/l malt extract and 50 g/l sucrose. Recovered somatic embryos exhibiting normal morphlogy were enlarged on MT basal medium containing 1,500 mg/l malt extract and 25 g/l sucrose (as recommended by A. Vardi, Volcani Center, Israel). Somatic embryo germination was attempted on a MT basal media containing 25 g/l sucrose, 1 mg/l giberellic acid (GA3) ± 15 mg/l coumarin, but embryo germination did not occur following two one-month passages. Ungerminated somatic embryos were sectioned and cultured on a MT basal medium containing 5.0 mg/l 6-benzylaminopurine (6-BAP). Resulting multiple shoots were rooted and transferred to soil as described previously (Grosser et al. 1987).

Analyses of regenerated somatic hybrid plants

Morphological characteristics of each parent and regenerated plants were observed and compared. Chromosome numbers of regenerated plants were determined by root-tip cytology performed according to the protocol described previously (Grosser et al. 1987).

Electrophoretic analysis of parental and regenerated hybrid plants was performed using 10% starch gels with histidine gel and tank buffers (Cardy et al. 1981). Crude leaf extracts were prepared by grinding wicks of Whatman filter paper (No. 1) into the lower surface of the leaf samples. Samples were run for 3 h with a constant current of 40 mA. Gels were sliced and stained for phosphoglucose mutase (PGM) (E.C.2.7.5.1) and malate dehydrogenase (MDH) (E.C.1.1.1.37) activity using standard recipes (Vallejos 1983).

Results and discussion

The selection scheme employed took advantage of two distinct types of cell lines. The *S. disticha* callus line was grown on a high auxin medium, and the callus tissue was capable of organogenesis. Previous control experiments had demonstrated that protoplasts isolated from this line did not resynthesize cell walls or undergo mitosis in the protoplast culture medium utilized (BH3 and MT basal media contain no growth regulators). In contrast, the habituated 'Hamlin' suspenion culture was initiated from an embryogenic callus culture grown on growth regulator free medium (no auxin). 'Hamlin' protoplasts isolated from this line were amenable to culture (mitosis and callus formation), but because of the age of the culture only malformed somatic embryos, incapable of further regeneration, were recovered from control cultures. Following the fusion of *S. disticha* with 'Hamlin', unfused *S. disticha* protoplasts gradually disintegrated whereas unfused 'Hamlin' protoplasts acted as nurse culture cells and provided an appropriate cell density to allow the survival and mitotic division of heterokaryons. The regeneration of only somatic hybrid plants was the result of complementation between the

parents, combining somatic embryogenesis and organogenesis.

Repeated efforts to isolate epicotyl callus-derived protoplasts of *S. disticha* gave inconsistent yields and the preparations contained variable levels of particulate debris. *S. disticha* protoplasts were approximately twice as large as corresponding 'Hamlin' protoplasts and usually contained clumps of globular storage bodies. *S. disticha* protoplast preparations were susceptible to over-digestion, but superior preparations were amenable to fusion, resulting in high fusion frequencies. In contrast, very clean high yield preparations of 'Hamlin' protoplasts were routinely isolated from habituated suspension cultures. *S. disticha* × 'Hamlin' heterokaryons could be identified visually on the basis of the presence of two distinct types of storage bodies within a fusion product, donated by each parent. The first mitotic division of protoplasts in fusion cultures was observed 12 days post-fusion.

Attempts to germinate well-formed somatic hybrid embryos recovered from fusion cultures were unsuccessful. Previously, we were able to regenerate shoots of *S. disticha* from epicotyl-derived callus via shoot bud induction (organogenesis) on MT basal medium containing 5.0 mg/l 6-BAP and 0.01 mg/l 2,4-D (data not shown). Therefore, ungerminated somatic embryos recovered from fusion cultures were sectioned and cultured on a MT basal medium containing 5.0 mg/l 6-BAP in an attempt to induce organogenesis. Shoot-bud induction and subsequent multiple shoot formation occurred from the meristematic areas of several of the dissected embryos. Hybrid shoots transferred to rooting medium rooted efficiently; over 70% of the shoots developed vigorous taproots within four weeks. It was interesting to note that somatic hybrid shoots rooted more efficiently than organogenically derived shoots of either parent. In previous attempts, we were able to root only two out of 100 *S. disticha* shoots on various media, some containing low levels of auxin.

We have also attempted to generate *Citrus* × *Severinia* somatic hybrids by fusing leaf protoplasts of *Severinia* with suspension culture derived protoplasts of 'Hamlin'. Although we recovered somatic embryos from such fusion cultures, plant regeneration was not successful. This may be due to some inhibitory metabolite present in the *Severinia* leaf protoplasts and/or differences in regeneration capacity between *Severinia* genotypes and/or tissue sources.

As of September, 1987, over 150 somatic hybrid plants had been produced and were growing in soil (Fig. 1). The number of fusion events contributing to this population was not determined. Since then, we have regenerated plants from physically separated fusion cultures.

Fig. 1. Population of intergeneric somatic hybrid plants (*Citrus sinensis* cv. 'Hamlin' × *Severinia disticha*)

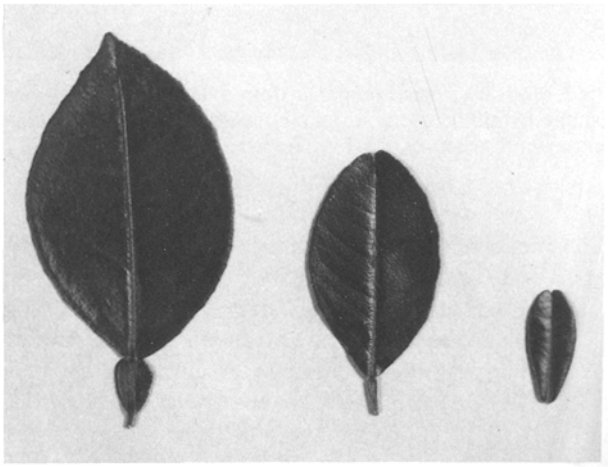

Fig. 2. Profiles of leaf morphology (from *left to right*) of *C. sinensis* cv. 'Hamlin', Somatic hybrid of 'Hamlin' and *S. disticha,* and *S. disticha*

Regenerated somatic hybrid plants are vigorous, uniform and exhibit a morphology intermediate to the parents. Distinct morphological characters unique to each parent were observed in the hybrid plants. These characters include pubescence and red pigmentation in developing leaves, from *S. disticha,* and a winged petiole, from 'Hamlin'. A profile of leaf morphology of the somatic hybrid and parents is provided in Fig. 2. Root-tip cytology performed on five of the hybrid plants revealed the expected amphidiploid chromosome number of 36 (Fig. 3), apparently 18 from each parent. This evidence suggests that the somatic hybrid plants have retained the entire nuclear genome of each parent. Some chromosomes were very small, and slight

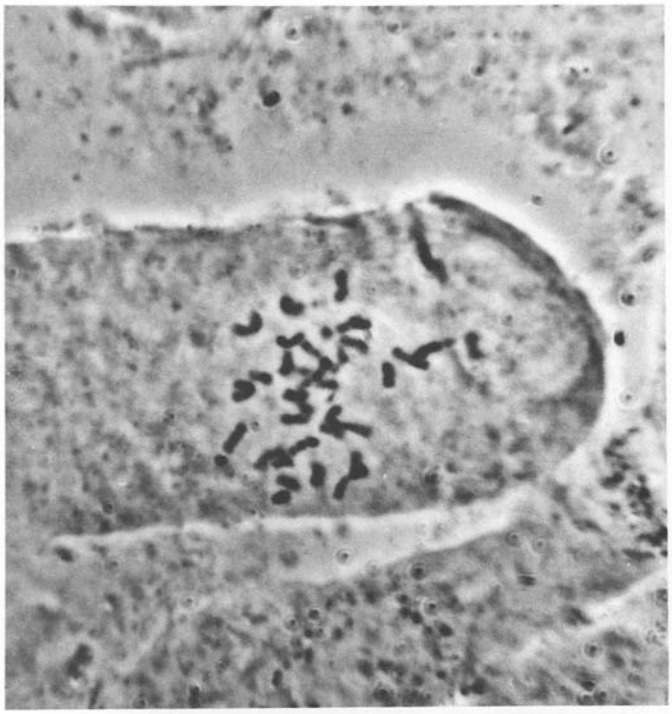

Fig. 3. Root tip squash prepared from a 'Hamlin' × *S. disticha* somatic hybrid, showing an allotetraploid chromosome number (magnification = 1,000×, oil immersion)

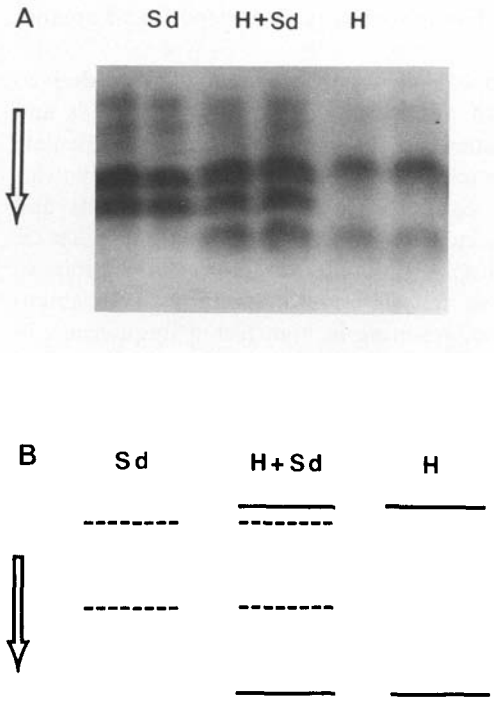

Fig. 4 A, B. Starch gel electrophoresis of PGM isozymes. Photo (**A**) and diagram (**B**) of zymograms of 'Hamlin' sweet orange (H), *Severinia disticha* (*Sd*), and somatic hybrid plants (*H* + *Sd*)

adjustments in the plane of vertical focus were required for accurate counting.

Starch gel electrophoresis of PGM and MDH isozymes was utilized to provide biochemical confirmation of the hybrid nature of the regenerated plants (Figs. 4 and 5). PGM is a monomeric enzyme coded for by a single locus in *Citrus* (Soost and Torres 1981; Torres et al. 1982). 'Hamlin' orange PGM zymograms revealed two bands, one each from the F and S alleles. Likewise, *S. disticha* produced two bands, but both were of intermediate mobility relative to the S and F alleles of 'Hamlin'. No effort was made to determine the allelic identity of the *Severinia* bands by comparison with known *Citrus* PGM genotypes. Nonetheless, the zymograms of regenerated hybrid plants revealed a complementary pattern of four bands corresponding with the bands observed in each of the parental zymograms. Note that the slow band of *S. disticha* migrates only slightly faster than the slow band of 'Hamlin', and these bands overlap in the hybrid zymogram.

In contrast with PGM, MDH isozymes are dimeric and controlled by two separate loci in *Citrus* (MDH-1 and MDH-2) (Soost and Torres 1981; Torres et al. 1982). The subunits of MDH-1 and MDH-2 do not dimerize with each other. 'Hamlin' orange MDH zymograms revealed two bands corresponding with the FF (MDH-1) and MM (MDH-2) homodimers. The *Severinia*

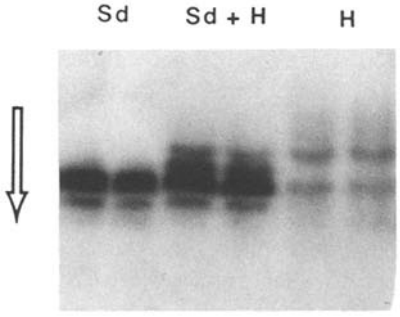

Fig. 5. Starch gel electrophoresis of MDH isozymes. The photo of the zymogram demonstrates a complementary banding pattern in somatic hybrid plants (*H* + *Sd*), produced between 'Hamlin' sweet orange (*H*) and *Severinia disticha* (*Sd*)

zymograms were more complex, and determination of the allelic constituents were not made. However, the 'Hamlin' orange and *Severinia* zymograms were distinguished by the unique FF band (MDH-1) of 'Hamlin' and a unique *Severinia* band that displayed greater mobility than the MM band (MDH-2) from 'Hamlin' (Fig. 5). Both unique parental bands were visible in the complex pattern produced by the regenerated somatic hybrid plants. The evidence provided by PGM and MDH isozyme systems confirmed the hybrid origin of the regenerated plants.

Evidence for the reproducibility of the described methods and results has been demonstrated by the regeneration of putative somatic hybrid plants between 'Hamlin' and *Severinia buxifolia* Poir. Ten. Hybrid plants are now being multiplied and grown in preparation for further analyses.

The horticultural evaluation of 'Hamlin' × *S. disticha* somatic hybrid plants is underway. In the event that this hybrid shows promise as a citrus rootstock, nursery propagation by the conventional technique of nucellar polyembryony probably would not be sufficient, because *S. disticha* is a monoembryonic species. However, in vitro shoot multiplication or the use of rooted cuttings could provide a suitable alternative method. If the somatic hybrid plants are fertile and sexually compatible with citrus types, they will provide a novel germplasm source for efforts in scion and rootstock improvement.

In conclusion, this research provides the first example of regenerated somatic hybrid plants produced between sexually incompatable woody genera. Accession of previously unavailable wild-relative germplasm for citrus rootstock improvment is now possible. These results suggest potential for generation of somatic hybrids between *Severinia* and other citrus types, and between citrus and other related genera of interest that are either sexually incompatible or difficult to hybridize by conventional methods.

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