Theor Appl Genet (1986) 71:691–697

Somatic hybrid plants between *Lycopersicon esculentum* and *Solanum lycopersicoides*

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Received September 30, 1985; Accepted October 7, 1985
Communicated by H. F. Linskens

Summary. Leaf mesophyll protoplasts of *Lycopersicon esculentum* ($2n=2x=24$) were fused with suspension culture-derived protoplasts of *Solanum lycopersicoides* ($2n=2x=24$) and intergeneric somatic hybrid plants were regenerated following selective conditions. A two phase selection system was based on the inability of *S. lycopersicoides* protoplasts to divide in culture in modified medium 8E and the partial inhibition of *L. esculentum* protoplasts by the PEG/DMSO fusion solution. At the p-calli stage, putative hybrids were visually selected based on their hybrid vigor and lime-green coloration in contrast to slower growing parental calli characterized by a watery, whitish-brown coloration. Early identification of the eight hybrid plants studied was facilitated by isozyme analysis of leaf tissue samples taken from plants in vitro at the rooting stage. Regenerated plants growing in planting medium were further verified for hybridity by 5 isozymes marking 7 loci on 5 chromosomes in tomato. These included Skdh-1 mapped to chromosome 1 of tomato, Pgm-2 on chromosome 4, Got-2 and Got-3 on chromosome 7, Got-4 on chromosome 8, and Pgi-1 and Pgdh-2 both on chromosome 12. Fraction I protein small subunits further confirmed the hybrid nature of the plants with bands of both parents expressed in all hybrids. The parental chloroplasts could not be differentiated by the isoelectric points of the large subunit. Seven of the eight somatic hybrids had a chromosome number ranging from the expected $2n=4x=48$ to $2n=68$. Mixoploid root-tip cells containing 48, 53, 54 or 55 chromosomes for two of the hybrids were also observed.

* Michigan Agricultural Experiment Station Journal Article No. 11736. Supported by Grant No. I-751-84R from BARD – The United States – Israel Binational Agricultural Research and Development Fund

Key words: Cell-fusion – Fraction-I protein – Isozymes – Protoplasts – Regeneration – Somatic hybrid plants

Introduction

The cultivated tomato, *Lycopersicon esculentum*, Mill., is an important horticultural crop grown throughout the world. Yields of this crop, however, are often decreased by plant and fruit sensitivity to cold temperatures especially in areas with short growing seasons (Herner 1982). No true chilling tolerant genotypes have been found among accessions of *L. esculentum* but are present among other *Lycopersicon* and *Solanum* species (Patterson et al. 1978; Scott and Jones 1982; Vallejos 1979).

One such species is *Solanum lycopersicoides* Dun., a perennial wild relative of the tomato native to the southern coast of Peru. In cold stress studies conducted at Michigan State University, selections of this species originating from higher altitudes have exhibited a degree of cold tolerance superior to *Lycopersicon* species (Herner and Kamps 1983).

S. lycopersicoides, however, is difficult to cross sexually with tomato. The cross can only be made unilaterally using tomato as the female parent and the intergeneric hybrid is highly sterile (Rick 1951). Backcrosses can sometimes be made with tomato used as the female parent (Rick 1951) but the resulting progeny are also sterile.

Present goals in our tomato cell genetics project are to develop chilling tolerant tomato germplasm and to study organelle genetics in this species. Based on the above considerations we devised a protoplast scheme for the production of somatic hybrids between tomato and *S. lycopersicoides*. The success in this endeavor was based on previous reports from this laboratory dealing with the regeneration of plants from tomato leaf protoplasts (Niedz et al. 1985), and cell culture and proto-

plast regeneration for *S. lycopersicoides* (Handley and Sink 1985 a, b). Herein the cell manipulations for the production of somatic plant hybrids between *S. lycopersicoides* (LA 1990) and *L. esculentum* 'Sub Arctic Maxi' and their verification by biochemical and cytological studies is reported.

Materials and methods

Plant material

Solanum lycopersicoides LA 1990 was obtained from C. M. Rick; Tomato Genetics Stock Center, University of California, Davis. The *Lycopersicon esculentum* 'Sub Arctic Maxi' was obtained from D. Emmatty, H. J. Heinz Co., Bowling Green, Ohio.

Protoplast isolation

Seeds of *L. esculentum* 'Sub Arctic Maxi' were sown in V.S.P. planting medium (Bay Houston Towing Co., Houston, Texas) and grown in a controlled environment chamber at 22 °C night temperature/27 °C day temperature under a photon fluence flux of 210 $\mu E\ m^{-2}\ s^{-1}$ provided by cool white fluorescent lamps (GE96T12CW) on a 16 h photoperiod. Seedlings were transplanted to 4-cell plastic trays and maintained under the above conditions. Plants were fertilized at each watering with 150 ppm 20N–8.6P–16.6K aqueous solution adjusted to pH 6.5 with phosphoric acid. Leaf harvesting, sterilization, and protoplast isolation procedures were followed as described (Niedz et al. 1985). Following the last wash, the leaf mesophyll protoplasts were collected, counted and resuspended in W5 solution (Menczel and Wolfe 1984) prior to fusion.

Suspension cultures of *S. lycopersicoides* LA 1990 were initiated and maintained as described (Handley and Sink 1985 b). Protoplasts were isolated from the 20th subculture, 5 days after the previous subculture. The suspension culture protoplasts were isolated, washed and counted as described (Handley and Sink 1985 b) and resuspended in W5 solution prior to fusion.

Protoplast fusion

A modification of the procedure of Menczel and Wolfe (1984) was used to fuse tomato mesophyll protoplasts with *Solanum* suspension culture protoplasts. A mixture was prepared which contained 2×10^6 mesophyll protoplasts and 9×10^6 suspension protoplasts in 12 mls of W5. A PEG fusion-inducing solution was prepared as described (Menczel and Wolfe 1984) using 10% DMSO. Fusion was initiated by placing 1 ml of the PEG/DMSO solution in a 16×100 mm test tube. To this fusogen 0.5 ml of the mesophyll and suspension protoplast mix was added and allowed to stand for 3 min. The entire mixture was agitated gently by hand, then allowed to stand another 5 min. This suspension was then diluted by gently adding 8.5 ml of W5 solution supplemented with 50 mM morpholinoethansulfonate (MES, pH 5.6) buffer. The protoplasts were incubated in this solution for 30 min at room temperature. The tubes were then centrifuged at 60×g for 5 min and the supernatant discarded. In each tube the pellet was resuspended in 4 mls of modified medium 8E (M8E) (Niedz et al. 1985) and 2 mls (approximately 1.5×10^6 protoplasts/ml) were pipetted into each of 2 15×60 mm plastic petri dishes. The dishes were sealed with Parafilm and placed in the dark at 28 °C. Unmixed protoplasts of both tomato and *Solanum* were incubated in the same PEG/DMSO solution, brought to volume with buffered W5 solution, centrifuged, and plated as above to serve as controls.

Protoplast culture

Two days after plating, the protoplast cultures were transferred to the light (3–4 $\mu E\ m^{-2}\ s^{-1}$, cool white, 16 h photoperiod) at 28 °C. Two weeks after plating, the cultures were moved to a higher light intensity of 20–28 $\mu E\ m^{-2}\ s^{-1}$ and each dish was fed with 0.2 mls of fresh M8E without 2,4-D (M8E-2,4-D). Dishes were fed every 2 weeks thereafter with 0.2 ml M8E-2,4-D containing decreasing levels of mannitol used as the osmoticum (from 0.5 M to 0.2 M). Seventy days after initial plating, the dishes were split in half and brought to the original volume with M8E-2,4-D, 0.2 M mannitol. Three months following plating, the entire contents of the 15×60 mm dishes were pipetted onto the surface of Whatman No. 1 filter paper discs overlaid on agar solidified MS3ZG regeneration medium (Handley and Sink 1985 b).

The small p-calli plated onto the MS3ZG medium rapidly resumed growth. As soon as these colonies were 2–3 mm in size they were removed and placed on MS3ZG medium without filter paper as described (Handley and Sink 1985 b). New colonies appeared on the filter paper plates over a two month period and as these reached 2–3 mm diameter they were individually selected and transferred to fresh MS3ZG medium. Subsequent subculturing on MS3ZG was done monthly.

Shoots arising from these calli were excised and rooted in vitro in Murashige and Skoog (1962) inorganic salts and vitamins containing thiamine-HCl at 1.0 mg/l (MSOT). Regenerated shoots were maintained on MSOT for initial isozyme analysis, rooting and growth prior to transfer to the greenhouse.

Biochemical analyses

Eight putative somatic hybrids, one sexual intergeneric (*L. esculentum* 'Sub Arctic Maxi'×*S. lycopersicoides*) hybrid plant and both parental-species were analyzed by starch gel electrophoresis. Samples were collected from shoots maintained on MSOT medium or plants growing in a greenhouse. Root or leaf samples were homogenized in a 0.1 M tris-HCl buffer pH 7.6 containing 0.5% 2-mercaptoethanol and 1% PEG (MW 3350). Enzymes were separated by electrophoresis on 12% starch gels. Glutamate oxaloacetate transaminase (Got), phosphoglucoisomerase (Pgi) and phosphoglucomutase (Pgm) were analyzed using a lithium borate buffer system (Scandalios 1969). Shikimic acid dehydrogenase (Skdh) and 6-phosphogluconate dehydrogenase (Pgdh) were analyzed using a tris citrate gel and electrode buffer modified from that of Meinzel and Markert (1967). Stain formulations were as described by Vallejos (1983). Skdh was analyzed in root samples and Got in leaf samples. The other enzymes were analyzed in both roots and leaves.

Isozymes localized in plastids were identified by preparing chloroplast extracts. Leaf tissue was homogenized by five 1 s bursts in a Waring blender in four volumes of homogenization buffer. The homogenization buffer was 10 mM phosphate buffer pH 7.4, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EDTA and 0.4 M sucrose. The homogenate was filtered through 4 layers of cheesecloth and 2 layers of Miracloth and then centrifuged at 2,000×g for 30 s. The pellet was resuspended in homo-

genization buffer and re-centrifuged. The final pellet was resuspended in the isozyme extraction buffer and analyzed by starch gel electrophoresis.

Samples for isoelectric focusing were prepared as described by Cammaerts and Jacobs (1980). Isoelectric focusing was performed on a 5% acrylamide gel containing 8 M urea and 2.67% ampholytes (equal parts Pharmalytes pH 5–8 and pH 3–10). Gels were focused for 3–5 h at 1,000 V. Protein was stained as described by Shepard et al. (1983).

Cytology and morphology

Shoots of the somatic hybrid plants exhibited some callus formation at the basal end during the rooting stage in vitro. Therefore, terminal shoot cuttings were taken from greenhouse-grown plants, and placed in moistened perlite in a controlled environment chamber. Subsequently, root tips were taken from the rooted cuttings or from plants transferred to planting medium. Root tips were pretreated for 3 h in p-dichlorobenzene, fixed in Carnoys solution, hydrolyzed in 1.0 N HCl at 60 °C for 10–12 min, and the cells smeared and stained in propio-orcein or aceteo-carmine. Pollen viability was assessed in aniline blue solution.

Results and discussion

Three fusion experiments were conducted and the results presented are for the second set which succeeded. The control plates of tomato mesophyll protoplasts and of *S. lycopersicoides* suspension culture protoplasts did not survive the PEG/DMSO fusion protocol. Protoplasts of the sexual intergeneric hybrid were not pretested for the selection system. No viable parental protoplasts were observed after one week in culture and no colonies subsequently appeared. However, calli formed following fusion and eventually grew into large calli. The p-calli were transferred from the liquid medium directly to MS3ZG regeneration medium based on previous studies with *S. lycopersicoides* (Handley and Sink 1985 b).

Calli arising on the MS3ZG medium overlaid with filter paper had a strikingly different morphological appearance than calli derived from each parent as observed in previous studies (Niedz et al. 1985; Handley and Sink 1985 b). Calli derived from tomato are characteristically light to dark brown in color with a watery appearance. Calli of *S. lycopersicoides* are yellow to light green and have the same appearance. These phenotypes are exhibited at the filter paper dish stage where the parental protoplasts may have appeared slowly and grew slowly or were not present. The majority of the calli, however, were very dense and hard; had a lime-green to whitish color and grew much more rapidly than was previously encountered with the parents. These calli with a unique phenotype were visually selected and transferred to MS3ZG regeneration medium without filter paper. Such calli continued to appear on the filter paper dishes over a period of two months and a total of 480 such colonies were retrieved.

These putative fusion calli responded much the same as did *S. lycopersicoides* by rapidly initiating shoots. This occurred in some instances after only 1 month on MS3ZG medium. After transfer to fresh MS3ZG medium these shoots continued to elongate. Six months after the original plating, 62 of the 480 calli had regenerated shoots. Thsese were excised and inserted in the MSOT medium in GA7 boxes. These shoots rooted, grew rapidly and exhibited a definite hybrid leaf morphology. After 10 months it was no longer feasible to maintain all 480 calli, so 100 calli were randomly selected and the remainder discarded. After 13 months all 100 have regenerated shoots and individuals that root are being transferred to the greenhouse.

The difference in rate of cell growth and regenerative morphology between the somatic hybrid and the two parents were quite apparent. The cell growth of the hybrid was at least twice as rapid as *S. lycopersicoides* and even more rapid than tomato. Thus, the hybrid calli exhibited a heterotic growth response in culture, a trait which was also used previously by Schieder (1980) for selection of *Datura* somatic hybrids. Also, the somatic hybrid calli had a yellow-green color during the shoot regeneration phase. In contrast, *S. lycopersicoides* tends to be dark green to almost bluish on regeneration medium and tomato is usually a medium to dark green.

Differences between tomato and *S. lycopersicoides* were found for each of the five enzyme systems studied. These five enzyme systems represent seven loci which have been mapped to five chromosomes in tomato.

Table 1. Cytology and seed set of *L. esculentum* + *S. lycopersicoides* somatic hybrids. NR = No rooting

Line	Chromosome no.	% Pollen fertility	Self fruit set
S. lycopersicoides	24	91	+
S. esculentum 'Sub-Arctic Maxi'	24	96	+
L. esculentum × *S. lycopersicoides*	24	2	–
23	68	30	5
36	64	8	–
47	60	29	–
57	53, 54	49	5
67	NR	–	–
69	48, 53, 54, 55	43	3
165	48	36	2
204	48	2	–

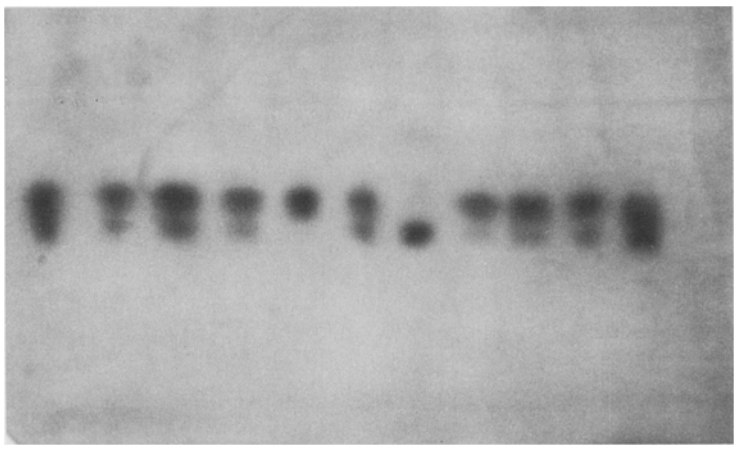

Fig. 1. Pgm-2 isozyme patterns (*left to right*): somatic hybrids 23, 36, 47, 57, *S. lycopersicoides*, sexual hybrid, tomato; somatic hybrids 67, 69, 165 and 204

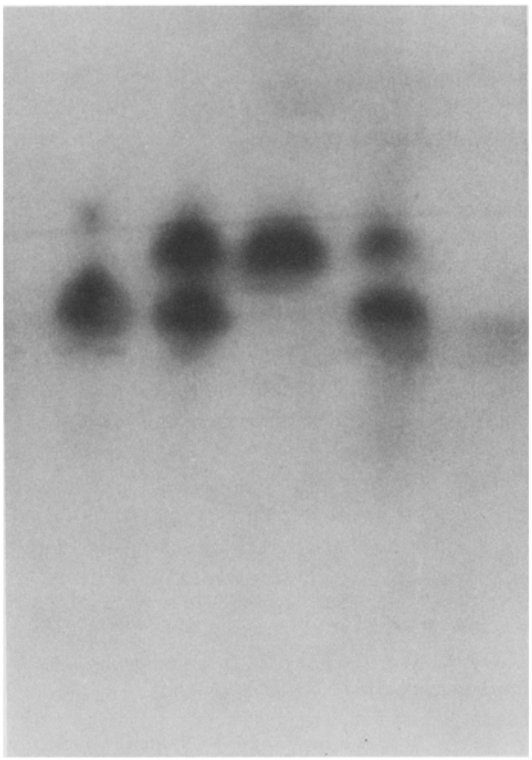

Fig. 2. Skdh-1 isozyme patterns (*left to right*): *S. lycopersicoides*, sexual hybrid, tomato, typical somatic hybrid

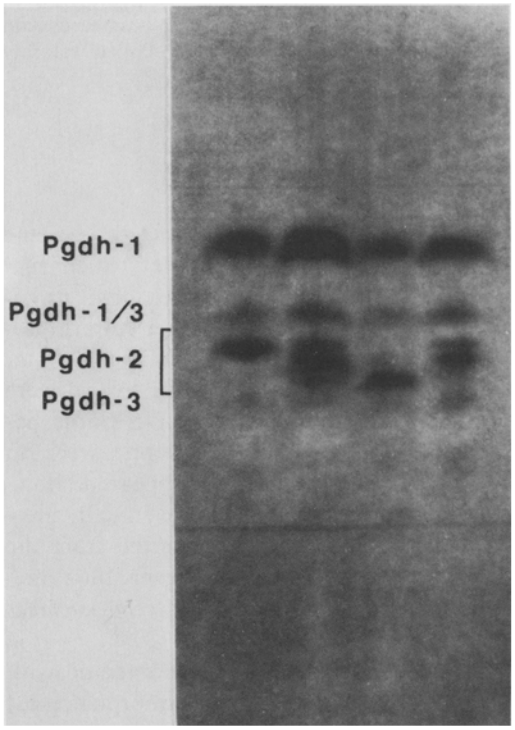

Fig. 3. Pgdh isozyme patterns (*left to right*): *S. lycopersicoides*, sexual hybrid, tomato, typical somatic hybrid. Interlocus heterodimer between Pgdh-1 and Pgdh-3 indicated by Pgdh-1/3

Pgm-2 has been mapped to chromosome 4 (Tanksley 1979) and is active as a monomer (Vallejos and Tanksley 1983). The parental species each expressed one band. In all somatic hybrids both of the parental bands were expressed (Fig. 1).

Skdh-1 was expressed in root samples (Fig. 2). This enzyme is also active as a monomer and has been mapped to chromosome 1 (Tanksley and Rick 1980). The absence of roots in hybrid 67 (Table 1) did not permit analysis for this enzyme.

Three forms of Pgdh have been reported in tomato (Tanksley and Kuehn 1985). All three forms of the enzyme are dimers and an interlocus heterodimer is formed between Pgdh-1 and Pgdh-3 (Tanksley and Kuehn 1985). Only one form of the enzyme differed between tomato and *S. lycopersicoides* (Fig. 3). Chloroplast preparations indicated that this form of Pgdh was localized to the chloroplast. Tanksley and Kuehn (1985) reported Pgdh-2 to be localized to plastids and mapped on chromosome 12. All hybrids had each of the

parental bands and a band of intermediate mobility as expected for a dimeric enzyme.

Pgi-1 is also active as a dimer (Tanksley 1980) and also mapped to chromosome 12 (Vallejos and Tanksley 1983). Three bands were detected for Pgi-1 for all of the hybrids (Fig. 4).

Four Got isozymes have been mapped in tomato. Got-2, Got-3 and Got-4 have been demonstrated to be dimers (Rick 1983). Got-1 is the fastest migrating form in tomato and Got-4 the slowest (Fig. 5). Got-1 was weakly expressed in both leaf and root extracts and no differences between the two species were detected. Both Got-2 and Got-3 have been mapped to chromosome 7 (Rick and Fobes 1977). Three bands were detected in all hybrids for both Got-2 and Got-3. Got-4 has been mapped to chromosome 8 (Tanksley and Rick 1980). In tomato Got-4 is a slow migrating enzyme. In *S. lycopersicoides* Got-4 migrates much faster, migrating further than Got-3 from either tomato or *S. lycopersicoides.* Got activity in a chloroplast preparation confirmed that this fast migrating form in *S. lycopersicoides* is Got-4. Three bands were obtained from chloroplast extracts of the sexual hybrid and a single band in each of the parents. The heterodimer for Got-4 from the chloroplast preparation migrated to the same position on the gel as the *S. lycopersicoides* homodimer for Got-3.

For all enzyme systems studied the putative hybrids expressed the expected hybrid isozyme patterns, except two of the eight somatic hybrid plants did not express the *S. lycopersicoides* form of Got-4. Got-4 is the only isozyme tested which has been mapped to chromosome 8 of tomato (Tanksley and Rick 1980). The reason for the lack of activity for this form of Got is not presently known.

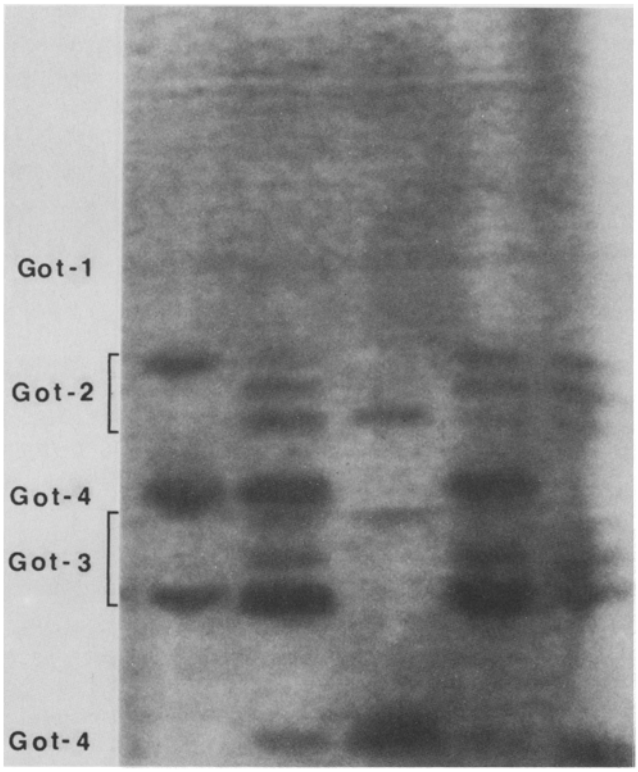

Fig. 5. Got isozyme patterns (*left to right*): *S. lycopersicoides,* sexual hybrid, tomato, typical somatic hybrid, somatic hybrid 204 missing the Got-4 band of *S. lycopersicoides.* The Got-4 heterodimer co-migrates with the Got-3 homodimer of *S. lycopersicoides*

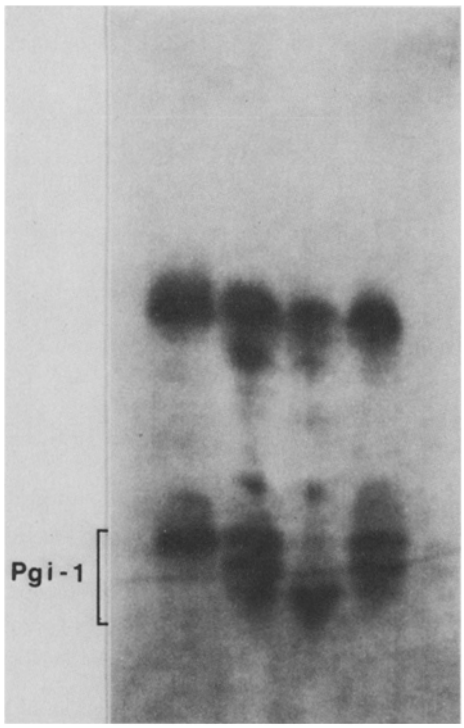

Fig. 4. Pgi-1 isozyme patterns (*left to right*): *S. lycopersicoides,* sexual hybrid, tomato, typical somatic hybrid

Fraction 1 protein (ribulose bisphosphate carboxylase oxygenase) is a multimeric enzyme expressed in the chloroplasts. The gene(s) for the large subunit (LS) is chloroplast encoded and the gene(s) for the small subunit (SS) are nuclear encoded. The loci of the small subunit have not been mapped in tomato. There were no differences between the two parental species in the isoelectric point of LS. Therefore it was not possible from these analyses to determine which chloroplast type is present in the hybrids. However, differences between species were found in the isoelectric points for SS (Fig. 6). Two double bands for the SS were present for *S. lycopersicoides* and tomato had three bands. The SS pattern for tomato was the same as that reported by Shepard et al. (1983). Seven of the somatic hybrids and a sexual hybrid were analyzed. All hybrids expressed the SS bands of both parents (Fig. 6).

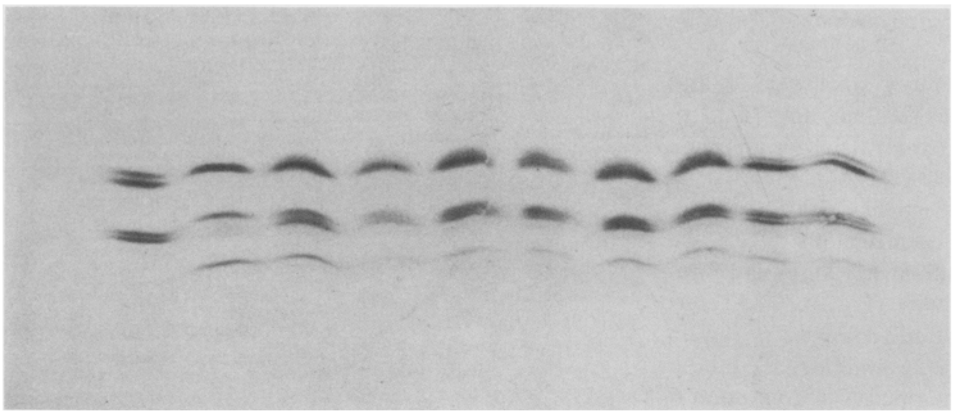

Fig. 6. Fraction I small subunit patterns (*left to right*): *S. lycopersicoides,* tomato, sexual hybrid, somatic hybrids 23, 36, 47, 57, 69, 165 and 204

Fig. 7. Leaf morphology (*left to right*): tomato, somatic hybrid, *S. lycopersicoides,* sexual hybrid

Fig. 8. Flower morphology (*left to right*): *S. lycopersicoides,* typical somatic hybrid, tomato

The seven somatic hybrid plants grown to flowering in the greenhouse are characterized by vigorous growth, branching and leaf morphology resembling *S. lycopersicoides* (Fig. 7). Leaves of similar physiological age of *S. lycopersicoides* and somatic hybrid plants had a rachis similar in length. Leaflets were sessile or with short petioles and toothed irregularly, but not as deeply as 'Sub-Arctic Maxi'. Flowers of the somatic hybrids were typically bright yellow, abundant, with triangular lobes like 'Sub-Arctic Maxi' but broader at the base (Fig. 8). The calyx lobes are reduced in length compared to the tomato parent. The anthers of the hybrids are 5 mm long, have 2 pores at the apex, and are not adnate, thus resembling *S. lycopersicoides.* In general, the somatic hybrids are phenotypically primarily like *S. lycopersicoides* as was observed by Rick (1951) for the unilateral sexual intergeneric hybrid.

The number of chromosomes for the seven somatic hybrids regenerated to intact plants varied from the expected $2n=4x=48$ to $2n=68$ (Table 1). Two plants, 57 and 69, had mixoploid root cells. Plant 57 had cells with approximately 53 and 54 chromosomes; whereas plant 69 had cells with approximately 48, 53, 54 and 55 chromosomes. Pollen fertility ranged from 2% for plant 204 to 49% for plant 57. To date, four of the hybrids have set limited fruit.

Research studies on the organelle genetics, molecular biology and potential use of these plants for breeding purposes are in progress.

Acknowledgements. The authors express appreciation to Dr. J. Martinez-Zapater and Dr. S. D. Tanksley for assistance and interpretation of the isozyme data.

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