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Interspecific somatic hybrid plants between eggplant (*Solanum melongena*) and *Solanum torvum**

A. Guri and K.C. Sink
Department of Horticulture, Michigan State University, East Lansing, MI 48824, USA

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Summary. Mesophyll protoplasts of eggplant (cv Black Beauty) and of *Solanum torvum* (both $2n=2x=24$) were fused using a modification of the Menczel and Wolfe PEG/DMSO procedure. Protoplasts post-fusion were plated at 1×10^5/ml in modified KM medium, which inhibited division of *S. torvum* protoplasts. One week prior to shoot regeneration, ten individual calluses had a unique light-green background and were verified as cell hybrids by the presence of the dimer isozyme patterns for phosphoglucoisomerase (PGI) and glutamate oxaloacetate transaminase (GOT). Hybridity was also confirmed at the plant stage by DNA-DNA hybridization to a pea 45S ribosomal RNA gene probe. The ten somatic hybrid plants were established in the greenhouse and exhibited intermediate morphological characteristics such as leaf size and shape, flower size, shape, color and plant stature. Their chromosome number ranged from 46–48 (expected $2n=4x=48$) and pollen viability was 5%–70%. In vitro shoots taken from the ten hybrid plants exhibited resistance to a verticillium wilt extract. Total DNA from the ten hybrids was restricted and hybridized with a 5.9 kb *Oenothera* chloroplast cytochrome f gene probe, a 2.4 kb EcoRI clone encoding mitochondrial cytochrome oxidase subunit II from maize and a 22.1 kb Sal I mitochondrial clone from *Nicotiana sylvestris*. Southern blot hybridization patterns showed that eight of ten somatic hybrids contained the eggplant cpDNA, while two plants contained the cpDNA hybridization patterns of both parents. The mtDNA analysis revealed the presence of novel bands, loss of some specific parental bands and mixture of specific bands from both parents in the restriction hybridization profiles of the hybrids.

Key words: Cell fusion – Isozymes – *Solanum melongena* – *Solanum torvum* – DNA-DNA hybridization

Introduction

Eggplant (*Solanum melongena* L.), an important non-tuberous solanaceous crop species, has to date not been used extensively in somatic fusions. Gleddie et al. (1986) are the only authors to report a successful fusion between eggplant and a wild *Solanum* species, *S. sisymbriifolium*. The latter species was reported to carry resistances for root knot nematode and for carmine spider mite. Another wild *Solanum* species related to eggplant, *S. torvum*, was found (Yamakawa and Mochizuki 1978) to be resistant to verticillium and fusarium wilts, bacterial wilt and root knot nematode. The resistance to verticillium wilt (VW) was also confirmed by McCammon and Honma (1983), and sterile sexual hybrids were obtained with difficulty only when eggplant was used as the female parent. Recently, Guri et al. (1987) developed a protocol to obtain plant regeneration from isolated mesophyll protoplasts of *S. torvum*, and herein we report the synthesis of somatic hybrid plants between eggplant and *S. torvum* that have the same resistance to VW as *S. torvum*. The hybridity of the putative somatic hybrids was verified by dimer isozyme analysis and DNA-DNA hybridizations using nuclear, chloroplast and mitochondrial specific probes.

Material and methods

Plant material

Eggplant seeds, cv Black Beauty, were obtained from A. H. Hummert Seed Co., St. Louis/MO, and *S. torvum* seeds were a

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gift from Professor K. Yamakawa (Vegetable and Crop Research Station, Tsue City, Japan). The procedure to obtain eggplant and *Solanum torvum* plants as sources for leaf protoplasts are described in Guri and Izhar (1984) and Guri et al. (1987), respectively.

Protoplast isolation and fusion

The procedures for isolation of leaf-derived protoplasts for eggplant and for *S. torvum* were done as described in Guri and Izhar (1984) and Guri et al. (1987), respectively. Prior to fusion, *S. torvum* and eggplant protoplasts were mixed 10:1, respectively, to a final density of 5×10^6 protoplasts/ml. A modification of the fusion procedure of Menczel and Wolfe (1984) as described by Guri et al. (1988) was used.

Protoplast culture and regeneration

After fusion, protoplasts were cultured in KM medium (Kao and Michayluk 1981) as modified by Guri and Izhar (1984) without agarose in 100 mm X-dishes and using the reservoir medium described by Shepard (1980). Protoplasts were initially cultured in the dark for 2 weeks at 27 °C and then exposed to dim light, 3–4 $\mu Em^{-2} s^{-1}$ (Cool White fluorescent tubes). Three and half-weeks post fusion, when protoplast-derived calluses (p-calluses) reached approximately 1.0 mm, the culture medium was replaced with C-medium (Shepard 1980). When the p-calluses reached 3–5 mm they were placed individually on Whatman # 1 qualitative filter paper layered on solidified MS (Murashige and Skoog 1962) salts and vitamins medium supplemented with 7% sucrose, 2 mg/l zeatin, 1 mg/l indoleacetic acid (IAA) (0.45 µm filter sterilized) and 6 g/l Noble agar (Difco). At 10 mm size, the p-calluses were transferred onto 3 regeneration media all containing MS salts and vitamins, 3% sucrose and 8 g/l agar (Sigma); the first contained 2 mg/l kinetin, the second, 2 mg/l 2ip and the third had 2 mg/l zeatin. Regenerated shoots were excised and inserted in MSOT medium (Handley et al. 1986) in Magenta boxes for rooting. Rooted plants were planted in a 1:1 mixture of peat and sand, covered with clear plastic for 2 weeks in a controlled environmental chamber (CEC) and transferred to the greenhouse.

Isozyme analyses

The procedures for electrophoresis and isozyme staining for phosphoglucoisomerase (PGI) and glutamate oxaloacetate transaminase (GOT) using 100 mg callus or leaf material as described by Scandalios (1969) and Guri et al. (1988) were used. GOT isozymes localized in plastids or in mitochondria were identified by preparing chloroplast and mitochondrial extracts as described earlier (Huang et al. 1976, Handley et al. 1986). The eggplant and *S. torvum* isozyme bands corresponding to *Got*-2 and *Got*-3 were designated according to the relative gel position of the parental and heterodimer bands.

DNA extraction and hybridization

DNA was extracted from leaves of both fusion parents and the ten somatic hybrid plants, originated from 10 independent fusion events, grown in a CEC. Nuclear DNA, cpDNA and mtDNA were extracted from the parents according to Palmer (1986) and Hanson et al. (1986), while total DNA was extracted from the parents and the somatic hybrids as described in Guri and Sink (1988). Restriction fragment electrophoresis, Southern hybridization, nick translation and photography were performed according to Maniatis et al. (1982). A nuclear specific 9.0 kb probe containing the 45S ribosome gene of pea (gift from W. F. Thompson) was used for nuclear DNA hybridizations. A 5.9 kb *Oenothera* chloroplast cytochrome f gene (gift from W. Bottomly; provided by B. B. Sears) was used as a probe for cpDNA. The mtDNA was probed with pZmE1, a 2.4 kb EcoRI clone encoding maize mitochondrial cytochrome oxidase subunit II (Fox and Leaver 1981), and Pmt SylSa8, a 22.1 kb SalI mtDNA clone from *Nicotiana sylvestris* (Aviv et al. 1984), gifts from T. Fox and D. Aviv, respectively. The hybridization of those probes to purified nuclear, cpDNA and mtDNA versus total DNA was compared for the two parents.

Cytology and pollen staining

Chromosome counts were done on root tip cells of in vitro growing somatic hybrids using the procedure described in Guri et al. (1987). Pollen grains were stained with 2% acetocarmine.

Verticillium wilt (VW) assay

Verticillium dahliae isolates were obtained from M. Lacey (Plant Pathology Department, M.S.U., East Lansing/MI). The fungus growth conditions were the same as reported by Nachmias et al. (1982). Fungus extract was obtained by removal of the mycelia by filtration through Whatman # 1 qualitative filter paper, centrifugation at 10,000 g for 20 min and filter sterilization. Preliminary experiments with VW resistant lines of *S. torvum* and tomato and VW sensitive cv Black Beauty showed that placing stem cuttings for 2–4 days in water containing a 50% fungus extract yielded a selective response. Cuttings (10 each) of eggplant, *S. torvum* and the somatic hybrids were tested as described above and compared to control cuttings that were placed in water.

Results

Four to six day post-fusion protoplasts began to divide, while in the control plates *S. torvum* self-fused protoplasts did not initiate p-calluses. The p-calluses obtained in the eggplant plus *S. torvum* fusions were individually placed 5½ weeks later on filter paper layered on the support medium, where they rapidly enlarged and were transferred onto the 3 regeneration media. Shoot regeneration did not take place on the 2 regeneration media with kinetin and 2ip; rather, after 3 weeks these calluses turned dark brown. However, on MS medium with zeatin the calluses retained their green color. Among the 78 retrieved p-calluses it was possible to distinguish 2 types. The predominant type was light green with dispersed dark green spots a week prior to shoot regeneration and subsequent high shoot regeneration frequency (85%) were later identified as eggplant callus. The other callus type, ten all together, had light green color, lacked the dark green spots and had a low shoot regeneration frequency (8%); these were confirmed as somatic cell hybrids. Isozyme analysis for the dimer patterns of PGI and GOT on callus extracts assisted with the early identification of the ten somatic hybrids 1 week prior to shoot regeneration. For PGI (Fig. 1), the somatic hybrid lanes contained the two parental bands of *Pgi*-1 and a heterodimer band not found in the mixed extracts of the parents. Eggplant and *S. torvum* have four GOT loci encoding the 4 GOT isozymes, however, variation with regard

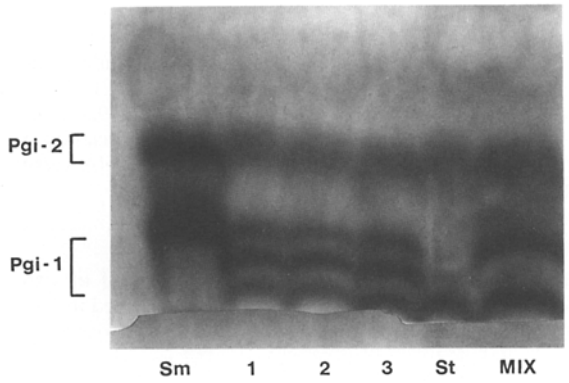

Fig. 1. Phosphoglucoisomerase (PGI) isozyme patterns for eggplant (Sm), somatic hybrids Smt-1 (1), Smt-5 (2), Smt-7 (3), *Solanum torvum* (St) and mixture of eggplant plus *Solanum torvum*

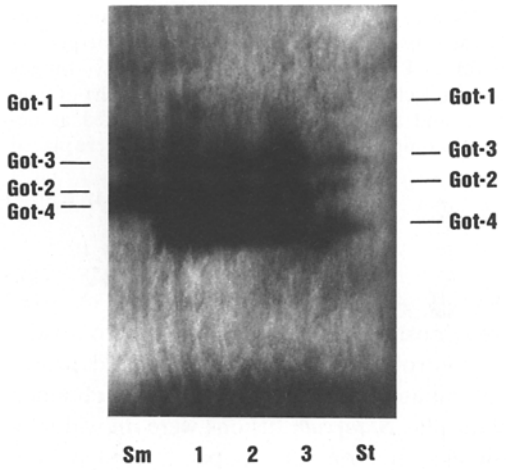

Fig. 2. Glutamate oxaloacetate transaminase (GOT) isozyme patterns for eggplant (Sm), somatic hybrids Smt-1 (1), Smt-5 (2), Smt-7 (3) and *Solanum torvum* (St)

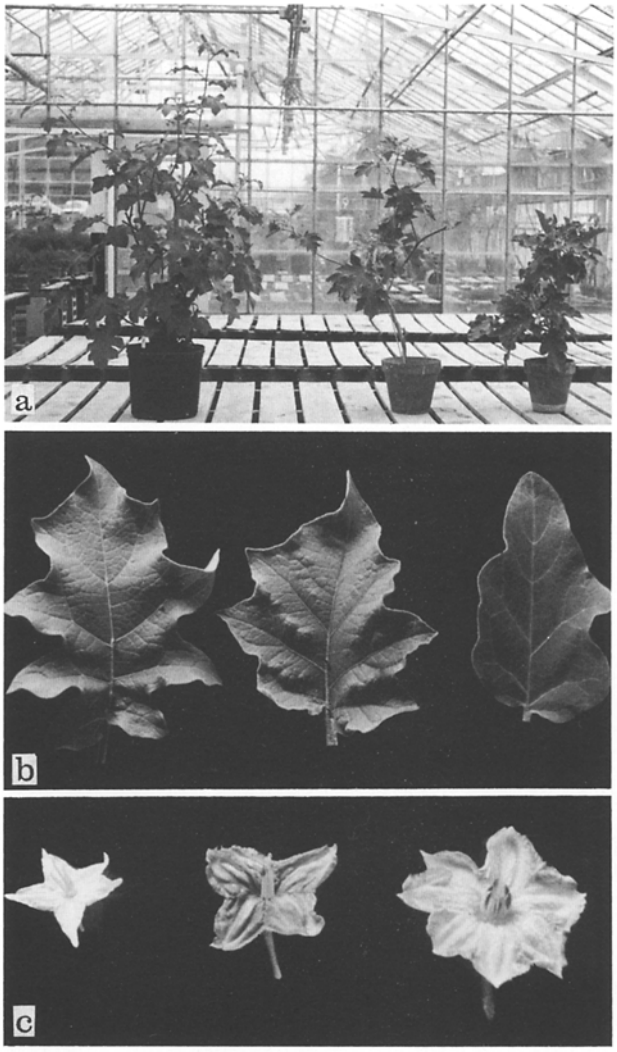

Fig. 3. **a** Plants, **b** leaves and **c** flowers of (*left*) *Solanum torvum*, (*center*) somatic hybrid and (*right*) eggplant

to band migration was only observed for one, *Got*-4. For this variable locus the hybrid lanes contained both parental bands and, in addition, a heterodimer band (Fig. 2). Shoots from the somatic cell hybrids easily rooted in MSOT medium and were transferred to the greenhouse.

The ten somatic hybrid plants exhibited intermediate whole plant morphology and leaf shape (Fig. 3a and b). All the hybrids except one had thorns along the stems and leaves, similar to *S. torvum*. The thorns were purple, unlike the bright color of those of *S. torvum*. The flower size of the hybrids was also intermediate. The flower color was a deeper purple than that of eggplant (Fig. 3c) and could be contrasted with the white flowers of *Solanum torvum*. In addition, the sepals were long, similar to those of eggplant, and the pistil extended beyond the anthers as in *S. torvum* flowers; whereas, pollen grain viability ranged from 5%–70%. Chromosome counts for 7 of the 10 somatic hybrids was $2n=2x=48$; 2 of the hybrids had 47 and 1 had 46 chromosomes. From general observations in the greenhouse, it appeared that the eggplant plants are very susceptible to spider mites while *S. torvum* plants are resistant. The somatic hybrid plants, on the other hand, exhibited partial resistance. The somatic hybrid cuttings were VW resistant, comparable to their VW resistant *S. torvum* parents, and they rooted after 1 week in the fungus extract solution. Cuttings of eggplant, on the other hand, died after 2–3 days.

Purified versus whole cell DNA's of each parent restricted with EcoRI and probed with 45S, cytochrome f, pZmE1 and Pmt SylSa8 were found to be identical (data not shown); therefore, whole cell DNA's were used for subsequent analysis. Total DNA extracted from the 10 somatic hybrids restricted with EcoRI and hybridized to

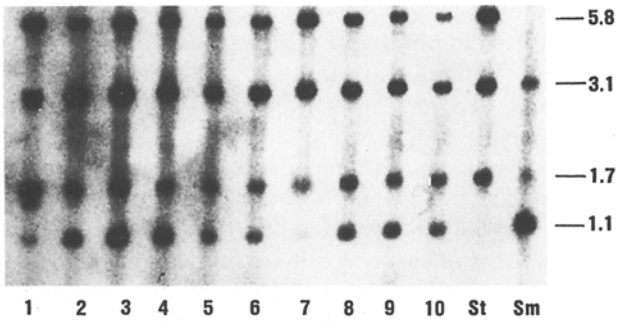

Fig. 4. Autoradiogram showing hybridization of ^{32}P labelled pea 45S probe to total DNA digested with EcoRI for (*left to right*) somatic hybrids Smt-1 to -10, *Solanum torvum* (St) and eggplant (Sm)

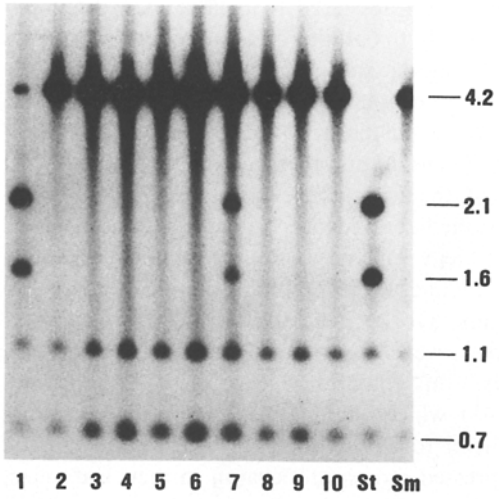

Fig. 5. Autoradiogram showing hybridization of ^{32}P labelled cp-cytochrome f gene to total DNA digested with EcoRV for (*left to right*) somatic hybrids Smt-1 to -10, *Solanum torvum* (St) and eggplant (Sm)

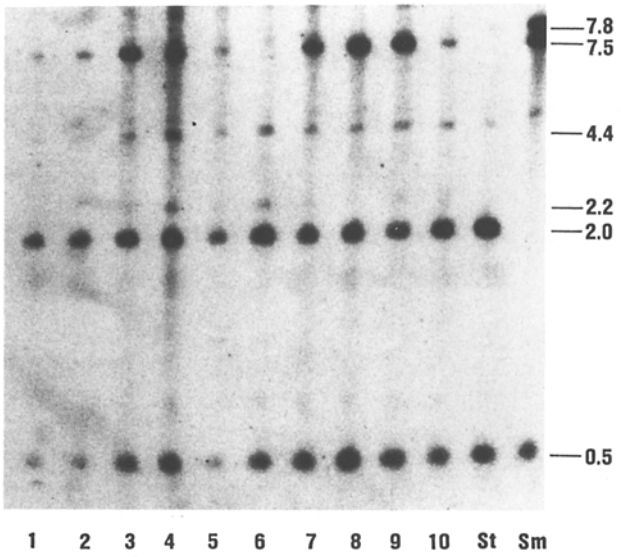

Fig. 6. Autoradiogram showing hybridization of ^{32}P labelled mt-cytochrome oxidase subunit II of maize to total DNA digested with EcoRI for (*left to right*) somatic hybrids Smt-1 to -10, *Solanum torvum* (St) and eggplant (Sm)

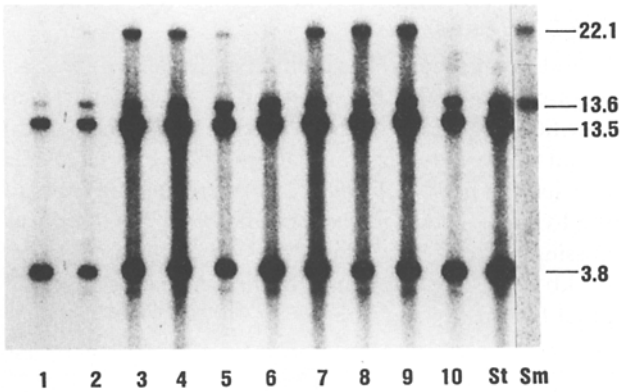

Fig. 7. Autoradiogram showing hybridization of ^{32}P labelled mt-cytochrome oxidase subunit II of maize to total DNA digested with EcoRV for (*left to right*) somatic hybrids Smt-1 to -10, *Solanum torvum* (St) and eggplant (Sm)

the 45S ribosome probe further confirmed the hybrid nature of 9 plants, as the diagnostic bands of both parents were present in the hybrid lanes (Fig. 4). The cpDNA specific probe encompassing the chloroplast encoded cytochrome f gene and hybridized to total DNA restricted with EcoRV revealed the presence of only the 4.2 kb eggplant specific band in eight somatic hybrids, while the other two (Smt-1 and -7) contained, in addition to the eggplant specific band, the two *S. torvum* specific bands of 1.6 and 2.1 kb (Fig. 5).

The mtDNA probe cytochrome oxidase subunit II, when hybridized to total DNA extracted from the parents and the somatic hybrids restricted with EcoRI, showed that the 7.8 kb eggplant specific band was missing in the lanes of all hybrids (Fig. 6). Conversely, the other eggplant specific band (7.5 kb) was found in different intensities in all hybrids except Smt-6. The *S. torvum* specific band of 2.0 kb was present in all hybrids as was the common band 0.5 kb. The *S. torvum* specific 4.4 kb band was not found in two hybrids (lanes 1, 2), while two hybrids (lanes 4, 6) had a 2.2 kb novel band. Hybridization of the above probe to total DNA restricted with EcoRV (Fig. 7) clearly revealed the 22.1 kb eggplant specific band in six hybrids (lanes 3–5, 7–9). The two *S. torvum* specific band 3.8 and 13.5 kb appeared in all hybrid lanes. The common band of 13.6 was also found in all hybrids. The second mtDNA specific probe, containing mitochondrial sequences for *Nicotiana sylvestris*, was hybridized to mtDNA restricted with HindIII (Fig. 8). The eggplant 2.1 kb specific band appeared in all

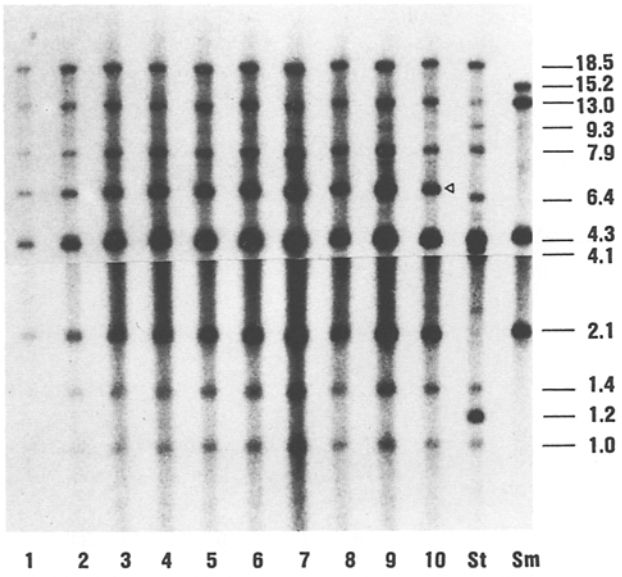

Fig. 8. Autoradiogram showing hybridization of ^{32}P labelled mtDNA of *Nicotiana sylvestris* to total DNA digested with Hind III for (*left to right*) somatic hybrids Smt-1 to -10, *Solanum torvum* (St) and eggplant (Sm)

hybrids while the other specific band (15.2 kb) was always missing. With this digestion *S. torvum* had seven specific bands, four of them (18.5, 7.9, 1.4 and 1.2 kb) found in the hybridization pattern of all hybrids, while two specific band (4.1 and 1.2 kb) were missing from all the hybrids. The *S. torvum* specific band of 6.4 kb was missing; instead, the hybrid lanes all contained a novel 6.7 kb band. The common hybridization bands appeared in all hybrids.

Discussion

In this study, and as in the case of tomato and *Solanum nigrum* fusions (Guri et al. 1988), we obtained somatic hybrid plants by taking advantage of the differential tissue culture requirements of the parental species. A previous study in our laboratory (Guri et al. 1987) showed that mesophyll-derived protoplasts of *Solanum torvum* did not divide to form p-calluses when cultured in medium (Guri and Izhar 1984) that promoted the formation of eggplant p-calluses. This is why we chose to fuse 1:10 (eggplant: *S. torvum*) protoplasts. Conversely, *S. torvum* p-calluses regenerated shoots on media that contained solely 2ip or kinetin as the cytokinin source, whereas, eggplant p-calluses failed to regenerate on these same media (Guri et al. 1987). The similar two-step selection approach used successfully with the fusion between tomato and *Solanum nigrum* (Guri et al. 1988) was only partially feasible in the present fusion. In the initial steps, presumably all *S. torvum* unfused and homokaryon protoplasts did not divide (no presence of *S. torvum* p-calluses). The growth of those p-calluses [eggplant and eggplant (+) *S. torvum*] that formed in the fusion plates ceased whenever they were placed on the above 2ip or kinetin shoot regeneration media. The phenotype of some of the calluses after placement on MS + 2 mg/L zeatin, prior to shoot regeneration, was strikingly different from that of eggplant calluses on the same medium. This differential appearance combined with the use of dimer isozyme assay at this stage enabled early identification of 10 somatic hybrid calluses among the 78 tested. Unlike our previous study in the fusion of tomato (+) *Solanum nigrum* and others by Handley et al. (1986) (tomato (+) *Solanum lycopersicoides*) and Kinstara et al. (1986) (tomato (+) *Lycopersicon peruvianum*) where shoot regeneration appeared to behave as a dominant trait, in this study the ability of somatic hybrid calluses to regenerate shoots was approximately tenfold lower than those of the eggplant. Such results could imply that genetic control of the pathway involved in callus organogenesis is different among these solanaceous species.

In general, the interspecific somatic hybrid plants exhibit an intermediate phenotype. The presence of thorns on stems and leaves is known to be a dominant encoded trait in the solanaceae (S. Honma, personal communication), and nine hybrids had thorns similar to *S. torvum*. One hybrid, however, was thornless, like eggplant, and interestingly this hybrid lacked 2 chromosomes ($2n = 2x = 46$), which might be those of *S. torvum* carrying the gene(s) for thorn formation. Likewise, all the hybrids expressed more anthocyanin in their leaf veins, thorns and flowers than either parent. Pollen grain viability of the hybrids ranged from 5%–70%, and the hybrids so far (similar to *S. torvum* plants) have not produced fruits by self-pollination under greenhouse conditions. It is possible that the somatic hybrids are self-incompatible and subsequently we shall attempt crosses among the somatic hybrids.

The inheritance of verticillium wilt (VW) resistance in *S. torvum* has not been genetically characterized. However, in tomato (Schaible et al. 1951) it was found that VW resistance is controlled by a single dominant gene. Therefore, it is not surprising that all the hybrids are VW resistant. The VW assay conducted in this study on cuttings of the somatic hybrids was found to differentiate very efficiently between VW resistant and sensitive species (data not shown). Tests with plants grown in soil infected with *Verticillium dahliae* spores is still needed to verify these results.

Plastid sorting following somatic fusion in the absence of directed in vitro selection appears to occur randomly or non-randomly, depending on the degree of taxonomic compatibility (Fluhr 1983). In this study, the eggplant hybridization pattern occurred in eight out of

ten somatic hybrids. Perhaps there is a higher compatibility between the eggplant plastome with the alloploid nuclear genome of the somatic hybrids than with that of *S. torvum*. This assumption is supported by the fact that sexual hybrids between these two species are only obtained when eggplant serves as female parent (McCammon and Honma 1983). The two somatic hybrids that contained both parental specific hybridization fragments could result from co-existence of both parental chloroplasts at the whole plant stage. Such a phenomenon was observed in other somatic fusion experiments (see review by Kumar and Cocking 1987).

The use of two mitochondrial specific probes combined with three restriction enzymes revealed novel bands and a mixture of eggplant and *S. torvum* specific bands in the somatic hybrids. Some of the parental specific bands were missing, but all the common bands were present. The presence of novel bands within mitochondrial restriction patterns or autoradiograms of somatic hybrids was noticed in many previous protoplast fusion studies as first reported by Belliard et al. (1979) in *Nicotiana*.

It is also possible that mtDNA rearrangements occurred through the tissue culture stages in the absence of cell fusion. In wheat callus, cultures initiated from immature embryos, mitochondrial rearrangements that included the appearance of novel bands, disappearance of bands and changes in band intensity partially occurred via intragenomic recombination (Rode et al. 1987). Similar observations were made on maize and soybean regenerates by Kemble et al. (1982) and Morgans et al. (1984), respectively. Likewise, protoclones of potato exhibited mtDNA rearrangement (Kemble and Shepard 1984). Whether such variation observed herein on the somatic hybrids is exclusive of that which may occur in the parents alone remains to be determined.

The recognition of hot spots along the mitochondrial genome that are involved in intragenomic recombination in *Brassica* species and maize, leading eventually to variation within the mitochondrial restriction patterns and/or autoradiograms, was discussed by Palmer and Herbon (1986) and Pring et al. (1987), respectively. Following intensive research, Rothenberg and Hanson (1987) and Vedel et al. (1986) detected restriction fragments that evolved through intergenomic recombination preceded by fusion of two different parental mitochondrion in *Petunia* and *Brassica* somatic hybrids, respectively. Some of the sites in which the intergenomic recombination occurred were not involved before in intragenomic recombination within *Brassica* or *Petunia*.

Using two mtDNA probes and three restriction enzymes, our results indicate the presence of both eggplant and *S. torvum* mitochondrial specific bands within the ten somatic hybrid hybridization autoradiograms as also found by Boeshore et al. (1983) in *Petunia* somatic hybrids, and more recently by Robertson et al. (1987) and Morgan and Maliga (1987) in *Brassica* somatic hybrid and cybrids, respectively. The latter were pursued further and a restriction map was constructed of the mt probe and the flanking regions associated with this probe in the two parents and the cybrids. Their results revealed mitochondrial intergenomic recombination within some of the cybrids. At this stage, our preliminary findings, in addition to intergenomic recombination, could also indicate an alloplasmic state where the mitochondria of both parents co-exist within the hybrid plants as found before in *Petunia* somatic hybrids by Izhar et al. (1983).

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