Comparison of *Lycopersicon peruvianum* Mill. and *L. chilense* Dun. for Development of Tomato Somatic Hybrids

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*Lycopersicon peruvianum* Mill. and *L. chilense* Dun., two species of ‘Peruvianum complex’ were compared for production and characterisation of tomato somatic hybrids. The double selection system “employing kanamycin resistance from tomato (*L. esculentum* Mill.) cultivar, Pusa Ruby and protoplast regeneration pathway from wild tomato parents” was used for identification of heterokaryons. Somatic hybrid plants were characterised by morphological markers, cytogenetic studies and biochemical analyses. The molecular confirmation of tomato somatic hybrids was obtained by PCR-based DNA markers. The importance of selectable marker (*nptII*) gene from cultivated species during characterisation of somatic hybrid plants was demonstrated.

**Keywords:** tomato, *Lycopersicon*, *L. esculentum*, *L. chilense*, *L. peruvianum*, somatic hybrid, protoplast fusion

**Introduction**

Wild relatives of genus *Lycopersicon* are the valuable source of economic traits in tomato breeding (Kalloo, 1991). Although, certain wild species of this genus are sexually incompatible with the cultivated tomato, *L. esculentum* Mill.; in particular, the ‘Peruvianum complex’ of the genus *Lycopersicon* is sexually isolated from the ‘Esculentum complex’ (Rick, 1979). For tomato improvement, therefore, two species of the ‘Peruvianum complex’, namely *L. peruvianum* Mill. and *L. chilense* Dun., are least exploited by plant breeders. In spite of such difficulties, tomato breeders have always been keen to include the genotypes of *L. chilense* and *L. peruvianum* into their available germplasm, not only because these genotypes represent an unexploited reservoir for important biotic and abiotic stresses but also for they are the genetically most diverse species in the genus (Breto et al, 1993). However, only limited success has been achieved by using embryo or immature seed culture following sexual interspecific hybridization (Patil et al, 1993). Most of the available germplasm has also been exhausted as a result of intensive tomato breeding programmes. Thus, it is obvious that tomato breeders require more broad-based genetic resources today than had been available in the past.

To circumvent sexual barriers of ‘Peruvianum complex’, somatic hybridization was used as a complementary tool in plant breeding. As a result, *L. peruvianum* has frequently been used since 1986 for the production of tomato somatic hybrids (Kinsara et al, 1986; Wijbrandi et al, 1990; San et al, 1990; Sakata et al, 1991). *L. chilense*, however, been involved more recently in a somatic hybridization programme (Bonnema & O’Connell, 1992), which resulted in only two somatic hybrid (SH) plants being regenerated and characterised by RFLP analysis.

The main objectives of the present study are to compare both species of ‘Peruvianum complex’ (*L. peruvianum* Mill. and *L. chilense* Dun.,) and to demonstrate the importance of using selectable marker during characterisation of somatic hybrid plants.
peruvianum and L. chilense) in the production of tomato somatic hybrids; to evaluate double selection system for regeneration of somatic hybrid shoots; to assess use of the selectable marker (nptII) gene in characterisation of SH plants; and to test PCR-based DNA markers (RAPD) for molecular confirmation of tomato SH plants.

Materials and Methods

Plant Material
Seeds of L. peruvianum (LA 2744) and L. chilense (LA 2930), obtained from Prof C M Rick, University of California, Davis, CA, and seeds of tomato cv Pusa Ruby (PR), provided by Tomato Improvement Project, Mahatma Phule Agricultural University, Rahuri, Maharashtra, India, were used for plant material.

Proposed Double Selection Strategy for Regeneration of Somatic Hybrid Shoots
Tomato cv. PR was utilised for Agrobacterium-mediated transformation (Patil et al, 2002). Kanamycin resistant transformants were obtained after leaf-disc transformation with a disarmed strain of A. tumefaciens containing plasmid co-integrate pMon 200 (Monsanto group, USA). This plasmid carried neomycin phosphotransferase (nptII) gene with nopaline synthetase promoter and terminator. Protoplast culture of parental Lycopersicon species (unpublished data) revealed that L. esculentum (cv. PR) protoplasts were kanamycin resistant but were recalcitrant for shoot regeneration. Whilst protoplasts of L. peruvianum and L. chilense were highly totipotent but proved kanamycin sensitive. Therefore, after addition of kanamycin sulphate (KS) to fusion-treated protoplast culture, only somatic hybrid P-calli were able to regenerate shoots.

Protoplast Source and Isolation
For protoplast isolation of L. esculentum (transformed) and L. peruvianum species, 14 d old axenic shoot cultures grown under 16 hrs photoperiod (40 μmol m⁻² s⁻¹) at 25°C on MS0 medium (3 shoots/175 ml glass jars containing 45 ml medium) were used as a source of leaf mesophyll protoplasts. Protoplast isolation was carried out using protocol of Patil et al (1994). However, long-term (>8 months-old) cell suspension cultures of L. chilense had to be initiated as a source of high yielding, viable protoplasts. Callus was induced from stem explants (0.5 cm in length) of axenic shoot cultures of L. chilense on MSP1 medium (MS0 medium supplemented with 2.0 mg l⁻¹ NAA + 0.5 mg l⁻¹ BAP) and cell suspension cultures were established from culture of singular cells in the same liquid medium (Patil et al, 2003). Suspension protoplasts were isolated according to Latif et al (1993).

Protoplast Fusion and Assessment of Fusion Products
The protocol of Menczel et al (1981) with some modifications was used for protoplast fusion. Protoplasts of L. esculentum and L. peruvianum, or L. esculentum and L. chilense, were chemically fused in 30% PEG MW 4000 (BDH, Poole, UK) in a 1:1 ratio (v/v) and with a final concentration of 1.0×10⁶ ml⁻¹. Fusion frequency was measured either in bright field illumination by various plastid contents of fusion bodies or by double labelling of protoplast with FDA and TRITC. Following protoplast fusion, protoplast viability was measured with FDA (Larkin, 1976), while plating efficiency of fusion products was determined as the percentage of dividing protoplasts after 7 d of culture.

Culture of Fusion Products
Following measurements on fusion frequency and protoplast viability, fusion products were cultured in TMp medium (Wijbrandi et al, 1990; 3.0 ml/5.0 cm Petri dish) and incubated in the dark at 25°C. On day 4, culture dishes were transferred to dim light (20 μmol m⁻² s⁻¹; 16 hrs photoperiod) and were diluted with 3.0 ml TMd medium (Wijbrandi et al, 1990) at 3 d intervals (e.g. day 4, 7, 10, 13, 16, 19, 22). Kanamycin sulphate was added to TMd medium either at the second medium dilution (7 d), with a final concentration of 25 μg ml⁻¹, followed by 50 μg ml⁻¹ KS since third medium dilution (10 d); or alternatively 50 μg ml⁻¹ KS was added directly from the third medium dilution (10 d). The purpose of addition of KS to protoplast culture at the earlier stage (7 d) was to inhibit the development of wild parental micro-calli. Since day 13, micro-calli (1-2 mm diameter) were transferred to agar-solidified TMc, callus inducing medium (Wijbrandi et al, 1990) containing 100 μg ml⁻¹ KS (20 calli/9.0 cm Petri dish with 20 ml medium) and incubated under continuous light (40 μmol m⁻² s⁻¹) at 25°C. After 28 d of culture, P-derived calli (1-2 cm diameter) were transferred to MS4 regeneration medium (MS0 medium supplemented with 0.1 mg l⁻¹ IAA and 2.0 mg l⁻¹ zeatin) containing 0.1 M mannitol and 100 μg ml⁻¹.
Shoot regeneration was observed within 1-4 subcultures (28 d) on MS4 medium lacking IAA and mannitol.

Characterisation of in vitro Grown Putative SH Plants

Regenerated shoots of putative somatic hybrids either from *peruvianum*-tomato somatic hybridization (PSH) or *chilense*-tomato somatic hybridization (CSH) were checked for kanamycin resistance and, therefore, the rooting response of regenerated putative SH shoots was investigated on agar-solidified MS0 medium containing 100 µg ml⁻¹ KS (3 shoots/175 ml glass jar containing 45 ml of medium) under a 16 hrs photoperiod (40 µmol m⁻²s⁻¹) at 25°C. In case of CSH, the rooting response of regenerated shoots was checked as an additional double selection to illustrate hybridity, kanamycin resistance used as selection for *L. esculentum* parent (cv. PR was transformed), while the supplement of 0.2 mg l⁻¹ IBA to MS0 medium was prerequisite for rooting of *L. chilense* axenic shoots, which were used as selection for *L. chilense* parent in CSH (unpublished data). To characterise the hybrid nature of in vitro grown putative SH plants, morphological markers such as leaf colour, shape, serration, size of the lamina together with stem thickness and brittleness of tissue were examined and compared with their parents.

Characterisation of ex vitro Grown Putative SH Plants

Randomly selected 32 putative SH plants from PSH experiment, and a complete set of 12 putative SH plants from CSH experiment, were transferred to the glasshouse. Three replications of each SH plants were prepared from 3 shoots regenerated from the same callus. Under glasshouse conditions [natural day light supplemented with 16 hrs photoperiod (40 µmol m⁻²s⁻¹) at 25±2°C], the growth of putative SH plants was compared with their parents and an intermediate morphology and dominant morphological markers from both parents were investigated to confirm hybridity. Plants were examined for vegetative growth, flowering, fruiting and self-fertility. Observations were recorded on leaf morphology (as described earlier), stem morphology (characters such as stem thickness, presence of anthocyanin pigments on stem surface, trichome growth, presence of bracts at internodes), floral morphology [e.g. inflorescence growth habit (branching or non branching), length of inflorescence stalk, anther-cone and stigma thickness, exertion of stigma over anther-cone and pollen viability] and fruit morphology [e.g. fruit size (equatorial x polar diameter), shape, fruit weight, colour of mature and ripe fruits, seed development (mature or immature), number of seeds per fruit]. Pollen viability was determined by staining freshly dehisced pollen in aceto-carmine and examining under the light microscope for deeply stained, fully rounded viable pollen. Two hundred pollen grains were examined for each replication.

Cytological Analyses

The number of chloroplasts per guard cell pair of stomata (No. Ch/GCP) was used as a rapid pre-screening method for estimating ploidy levels (Wijbrandi *et al.*, 1990). Root squash analysis was performed using protocol of Roberts & Short (1979) to count chromosomes.

Isoenzyme Analysis

Leaf tissues (1.0 g⁻¹ f.wt.) from ex vitro grown plants of putative SH plants and their parents were used for isoenzyme analysis. Acid phosphatase and leucine aminopeptidase activities were analysed by electrophoresis of 100 µl crude protein extracts with running buffer (0.025 M Tris base and 0.1 M glycine) and 5 µl marker dye [1.0% (w/v) bromophenol blue in 20% (v/v) glycerol]. Isoenzymes were separated by a DISC-PAGE. A vertical twin slab gel unit (Model 2001, LKB, Bromme, Sweden) was used with 12% (w/v) polyacrylamide separating gel and 4% (w/v) polyacrylamide stacking gel. Electrophoresis was carried out at 4°C for approximately 15 h at a constant 70 V current. Following electrophoresis, isoenzyme staining was carried out by incubating gels in the corresponding staining solution (Tanksley & Orton, 1983).

NPTII Assay

NPTII activity was measured from 5.0 µg plant protein using protocol of Tomes *et al.* (1990). Kanamycin resistant strain of *A. tumefaciens* and transformed tomato cv. PR, both were used as positive controls, while non-transformed *L. peruvianum* and *L. chilense* were used as negative controls.

PCR Analysis for nptII Gene

Presence of nptII, selectable marker gene in transformed tomato cv. PR and in tomato SH plants was confirmed by the PCR analysis. In this analysis,
two primers specific to nptII gene were used. The forward primer had a sequence of 5' GTC CCT TGG TCG TGC ATT TCG 3', while a reverse primer had a sequence of 3' GTC ATC TCA CCT TGC TGC TCC C 5' and 540 bp DNA segment of nptII gene was amplified. Plant DNA was extracted using leaf samples from ex vitro grown plants following method of Edwards et al (1991). Controls were established in the same manner as described for NPTII assay.

**RAPD Analysis**

RAPD being a PCR-based analysis, PCR protocol was utilised in with following modifications: 1) the plant genomic DNA was extracted as described by Doyle & Doyle (1989) and 1.0 µl of the extracted DNA (40 µg ml⁻¹) was added to the reaction mixtures; 2) a single random primer having 10 bp (1.0 µl) was added to reaction mixture; 3) controls were prepared without plant DNA; 4) three different thermal cycles (e.g. 93°C for 30 sec, followed by 37°C for 40 sec and 72°C for 1 min 20 sec) were used for amplification of DNA segments, this programme was being linked with two other programmes (37°C for 5 min followed by 4°C for 10 min) to terminate the reaction and; 5) for electrophoresis, 25 µl sample was loaded into individual wells of the 1.0% (w/v) agarose (Sea Kem, FMC, Rockland, USA) gel (20x25 cm) and electrophoresis of DNA samples was carried out at 35 V (0.92 V/cm) for 5 hrs.

Three random primers (Operon Technologies Inc, Atlantic Avenue, Almanda, CA 94501, USA) were used having the following sequence: No.7 (CTA CGC TCA C); No.8 (TCC GCA GTA G); and No. 10 (TGG TCG GGT G). Even though sequence of these primers was known, the homology of the primer if any, with a gene or repeated sequence in the plant genome was unknown. Any bands subsequently observed in the gel were used for comparison of plant genotypes. A standard calibration curve was drawn by plotting the logarithmic values of the molecular weight (bp) of marker bands of e DNA on the Y axis against the distance travelled (mm) by respective bands on the X axis. Using this standard curve, the molecular weight of electrophoretically separated plant DNA was determined from their distance travelled on a gel.

**Results**

**Shoot Regeneration from K' Hybrid P-calli: A Double Selection Strategy**

Upon protoplast fusion of *L. esculentum (+) L. peruvianum* or *L. esculentum (+) L. chilense*, optimal results were obtained in terms of fusion frequency, post-fusion protoplast viability and plating efficiency (Table 1).

| Table 1—Chemical fusion between *L. esculentum* (P1) and *L. peruvianum* (P2) or *L. esculentum* (P1) and *L. chilense* (P3) protoplasts, recovery of P-calli and regeneration of tomato somatic hybrids following a double selection system |  |
|---|---|---|---|---|---|---|
| Protoplast fusion | Fusion frequency (%) | Post-fusion viability (%) | Plating efficiency (%. 7d) | Total no. of P-micro-calli obtained | Kanamycin resistant and sensitive P-calli | Selection of hybrid P-calli | P-calli regenerated shoots (putative SH plants) |
| **Fusion:** |  |
| P1(+)+P2 | 24.6±3 | 70.4±3 | 57.2±5.8 | 885 | 302+583 | 108 | 96 |
| Controls |  |
| P1 | -- | 96.2±4 | 66.1±6.3 | >300b | 300+0 | -- | -- |
| P2 | -- | 98.3±3 | 74.3±5.9 | >300b | 0+300 | -- | -- |
| P1+P2 | -- | 95.1±4 | 68.7±5.4 | >300b | 107+193 | -- | -- |
| Mix |  |
| **Fusion:** |  |
| P1(+)+P3 | 31.7±3 | 74.8±4 | 60.1±3.9 | 779 | 212+567 | 80 | 12 (46c) |
| Controls |  |
| P1 | -- | 94.9±3 | 67.8±3.7 | >300b | 300+0 | -- | -- |
| P3 | -- | 97.2±4 | 78.9±5.8 | >300b | 0+300 | -- | -- |
| P1(+)+P3 | -- | 95.3±5 | 70.8±4.7 | >300b | 136+164 | -- | -- |
| Mix |  |

*Heterokaryon frequency for P1(+)+P3; b300 micro-calli from each control were transferred to callus inducing medium containing 100 µg ml⁻¹ kanamycin sulphate; ccalli underwent caulogenesis and produced leaf-like structures. Data is based on 3 experiments and each experiment replicated 3 times.*
Within 13-22 d following protoplast fusion, 885 and 779 micro-calli from PSH and CSH, respectively were transferred from liquid protoplast culture medium (TMP+TMd) onto agar-solidified TMc, callusing medium containing 100 \( \mu g \) ml\(^{-1}\) K.S. When kanamycin selection pressure was applied to fusion-treated protoplast cultures at earlier stage i.e. 7 d, all developing micro-calli were consistently found kanamycin resistant on TMc medium. Whilst micro-calli that developed from the kanamycin-treated fused protoplast at later stage, i.e. 10 d, were segregated into kanamycin resistant (K') and sensitive (K\(^s\)) calli on TMc medium. Following 28 d culture on TMc medium, kanamycin resistant P-calli were further divided under two distinct types based on callus colour, structure and growth rate. The pale-green coloured, compact, K' calli growing very slow (<1.0 cm diam in 28 d) were isolated as \( L. \) esculentum homokaryotic calli. On the contrary, dark-green coloured, semicompact (in PSH) or nodular (in CSH), K' calli growing very fast (>1.2 cm diam in PSH and >2.0 cm diameter in CSH, within 28 d culture) were recognised as somatic-hybrid P-calli and transferred separately onto regeneration medium. These heterokaryotic, K' P-calli were phenotypically alike with calli of their wild parents growing on kanamycin-free (control) medium. After 28 d culture on MS4 medium, in PSH experiment, out of 108 putative hybrid calli, 96 calli (89%) underwent shoot regeneration (Table 1). While in CSH experiment, although 80 putative hybrid calli were transferred on regeneration medium, in total 58 calli (72.5%) differentiated but 46 calli (57.5%) produced leaf-like structures (caulogenesis) and only 12 calli (15%) showed shoot regeneration. It is worthy to note that in both fusion studies (PSH and CSH), no shoot regeneration was observed either from separated \( L. \) esculentum homokaryotic, P-calli from fusion-treated protoplast cultures or from controls (unfused parental individual or mixed protoplasts). It further demonstrates a tight control of double selection system on selective shoot regeneration of putative somatic hybrid plants.

**Characterisation of in vitro Grown Putative SH Plants**

In vitro grown regenerated plants from both PSH and CSH were K' but regenerants from CSH, in particular, were further characterized on their rooting response and needed supplement of IBA to MS0 medium for root induction. Regenerated in vitro grown plant population from each fusion experiment was phenotypically uniform, displaying distinct intermediate morphology when compared to their parents. Specifically, dark-green leaf colour and broad leaf-lamina were the dominant features of \( L. \) esculentum, while shallow (in PSH) to very deep (in CSH) leaf serration were the dominant characters of wild parents. Nevertheless, stem thickness of both SH plants was the intermediate morphological marker.

**Characterisation of ex vitro Grown Putative SH Plants**

All SH plants of PSH and CSH grew normally under glasshouse conditions, producing uniform vegetative growth followed by flowering and fruit set. Among the SH plant population, individual variability was not noticed for chimeric arrangement. On the contrary, an intermediate morphology along with dominant markers from both the parents was distinctly expressed in SH plants, which provides obvious evidence for their hybridity (Table 2). It was interesting to note that similar dominant markers were expressed for parent \( L. \) esculentum in both SH plant populations (Table 2). However, overall morphology of SH plants from PSH and CSH were quite different from each other. The prominent features of SH plants from PSH were shallow leaf serration, presence of bracts at internodes, linear inflorescence, uniform fruit size and 3-12 mature seeds per fruit (i.e. self fertile) (Fig. 1). Whilst main phenotypical characteristics of SH plants from CSH were deep leaf serration, thick midrib of leaf, absence of bracts at internodes, brittle plant tissues, branched inflorescence, variable fruit shapes (round, flat, oblong and pear) with immature seed developments, i.e. self-sterility. Nevertheless, yellowish-orange ripe fruits were the undeviating phenomenon in both hybrid plants as a distinct intermediate morphology (Fig. 2).

**Cytological Analyses**

In comparison to their parents, both the putative SH plant populations had a higher number of chloroplasts per guard cell pair (No. Ch/GCP), showing a sign for higher ploidy level (Table 3). SH plants from PSH and CSH contained different No. Ch/GCP (21.4±3.6 and 32±5.3 respectively) at tetraploid level, although root squash analysis revealed that they contained similar chromosome number i.e. 2n=4x=48. In both somatic hybridisation studies, even though most of the SH plants were tetraploid, aneuploidy was also observed to some extent.
Table 2—Comparison of two wild species of genus *Lycopersicon*, viz. *L. peruvianum* and *L. chilense*, in the production of tomato somatic hybrids

<table>
<thead>
<tr>
<th>Character</th>
<th><em>L. peruvianum (+) L. esculentum</em> somatic hybridization</th>
<th><em>L. chilense (+) L. esculentum</em> somatic hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoplast source</td>
<td>Mesophyll (K²) + mesophyll (K')</td>
<td>Suspension (K⁵) + mesophyll (K')</td>
</tr>
<tr>
<td>Selection for heterokaryotic calli</td>
<td>Dependent on callus colour, structure and relative fast growth of K' calli</td>
<td>Distinct with nodular, K' calli with very fast growth rate</td>
</tr>
<tr>
<td>Shoot regeneration ability of heterokaryotic calli</td>
<td>Excellent (89%) with 4.9±2.3 shoots/regenerating callus</td>
<td>Poor (15%); although 72.5% calli differentiated, 57.5% calli showed caulogenesis, 2.1±1 shoots/callus</td>
</tr>
<tr>
<td>Rooting of SH shoots</td>
<td>MSO medium*</td>
<td>MSO medium* + 0.2 mg/l IBA</td>
</tr>
<tr>
<td><em>In vitro</em> SH plants</td>
<td>Distinct intermediate-morphology</td>
<td>Distinct intermediate-morphology</td>
</tr>
<tr>
<td><em>Ex vitro</em> plant growth</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Flowering of SH plants</td>
<td>Normal; unbranched inflorescence</td>
<td>Normal; branched inflorescence</td>
</tr>
<tr>
<td>Fruiting of SH plants</td>
<td>Normal with uniform fruit shape</td>
<td>Normal with variable fruit shape</td>
</tr>
<tr>
<td>Fertility of SH plants</td>
<td>Self-fertile with mature seed set</td>
<td>Self-sterile with immature seeds</td>
</tr>
<tr>
<td>Dominant morphological markers for <em>L. esculentum</em></td>
<td>Dark green coloured leaf, broad leaf lamina (3.5x5.0 cm), thicker stem (10.0 cm) with anthocyanin pigmentation, thicker anther-cone (3.2 mm) and stigma (0.6 mm), bold seeds.</td>
<td>Dark green coloured leaf, broad leaf lamina (3.0x4.3 cm), thicker stem (8.9 cm) with anthocyanin pigmentation, thicker anther-cone (3.4 mm) and stigma (0.5 mm).</td>
</tr>
<tr>
<td>Intermediate morphology for hybrid plants</td>
<td>Elongated shape of terminal leaflet, presence of bracts at base of the internodes, dwarf but dense trichome growth, exserted stigma and non-hairy seed coat.</td>
<td>Deep leaf serration, prominent midrib of thicker leaf, brittle stem, dense but dwarf trichome growth, exserted stigma, nipped blossom end of fruit surface</td>
</tr>
<tr>
<td>Heteromorphic isozyme system</td>
<td>Leucine aminopeptidase</td>
<td>Acid phosphatase</td>
</tr>
<tr>
<td>RAPD analysis</td>
<td>Hybridity confirmed but bands for <em>L. esculentum</em> were faintly stained</td>
<td>Prominent bands observed from both the parents; an additional hybrid band was also appeared</td>
</tr>
<tr>
<td>NPTII assay and PCR analysis for nptII gene</td>
<td><em>L. esculentum</em> genome confirmed in SH plants by presence of selectable marker gene</td>
<td><em>L. esculentum</em> genome confirmed in SH plants by presence of selectable marker gene</td>
</tr>
</tbody>
</table>

*MSO medium supplemented with 100 μg/ml kanamycin sulphate; K'=kanamycin resistance; K⁵=kanamycin sensitive, SH=somatic hybrid. A double selection system employed in both somatic hybridization studies (kanamycin resistance from *L. esculentum* and efficient shoot regeneration from wild parent).

Isoenzyme Analysis

In isoenzyme characterisation of SH plants from PSH, the bands derived from acid phosphatase could not differentiate parental and putative SH plants. However, leucine aminopeptidase was found to be useful as characteristic bands were obtained for parental species and summation of such bands were observed in all putative SH plants (Fig. 3A). Whereas, SH plants from CSH were easily characterised by acid phosphatase profile as hetero-morphic dimer pattern was observed and thus, their hybridity was confirmed (Fig. 3B).

RAPD Analysis

In characterisation of SH plants from PSH, RAPD markers were dominated by wild parent (Fig. 3C) as *L. peruvianum* specific bands (e.g. for primer 7: bands of 970 and 920 bp; for primer 8: bands of 1690, 1666, 1600, 860 and 820 bp; for primer 10: a band of 746 bp) were strongly amplified in SH plants, whereas specific bands for *L. esculentum* (e.g. for primer 7: bands of 2166 and 1800 bp; for primer 8: a band of 1150 bp) were weakly amplified. In characterisation of SH plants from CSH, however, these RAPD markers discriminated both the parents and a
Table 3—Mean number of chloroplasts per guard cell pair in tomato somatic hybrids in comparison to their parents

<table>
<thead>
<tr>
<th>Plant material (chromosome count)</th>
<th>No. of somatic hybrid plants</th>
<th>No. of chloroplasts/guard cell range (mean±standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parents (diploid; 2n=2x=24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. esculentum</td>
<td>06-09 (7.1 ± 0.9)</td>
<td></td>
</tr>
<tr>
<td>L. peruvianum</td>
<td>10-14 (12.0 ± 2.3)</td>
<td></td>
</tr>
<tr>
<td>Somatic hybrids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetraploid (2n=4x=48)</td>
<td>29</td>
<td>18-24 (21.4 ± 3.6)</td>
</tr>
<tr>
<td>Aneuploidy at tetraploid level</td>
<td>3</td>
<td>16-20 (18.2 ± 2.7)</td>
</tr>
<tr>
<td>Somatic hybridization of L. esculentum with L. chilense</td>
<td></td>
<td></td>
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<tr>
<td>Parents (diploid; 2n=2x=24)</td>
<td></td>
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<tr>
<td>L. esculentum</td>
<td>06-09 (7.1 ± 0.9)</td>
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</tr>
<tr>
<td>L. chilense</td>
<td>14-18 (14.7 ± 2.5)</td>
<td></td>
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<tr>
<td>Somatic hybrids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetraploid (2n=4x=48)</td>
<td>10</td>
<td>29-36 (32.0 ± 5.3)</td>
</tr>
<tr>
<td>Aneuploidy at tetraploid level</td>
<td>2</td>
<td>24-30 (28.4 ± 3.9)</td>
</tr>
</tbody>
</table>

Data is based on 3 replications and 3 samples/replication.

summation of specific parental bands were strongly amplified in SH plants (Fig. 3D). Profile of primer 7 showed that bands of 1500, 1350, 1300 and 1050 bp were L. esculentum specific, whilst a band of 600 bp was of L. chilense specific. Similarly, banding pattern of primer 8 revealed that a 460 bp band was specific to L. esculentum, whereas 1800, 1690, 1500, 1370, 860 and 820 bp bands were unique to L. chilense. More importantly, a hybrid-specific band (1666 bp) was observed from primer 10 (Fig. 3D).

**NPTII Assay and PCR Analysis for nptII Gene**

The NPTII activity and actual presence of nptII gene was detected in SH plants of both PSH and CSH by NPTII assay and PCR analysis (Fig. 3E & F). Thus, selectable marker gene furnished the evidence of L. esculentum genome presence within SH plants. Such confirmation was essential for the characterisation of SH plants from PSH, particularly when the DNA segments specific to L. esculentum were weakly amplified in the samples of somatic hybrid by RAPD analysis.

**Discussion**

'Peruvianum complex' of the genus, Lycopersicon is separated from its 'Esculentum complex' for sexual barriers and genetic diversity. It was, therefore, necessary to compare L. peruvianum and L. chilense, two species of 'Peruvianum complex' for the development of tomato somatic hybrids. In PSH, protoplast source of both parental species was mesophyll and the protoplast isolation was convenient. Whereas in CSH, an initiation of cell suspension cultures from L. chilense, the wild parent, was the prerequisite for a source of high-yielding and viable protoplasts. However, the selection of hybrid calli in CSH was more distinct as compared to PSH. Nodular callus structure and fast growth rate were the dominant features of L. chilense, which can effectively be exploited in identification of hybrid calli. Nevertheless, an excellent regeneration capacity of L. peruvianum mesophyll protoplasts resulted in recovery of more SH plants in PSH. Whilst caulogenetically influenced shoot regeneration was characteristic of suspension protoplast of L. chilense and consequently, hampered the regeneration of somatic hybrid shoots in CSH. These results demonstrated that the regeneration capacity of wild parental protoplast can effectively be used in tomato somatic hybridization and confirmed the findings of Kinsara et al (1986) and Wijbrandi et al (1990). Furthermore, double selection system (i.e. antibiotic resistance from transformed, cultivated parent and efficient shoot regeneration from protoplasts of wild parents) demonstrated total control on selectivity of somatic hybrids.

In the present study, putative SH plants from both PSH and CSH were identified in in vitro cultures because of their discrete morphology. Moreover, SH plants from CSH were easily characterized based on a double selection of their rooting response. Also ex vitro-grown SH plants were verified for their hybridity depending on expression of dominant morphological markers from both the parents along with intermediate morphology. Apart from inflorescence type, the expression of all other morphological markers of L. esculentum was similar in both PSH and CSH. Therefore, these tomato markers along with dominant markers for wild parents can efficiently be utilised in future tomato somatic hybridization programme. It was interesting to note that although both SH plants were self-compatible and resulted in fruit set but mature seed development was observed only in PSH, while immature seeds developed in CSH. These results are in agreement with Kinsara et al (1986), Wijbrandi et al (1990) and Sakata et al (1991) for PSH; and with Bonnema &
Fig. 1—Morphological characterisation of tomato somatic hybrid of *L. esculentum* (+) *L. peruvianum*

A. *Ex vitro* grown plant of *L. esculentum* (T-11, transformed tomato cv PR; 45 d) [x 0.06].

B. *Ex vitro* grown plant of *L. esculentum* (+) *L. peruvianum* somatic hybrid (45 d) [x 0.06].

C. *Ex vitro* grown plant of *L. peruvianum* (LA 2744; 45 d) [x 0.06].

In all plates, left to right: *L. esculentum*, somatic hybrid and *L. peruvianum*

D. Typical leaf morphology of the somatic hybrid in terms of colour, lamina size and shape, and extent of margin serration [x 0.13].

E. Presence of bracts at the base of axillary nodes: note the presence of bracts (arrowed) in *L. peruvianum* and somatic hybrid plants [x 0.36].

F. Inflorescences: note the non-branching (linear) type of inflorescence in *L. esculentum* and the somatic hybrid and an intermediate inflorescence stalk length also for somatic hybrid [x 0.12].

G. Floral forms: note the comparatively larger flower size for the somatic hybrid [x 0.5].

H. Pollen viability of a tetraploid (2n=4x=48) somatic hybrid (70.1±3.2%) [x 50].

I. Pollen viability of a hexaploid (2n=6x=72) somatic hybrid (39.2±2.9%) [x 50].

J. Fruits at maturity: note the distinctive yellowish-orange fruit ripening of the somatic hybrid than the yellow and red coloured fruit ripening of *L. peruvianum* and *L. esculentum* respectively. Note also a typical intermediate fruit size for the somatic hybrid [x 0.52].

O’Connell (1992) for CSH. The genetic sterility of CSH plants, reported by Bonnema & O’Connell (1992), was of pre-zygotic nature as they found seedless fruits, whereas in the present study no mature seed development was observed, so it was of post-zygotic nature. These differences might be due to polymorphism of *L. chilense*.

Cytogenetic analyses of the somatic hybrids revealed that most of them were amphi-diploid in nature, which gives conformity to the somatic
hybridization; as following diploid protoplast fusion, tetraploid SH plants are expected. Aneuploidy was, however, observed at the tetraploid level in both SH combinations, which resulted in genetic sterility in PSH and into variable (oblong-pear) fruit shapes in CSH. Spontaneous elimination of chromosomes from hybrid cells is a common phenomenon in tomato somatic hybridization (Wijbrandi et al, 1990; Guri et al, 1991; Jourdan et al, 1993).

The differences between PSH and CSH existed even in the characterisation of tomato somatic hybrids by isoenzyme and RAPD analyses. In the present

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**Fig. 2—Morphological characteristics of tomato somatic hybrid of *L. esculentum (+) L. chilense***

A. *Ex vitro* grown plant of *L. esculentum* (T-11, transformed tomato cv PR; 45 d) [× 0.06].

B. *Ex vitro* grown plant of *L. esculentum (+) L. chilense* somatic hybrid (45 d) [× 0.06].

C. *Ex vitro* grown plant of *L. chilense* (LA 2930; 45 d) [× 0.06].

In all plates, left to right: *L. esculentum*, somatic hybrid and *L. chilense*

D. A distinct intermediate leaf morphology of the somatic hybrid showing an expression of dominant markers from both parents: the broad leaf lamina and dark green leaf colour for *L. esculentum* with a characteristic, deep leaf serration for *L. chilense* [× 0.2].

E. Inflorescences: the somatic hybrid produces a branched inflorescence similar to *L. chilense* parent [× 0.12].

F. Dominant features of anther-cones: Thickened anther-cone and broad stigmatic surface expressing as markers for *L. esculentum*, whereas anther-cone with green tip (and base) and exserted stigma (arrowed) representing markers for *L. chilense* [× 1.5].

G. Relatively long but sparse trichome growth of *L. esculentum* [× 7.8].

H. Intermediate trichome form of the somatic hybrid [× 6.8].

I. Short-length but dense trichome distribution typical of *L. chilense* [× 8.8].
A markers, instead of more commonly used RFLP-based DNA markers, were used for molecular confirmation of tomato somatic hybrids. RAPD analysis was useful for characterisation of SH plants because of its speed, technical simplicity and ease in identification of polymorphism.
In most of the somatic hybridization studies, the selection system is dependent on an efficient regeneration ability of the wild parental protoplasts (Kinsara et al., 1986; Wijbrandi et al., 1990). The presence of selectable marker gene, especially from cultivated crop species, is more meaningful in characterisation of SH plants because SH plants are usually phenotypically dominated by wild parents (Kalloo, 1991); whereas protein (isoenzyme) and DNA markers are usually dominated by wild parents. Accordingly, in the present study, presence of tomato (L. esculentum) genome in tomato SH plants is demonstrated employing NPTII assay and PCR analysis for nptII gene.

References


