4. ANALYSIS AND CHARACTERIZATION OF SOMATIC HYBRID PLANTS

4.1 Introduction

Effective selection of the heterokaryon and the determination of true somatic hybrids are very important in the production of somatic hybrid plants. Different kinds of selectable markers are utilized in tomato at several steps. Some are expressed at the early stage and would thereby enable the development of selection strategy before the plants regenerated. For example, antibiotic resistance induced to one of parental plants (Guri *et al.* 1991, Schoenmakers *et al.* 1994), mutant character (Schoenmakers *et al.* 1992, Adams and Quiros, 1985) were reported as available selectable markers. Even if the selection of heterokaryon during protoplast culture and plant regeneration period are not completed, those selection markers would help to reduce the number of non- heterokaryon.

Morphology is normaly distinct from both parents and possible to be utilized as a criterion to identify the hybridity and characterization of the regenerated plants (Wijbrandi *et al.* 1990, Hossain *et al.* 1994). Several morphological characters are enable to be classified into either each parental type or intermediate type. However, due to polyploidy, chromosome rearrangement, mutation among others, unpredictable unique morphology are possibly observed and it is sometimes not sufficient to determine the hybridity based on only morphological analysis.

Isozyme analysis has often been used for hybrid determination (Handley *et al.* 1986, Wijbrandi *et al.* 1990, Schoenmakers *et al.* 1993). Molecl cular markers such as restriction fragment length polymorphysms (RFLPs), PCR-baced markers can also be effective for not only hybridity determination but also more details of genetical composition.

In CHAPTER 4, the confirmation of the plant hybridity and the characterization of the somatic hybrids were attempted by observation of their apparent phenotypes, cytological study, analysis of genotypes using molecular techniques. The pollen viability

was also determined and the progeny was developed.

4.2 Materials and Methods

4.2.1 Material plant and growth condition

Putative somatic hybrids and their fusion parents were transferred in pots. They were maintained in the greenhouse throughout the year. In summer, the plants were shielded by black mesh sheets to reduce light intensity and temperature in the greenhouse. While, in winter, the greenhouse was heated to maintain a temperature higher than 8°C.

4.2.2 Morphology

An assessment of the morphological characteristics was carried out in putative somatic hybrids and their fusion parents. The growth habit and the distinct features of the inflorescence, the anther, the leaf and the flower were examined and compared with the parents.

4.2.3 Isozyme analysis

The isozyme patterns of the regenerants and parents were analyzed by starch gel electrophoresis. Young leaves and shoot bud materials (0.1-0.2 g) of the parental and the regenerated plants were collected and kept in bottles in ice. The materials were frozen with liquid nitrogen and homogenized with a pestle and a mortar. Isozymes were extracted in a buffer (2.24 % Tris-HCl, 34.2 % sucrose, 0.44 % 2-mercaptoethanol, pH 8.5) and 10 mg of polyvinylpyrolidone were added to each extraction to remove phenolic compounds. The extract was transferred in eppendorf tubes and centrifuged at 6000 rpm for 5 min. The supernatant was used for the analysis. The paper wicks (5 x 6 mm, TOYOBO FILTER PAPER) absorbing the supernatant were set into 12 % horizontal starch gel and isozymes were separated by electrophoresis (Tanksley 1979) at 4°C for 4

to 7 h at 50 mA after 30 min at 30 mA. The gel was sliced into 2 sheets and stained the isozymes PGI (phosphoglucoisomerase), PGM (phosphoglucomutase) and SKD (Skimic dehydrogenase) according to Vallejos (1983).

4.2.4 Cytological study

(1) Mitotic metaphase chromosome preparation

Chromosomes were counted at mitotic metaphase stage according to the method of Fukui *et al.* (1994) which was slightly modified and applied for this study.

Young shoots of somatic hybrids were cut in the greenhouse and put in flasks with water for rooting. The roots measuring 1 to 3 cm were collected and treated with a spindle inhibitor 2 mM 8-hydoxyquinoline solution at room temperature for 2.5 to 3 hours. The root tips were transferred into ethanol: glacial acetic acid (3:1) at 4°C for 1 h to several weeks. The root tips were stored either in 70 % ethanol at 4°C or used immediately.

Fixed root tips were put in a glass petri dish with distilled water for 20 to 30 min for washing. Meristematic portions were dissected on a glass slide, put into an Eppendorf tube containing the enzyme mixture (2% Cellulase Onozuka RS, 0.3 % Pectolyase Y-23, 1.5 % Macerozyme R200, 10 mM EDTA, 30 mM citrate buffer, pH 4.2), and incubated for 30 min at 37 °C. Digested root tips were carefully transferred with a Pasteur pippete into a glass petri dish containing distilled water and incubated for 15 min. A root tip was put on a clean glass slide with a Pasteur pipette and excess water was removed with a small piece of filter paper. One or 2 drops of fresh ethanol: glacial acetic acid (3:1) were added onto a root tip to macerate and cells were spread with a pair of fine forceps. The materials were air-dried properly and stained with either 4 % giemsa solution or 1 % acetocarmine solution.

(2) Meiotic chromosome preparation

The flower buds of somatic hybrids were directly fixed in ethanol: glacial acetic acid (3:1) for 24 h and transferred in 70 % ethanol. An anther was washed in a glass petri dish containing distilled water for 15 min and transferred on a clean glass slide. A few drops of acetocarmine solution was added on an anther. The anther was broken by a maceration needle. After large debris were removed, PMCs (pollen mother cells) on a glass slide were covered with a cover glass and carefully squashed. The slide glass was heated in an alcohol lamp and cooled down several times to stain the chromosomes deeply.

4.2.5 RAPD analysis

(1) DNA extraction

Total DNA was extracted according to Draper and Scott (1992).

Fresh leaves (0.2g) were collected from somatic hybrids and their fusion parents growing in the greenhouse and frozen in a mortar containing liquid nitrogen. A small amount of quart sand was added and tissues were pulverized to an extremely fine powder whilst still frozen. 600 μl of extraction buffer was added and the tissues were homogenized further. The suspension was transferred in Eppendorf tubes. 40 μl of 20% SDS was added and mixed by vortexing for a few seconds. Tubes were incubated at 65°C for 10 min with occasional inversion. 250 μl of 5M potassium acetate was added, mixed well and incubated in ice for 30 min. The protein and SDS were precipitated by centrifugation at 14000 rpm for 20 min at 4°C. The supernatant was poured through a mira cloth into a clean Eppendorf tube and 400 μl of cold isopropanol stored at -20°C was added. Both the supernatant and isopropanol were mixed carefully by inverting the tube for 10 min and incubated at -20°C for 30 min. The precipitated DNA was pelleted at 14000 rpm for 15 min at 4°C. The supernatant was poured off and the pellet was partially dried by inverting the tube on a paper towel for 10 min. DNA was gently dissolved with 140 μl of TE buffer (50, 10) and centrifuged for 10 min at 14000 rpm to remove insoluble

contaminants. The supernatant was transferred into a new tube and 15 µl of 3M sodium acetate was added and mixed well. DNA was precipitated with 100 µl of cold isopropanol in ice. DNA was pelleted by centrifugation at 14000 rpm for 5 min. The supernatant was poured off carefully. The pellet was vacuum-dried for 5 min after washing with cold 80% ethanol (-20°C). DNA was dissolved in 50 µl of TE (10, 1) buffer. The concentration of DNA was determined at 260 nm (1 OD_{260} unit= 50ng/µl).

(2) Polymerase chain reaction (PCR)

PCRs were performed in a volume of 25 µl containing 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 2.0 mM of $MgCl_2$, 0.2 mM each of dNTPs, 0.4 µM random primer, 25 ng of template DNA and 0.625 unit of *Taq* polymerase (Takara, Ohtsu). Eight random decamer primers Kits A and K (Operon Technologies, USA) were used to develop the RAPD markers (Williams *et al.* 1990). PCR amplification was carried out with a thermal cycler Gene Amp PCR System 2400 (PERKIN ELMER, USA) programmed for 3 min preheating at 94°C followed by 45 cycles each consisting of 1 min at 94°C, 1 min 30 sec at 45°C, and 2 min at 72°C for denaturation, annealing, and reaction extension steps, respectively. The PCR products were resolved by electrophoresis in agarose gels (2 % agarose in TAE buffer) and were stained with ethidium bromide solution (10 mg/ml).

4.2.6 Chloroplast DNA analysis

(1) DNA digestion and electrophoresis

Total DNA (2.0 µg) of the regenerated plants and their fusion parents were digested with 1 to 2 µl of restriction enzymes in the buffer provided by the supplier (Takara, Ohtsu). 20 µl of the reaction mixtures in Eppendorf tubes were incubated at a suitable temperature for each enzyme reaction for 5 to 7 h. Restriction fragments were separated by electrophoresis in 0.7 % agarose in TAE buffer. The gel was stained with ethidium bromide for 20 min and photographed under UV.

(2) Vacuum blotting

Restriction fragments were transferred from agarose gel onto a nylon filter membrane (Micro Separations, USA) by a Vacu Gene XL (Pharmacia, Sweden) following the manufacture's instructions. Vacuum blotting was performed for 20 min in the depurination solution, 20 min in the denaturation solution, 20 min in the neutralization solution and 1 h in the transfer solution. DNA transferred was bonded on the filter by UV for 3 min.

(3) Probe preparation

The chloroplast DNA probe

The chloroplast DNA (cpDNA) probes, pTBa1 and pTB13 from tobacco (Sugiura *et al.* 1984) were used for the determination of the chloroplast types in the putative somatic hybrids.

Transformation and plasmid culture

Competent cells in an Eppendorf tube were taken out from a -70 °C freezer and kept in ice. 50 µl of competent cells was transferred in a pre-chilled Eppendorf tube. One to 3 µl of plasmid DNA which was added into a tube containing competent cells were mixed well by inversion. The competent cells and plasmid DNA mixture in a tube were incubated on ice for 30 min. The tube was transferred into 42 °C water bath and incubated for exactly 90 sec. The tube was transferred on ice immediately and chilled for 2 min. 400 µl of pre-warmed LB medium (37 °C) was added to the tube. The transformed cells were shaken (200 rpm) at 37 °C for 45 min. 200 µl of culture was spread onto LB-bacto agar medium containing the 20 µg/ml of ampicillin in a 9 cm petri dish. The plate was inverted and incubated for 12 to 16 h at 37 °C. The colonies containing plasmid DNA were cultured. A single colony was taken with a micro pipette and put into 2 ml of liquid LB-antibiotic medium in a sterile test tube. The cells were multiplied by rapid shaking

overnight. Two to 5 µl of culture was added in 2ml of LB-antibiotic medium (ampicillin 20 µg/ml) and cultured for 8 h in a shaker at 37°C.

Plasmid isolation by lysozime alkali lysis method

Transformed bacterial cultures (100-200 µl) were added in 25 ml of liquid LB-antibiotic medium and cultured overnight at 37°C by shaking. The cultures were decanted in a tube and centrifuged for 25 min at 1000 rpm. The supernatant was discarded completely. The pellet was resuspended with 1 ml of solution I and was collected at 14000 rpm for 5 min at 4°C. The supernatant was poured off and the excess supernatant was also pipetted out. The tube was kept in ice. Ice cold solution I (200 µl) was added and mixed well by a vortexing. After addition of freshly prepared lysozime solution, it was mixed gently and incubated in ice for 10 min. Solution II (200 µl) was added and mixed by inversion. It was kept in ice for 5 min. Cells were centrifuged at 14000 rpm for 10 min at 4°C. The supernatant (400 µl) was transferred into a new Eppendorf tube. Plasmid DNA were precipitated by adding 800 µl ice cold isopropanol and collected for 10 min at 14000 rpm. The pellet was washed with 80 % ethanol and completely dried by a vacuum. DNA was resuspended in TE (10,1) buffer (200µl) and RNase-treated for 30 min at 37°C. After phenol/chloroform/isoamyl alcohol washing, DNA was precipitated in ice cold isopropanol. The pellet was rinsed with 80 % ethanol and dried by a vacuum. Finally, plasmid DNA was resuspended in 30 µl TE (10,1) buffer.

Check of plasmid DNA

A small amount of plasmid DNA (ex. 0.1 to 5 µg) was digested with the restriction enzyme for 2 h at 37°C. The DNA fragments was electrophoresed and stained in ethidium bromide solution. The size of DNA was confirmed.

Digestion and purification of plasmid DNA

A large amount of plasmid DNA (40 μg) was digested with 1 μl of the restriction enzyme in 20 μl of reaction mixture at 37°C (30°C for *Bam*HI digestion) overnight. Digested DNA was separated in agarose gels. After staining with ethidium bromide solution, the band containing the desired DNA fragment was cut and DNA was collected into a tube with a filter membrane. The gels were kept at room temperature after incubation for at least 2 h in a -20°C freezer and centrifuged for 20 min at 14000 rpm at 4°C. TE (100 μl) solution was added and the tube was centrifuged again for 10 min at 4°C. The filter was discarded and 3 M NaAC (1/10 volume) and an equal volume of ice cold isopropanol were added. The tube was kept at -20°C for at least 2 h and centrifuged for 10 min at 14000 rpm. The supernatant was decanted carefully. The pellet was washed by the same volume of 80 % ethanol and the excess ethanol was pipetted out. DNA was vacuum-dried for 10 min and dissolved with 20 μl of MilQ water.

DNA labeling

The probe DNA was labeled with a non-radioactive digoxigenin-dUTP (Boehringer Mannheim, Germany).

DNA was denatured by heating in boiling water for 10 min and chilled quickly in ice for 5 min. Fifteen μl of freshly denatured DNA (3 ng/μl), 2 μl of hexanucleotide mixture and 2 μl of dNTP mixture were mixed in an Eppendorf tube and made up to 29 μl with sterile distilled water. Following the addition of 1 μl klenow enzyme, the DNA was incubated for at least 1h at 37°C. The reaction was stopped by adding 2 μl of EDTA solution (0.2 M, pH 8.0). Labeled DNA was precipitated with 2.5 μl of LiCl (4.0 M) and 75 μl prechilled (-20°C) 99 % ethanol and mixed well. DNA was left at -20°C for 2 h to induce precipitation and centrifuged for 20 min at 12000 rpm. DNA pellet was washed with 70 % cold ethanol, dried under vacuum and dissolved in 50 μl of TE (10, 1) buffer.

(4) Southern hybridization and immunological detection

Preparation of hybridization probe

Labeled DNA probes in Eppendorf tubes were denatured for 5 min in boiling water and immediately transferred in ice for 5 min. The probes were kept in ice until required.

Pre-hybridization and hybridization

The filter in which the DNA was transferred onto was pre-hybridized in a sealed plastic bag with 20 ml hybridization solution (26 ml for 132 cm^2 filter) at 42°C overnight. Denatured probe DNA (20 µl) was added in preheated hybridization solution (42°C) and mixed well. Pre-hybridization solution was replaced with hybridization solution containing probes and the plastic bag was sealed and incubated for at least 6h at 42°C. The filter was carefully taken out from a plastic bag and washed with 2 x SSC; 0.1 % SDS twice for 5 min each at room temperature and with 0.1 x SSC; 0.1% SDS twice for 15 min each at 42°C. The filter was used directly for detection.

Immunological detection

The detection of cpDNA fragments was carried out using the DIG nucleic acid detection kit (Boehringer Mannheim, Germany). The filter was washed briefly with washing buffer 1. The buffer 1 was replaced with about 100 ml of buffer 2 and the filter was incubated for 30 min. Antibody-conjugates (Boehringer Mannheim, Germany) were diluted to 150 mU in 20 ml of buffer 2 and the filter was treated for 30 min to 1.5 h. Unbounded antibody-conjugates were removed by washing 2 times with 100 ml of the buffer 1 for 15 min each and then equilibrated with 20 ml of the buffer 3 for 20 min. The filter was placed in a plastic bag and 10 ml of the color solution was added. The filter was kept in the dark until the expected bands were detected. The reaction was stopped by washing the filter for 15 min with 50 ml of buffer 4.

All steps were performed at room temperature with mild shaking except further

color development.

4.2.7 Pollen viability and pollination

The putative somatic hybrids flowered most profusely during winter and early spring. Freshly collected pollen was used for the determination of the pollen stainability. Pollen of the parents and the regenerants was stained with 0.05% aniline blue (0.1N potassium phosphate) or 1% acetocarmine for 10 min. The number of stained pollen was counted. Five flowers of each plant were examined.

All flowers for self-pollination were covered with paper bags 2 to 3 days before flowering until the petals dried to prevent flowers from pollen contamination with other pollen. When the fruits set, they were kept on the plant until maturity. The seeds were cultured on a wet filter paper in a petri dish and incubated at 25°C to determine the seed viability .

4.3 Results

4.3.1 Morphology

Eighty five putative somatic hybrids were initially acclimatized and grown in the green house. However, 53 putative hybrids died and only the remaining 32 plants were maintained for later analysis.

Half of the putative somatic hybrids showed morphological abnormalities. Although these plants *in vitro* were vigorous in growth, they displayed a retarded growth and developed local necrosis under greenhouse condition. They had weak prostrate stems and ill-developed root systems. Their leaves were very thin, highly serrated and they produced no flowers. Most of these poorly growing plants failed to sustain prolonged growth (**Table 4-1** and **4-2**).

The other half of the hybrids showed more highly heterotic and vigorous growth than the parents. They also expressed some of the parent species-specific characters. The stems had prominent internodes and hairs that had near resemblance to the F_1 plant. While the anther shape, anther color, perennial growth habit, and the days to flowering were closer to that of the Sl plant. Flowers were bright yellow, more or less intermediate. Inflorescence of the F_1, Sl and putative somatic hybrids were all of cyme type; the F_1 tended to be in a single cluster, the Sl and the somatic hybrids are in double cluster. The number of flowers per cluster was 6 to 8 in F_1, 8 to 10 in Sl and 4 to 8 in putative somatic hybrids (**Table 4-1**). These plants exhibited two distinct types of leaf morphologies (**Fig. 4-1 A and B**). In one group, the leaflets were highly serrated and light green and resembled Sl. Whereas, in another group, the leaflets were dense, thick and deep green without intermediate leaf-foliation, and not so highly serrated as the former type (**Fig. 4-1 B, Table 4-2**). Flowers of the former type resembled to Sl, and the latter types were intermediate, but larger than the parental one (**Fig. 4-1** C).

Table 4-1. Morphological characters of F_1 parent, *S. lycopersicoides* parent and somatic hybrids.

Characters	F_1 (Le x Lp)	Somatic hybrid	*S. lycopersicoides*
Habit	Perennial, bushy prostrate stem	Perennial, erect stem or prostrate	Perennial, bushy shrub, erect stem
Leaf	Pinnately compound, undulate leaflets	highly serrated or not so highly serrated leaflets	Highly pinnately compoumd, dentated leaflets
Petal color	Dark yellow	Bright yellow	Bright yellow
Anther shape and color	Adnate, dark yellow	Non-adnate, ivory	Non-adnate, white
Inflorescence	Cyme, single cluster	Cyme, double clusters	Cyme, double clusters
Number of flower per cluster	6-8 flowers	4-8 flowers	8-10 flowers
Pollen fertility	Fertile	Fertile	Fertile
Fruit color and size	Brownish yellow 1.5 - 2.0 cm	Pale green 1.0 - 1.5 cm	Pale green 0.6 - 0.8 cm

Le: *L. esculentum* cv. Early pink, Lp: *L. peruvianum* var. *humifusum* LA2153.

Table 4-2.

Morphology and isozyme analysis of somatic hybrids between F1 and *S. lycopersicoides.*

Plant accession	Morphology Habit[1)]	Leaf[2)]	Isozyme[3)]
e1	E	C	PGI
e2	E	C	PGI
e3	E	B	PGI
e4	E	C	PGI
e6	E	B	PGI
e11	E	B	PGI
f	E	B	PGI
g2	E	C	ND
g3	E	C	ND
g4	E	C	ND
g5	E	C	ND
g6	E	B	PGI
C11	P	A	SKD
C12	P	A	SKD
C13	P	A	SKD
C14	P	A	SKD
C16	P	A	SKD
C18	P	A	SKD
C110	P	A	PGM
C111	P	A	PGM
C112	P	A	PGM
C113	P	A	PGM
C114	E	A	PGM
C21	P	A	PGM
C22	P	A	PGM
C24	P	A	PGM
C25	P	A	PGM
C26	P	A	PGM
C27	P	A	PGM
C31	P	A	PGM
C48	E	B	PGM
d	E	C	ND

[1)] E: erect type, P: prostrate type. [2)] A: highly serrated, thin and yellowish green color. B: highly serrated, thick and light green. C: not so highly serrated, thick, deep green color. [3)] Isozymes which represented both parental polymorphic bands. PGI: phosphoglucoisomerase, SKD: skimic dehydrogenase, PGM: phosphoglucomutase, ND: not determined.

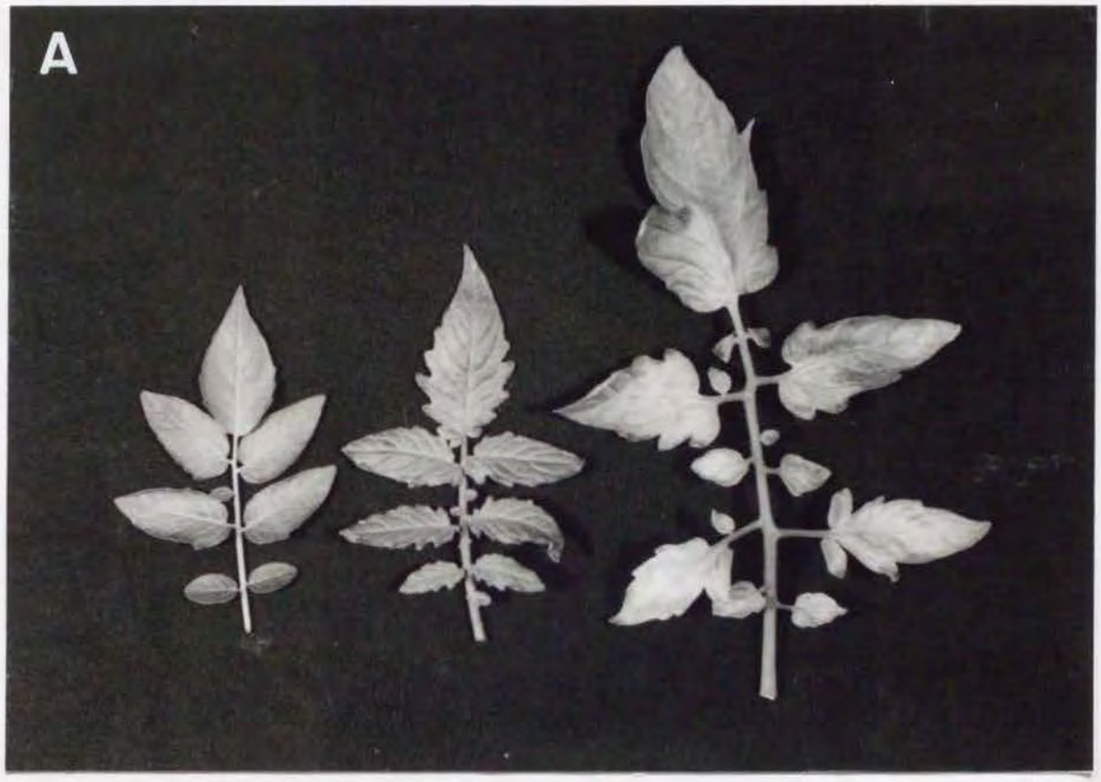
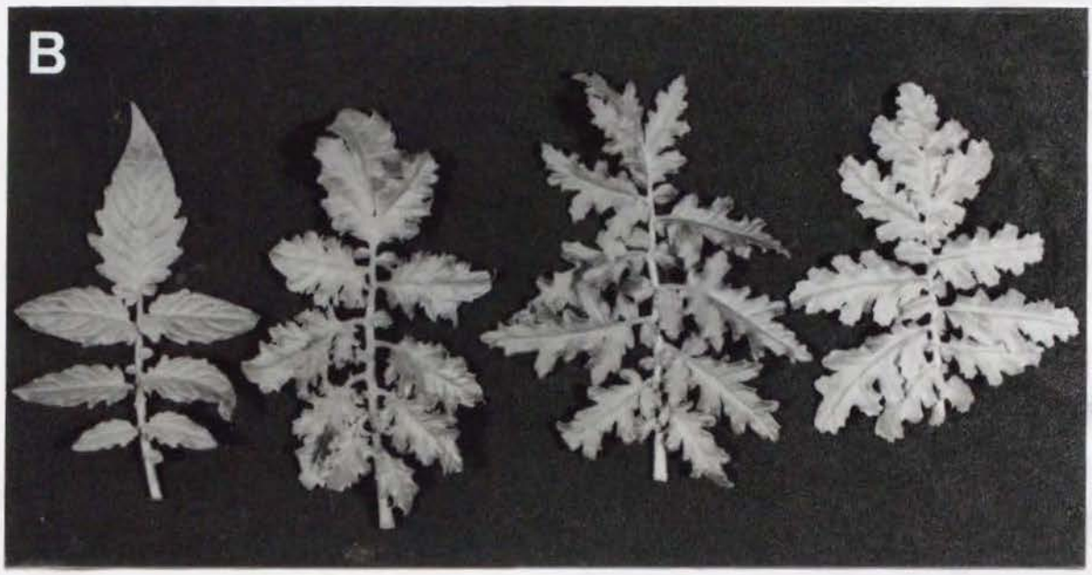


Fig. 4-1 Leaf and flower morphologies of somatic hybrids and their fusion parents. **A.** Lp: *L. peruvianun* var. *humifusum* LA2153, Le: *L. esculentum* cv. Early Pink, F1: Le x Lp and Sl: *S. lycopeprsicoides* LA2386. **B.** e2 and g6: two typical leaf morphology of somatic hybrids. **C.** Lp: *L. peruvianun* var. *humifusum* LA2153, Le: *L. esculentum* cv. Early Pink, F1 : Le x Lp, Sl: *S. lycopeprsicoides* LA2386, e2 and g6: two distinct flower morphology of somatic hybrids.



F1 e2 g6 Sl

F1 e2 g6 Sl

4.3.2 Isozyme analysis

PGI, PGM and SKD were analyzed to identify the hybridity of 32 putative somatic hybrids. The hybridity of the plants were confirmed by either electrophoretic pattern of PGI-1, PGM-2 or SKD. At first, parental electrophoretic patterns were confirmed. All three isozyme activities produced different molecular sizes of bands between F1 and Sl. However, the three forms did not show parental difference between Le and Lp. It is supposed that somatic hybrids have two specific bands derived from F1 and Sl because PGM-2 and SKD were monomers. In case of the isozyme PGI-1, somatic hybrids would probably show not only the parental homodimeric bands, but also heterodimeric bands because of dimer. Out of the 32 putative somatic hybrid tested, 28 plants possessed both the parental bands by assessment of the three isozymes (**Table 4-2**). The putative somatic hybrids detected by PGM-2 and SKD had two bands from the parents and the ones detected by PGI-1 had the novel heterodimer band in addition to the two parental bands.

4.3.3 Cytological study

Meiotic and mitotic chromosomes were investigated to determine the ploidy level and the degree of chromosomal association in the 20 somatic hybrids. Eleven of the somatic hybrid plants did not produce their flowers, hence only the root tip cells were used for the analysis. Chromosome numbesr were counted from the metaphase and anaphase-1 PMCs and the metaphase of root-tip cells (**Fig. 4-2**). Four tetraploid (2n=4x=48), 5 hexaploid (2n=6x=72), and one octoploid (2n=8x=96) somatic hybrid plants were found. Additionally, 9 hybrids were aneuploids at the tetraploid level and an aneuploid at the hexaploid level (**Table 4-3**).

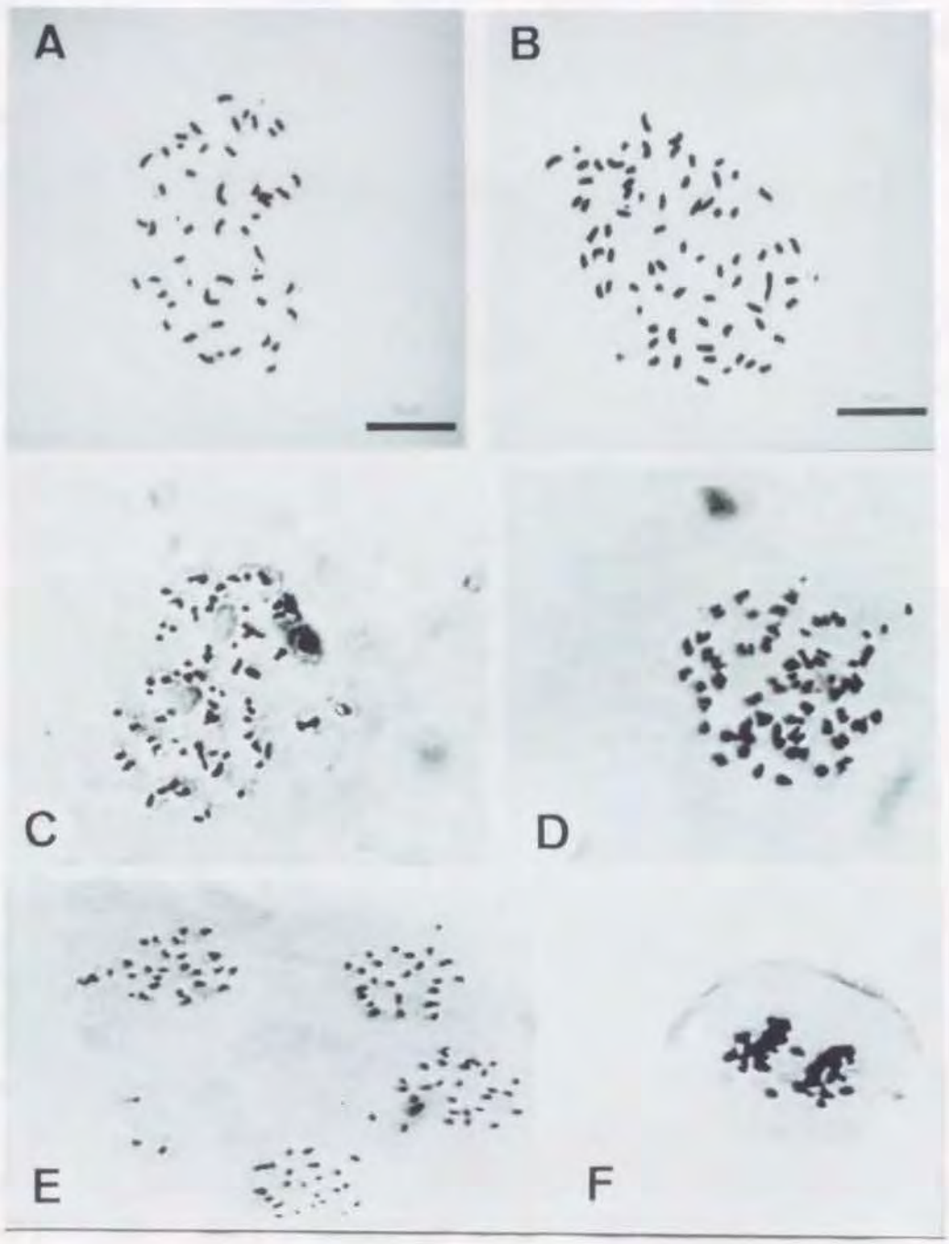


Fig. 4-2 Cytological analysis of somatic hybrids in mitotic and meiotic cell division. Chromosomes in somatic hybrids were counted at metaphase in root tip cells (**A** and **B**) and at telophase-II in PMCs (**E**). **C** and **D** show metaphase-I, and **F** shows anaphase-I in mitotic cell division. Chromosomes in **A** and **B** were stained with gimsa, in **C-F** were stained with acetocarmine solution.

Table 4-3. Cytology, morphology, chroloplast analysis and pollen fertility of the randomly selected somatic hybrid plants.

Plant accession	Leaf type[1)]	Chromosome number	Chroloplast DNA type[2)]	Pollen fertility (%)[3)]	Hybridity[4)]
e1	C	72	no data	65.3	I
e2	C	72	Le	69.5	I
e3	B	48	SL	55.8	I
e4	C	72	no data	82.0	II
e6	B	72	SL	70.0	II
e11	B	48	Le	77.4	II
f	B	48	Le	42.0	I
g3	C	96	Le	nf	II
g6	B	72	Le	46.1	I
$C_1$1	A	44	Le	nf	I
$C_1$3	A	34	no data	nf	-
$C_1$6	A	42	SL	nf	I
$C_1$8	A	38	Le	nf	I
$C_1$13	A	43	Le	nf	II
$C_2$1	A	40	Le	nf	I
$C_2$2	A	68	Le	nf	I
$C_2$4	A	35	SL	nf	II
$C_2$5	A	34	no data	nf	II
$C_2$6	A	36	no data	nf	I
$C_4$8	B	48	SL	60.0	II
L. esculentum		24		95	
L. peruvianum		24		95	
S. lycoperscoides		24		93.3	

[1)]Leaf morphology in somatic hybrids was classified into three types. A: highly serrated, thin and yellowish green color. B: highly serrated, thick and light green. C: not so highly serrated, thick, deep green color. [2)]Le: *L. esculentum*, SL: *S. lycopersicoides*. [3)] nf: no flower produced. [4)]Hybridity of somatic hybrids was assessed based on morphology, chromosome number and RAPD analysis using three primers (OPA-11, OPA-16 and OPK-13). I: three parental hybridity confirmed. II: two parents hybridity (Le+Sl or Lp+Sl) confirmed. -: hybridity not confirmed.

4.3.4 RAPD analysis

Out of the 8 primers tested, 3 primers (OPA-11, OPA-16 and OPK-13) produced predominant parental polymorphic bands (**Table 4-4, 4-5** and **Fig. 4-3**). Eleven out of the 20 selected somatic hybrids clearly had at least more than one band in each of the 3 kinds of parental specific bands generated by these three primers (**Table 4-5**). The three parental hybridity of the other 9 plants was not ascertained because of the disappearance of parental bands or the appearance of unique banding patterns in the RAPD profiles. Nevertheless, 7 of these 9 plants could be confirmed as hybrids between the F_1 and Sl parents. Hence, eighteen of the 20 putative somatic hybrids tested were thought to be true somatic hybrids on the basis of the RAPD profiles (**Table 4-5**).

4.3.5 Chroloplast DNA analysis

The cpDNA type of the somatic hybrids was determined by Southern hybridization using tobacco cpDNA as probe. For the determination of parental RFLPs, total DNAs from Le and Sl were digested with 8 restriction enzymes *Eco*RI, *Hind*III, *Kpn*I, *Xho*I *Sal*I, *Pst*I and *Hae*III, electrophoresed and subsequently blotted. Three combinations of probe-restriction enzyme provided reliable RFLP patterns. When the pTBa1 probe (19.6 kb *Bam*HI fragment) were hybridized to *Hae*III-digested DNA, 1.0kb of Le, 2.0kb of Sl specific RFLP fragments were recognized (**Fig. 4-4-1**). When pTB 13 (10.4 kb *Bam*HI fragment) probe was applied, *Kpn*I and *Hae*III provided reliable RFLP patterns. The pTB 13 and *Hae*III combination revealed useful RFLPs: 2.09 kb of a Le specific fragment and 0.82 kb of a Sl specific fragment (**Fig. 4-4-2 A**). Using the pTB 13 and *Kpn*I combination, the Le parent showed three specific bands of 5.9, 5.0 and 2.2 kb, and the Sl parent showed three specific bands of 6.8, 5.8 and 2.3 kb (**Fig. 4-4-2 B**). The 20 somatic hybrids were analyzed according to the above probe-restriction enzyme combinations. Consequently, somatic hybrids contained bands specific of either Le or Sl.

Table 4-4. Sequence of 10- base random primers.

Primer code	5' to 3'
OPA-11	CAATCGCCGT
OPA-16	AGCCAGCGAA
OPK-13	GGTTGTACCC

Table 4-5. Appearance of parent-specific RAPD fragments generated by three random primers in the somatic hybrids

Primers	OPA-11		OPA-16	OPK-13	
Plant accession	Lp (kb)	Sl (kb)	Le (kb)	Lp (kb)	Sl (kb)
e1	0.61	0.32, 0.27	0.80	0.54	1.50
e2	0.61	0.32, 0.27	0.80	0.54	1.50
e3	0.61	0.32, 0.27	0.80	0.54	1.50
e4	-	0.67	0.80	-	1.50
e6	0.61	-	-	0.54	1.50
e11	0.61	0.32, 0.27	-	0.54	1.50
f	0.61	0.32, 0.27	0.80	0.54	1.50
g3	-	0.32, 0.27	-	0.54	1.50
g6	0.61	-	0.80	0.54	1.50
$C_1$1	-	0.67	0.80	0.54	1.50
$C_1$3	-	0.67	-	no data	no data
$C_1$6	-	0.67	0.80	0.54	1.50
$C_1$8	-	0.67	0.80	0.54	1.50
$C_1$13	no data	no data	no data	0.54	1.50
$C_2$1	-	0.67	0.80	0.54	1.50
$C_2$2	-	0.67	0.80	0.54	1.50
C24	no data	no data	0.80	-	1.50
$C_2$5	-	0.67	0.80	-	1.50
$C_2$6	-	0.67	0.80	0.54	1.50
$C_4$8	no data	no data	0.80	no data	no data

Le: *L. esculentum*, Lp: *L. peruvianum*, Sl: *S. lycopersicoides*.

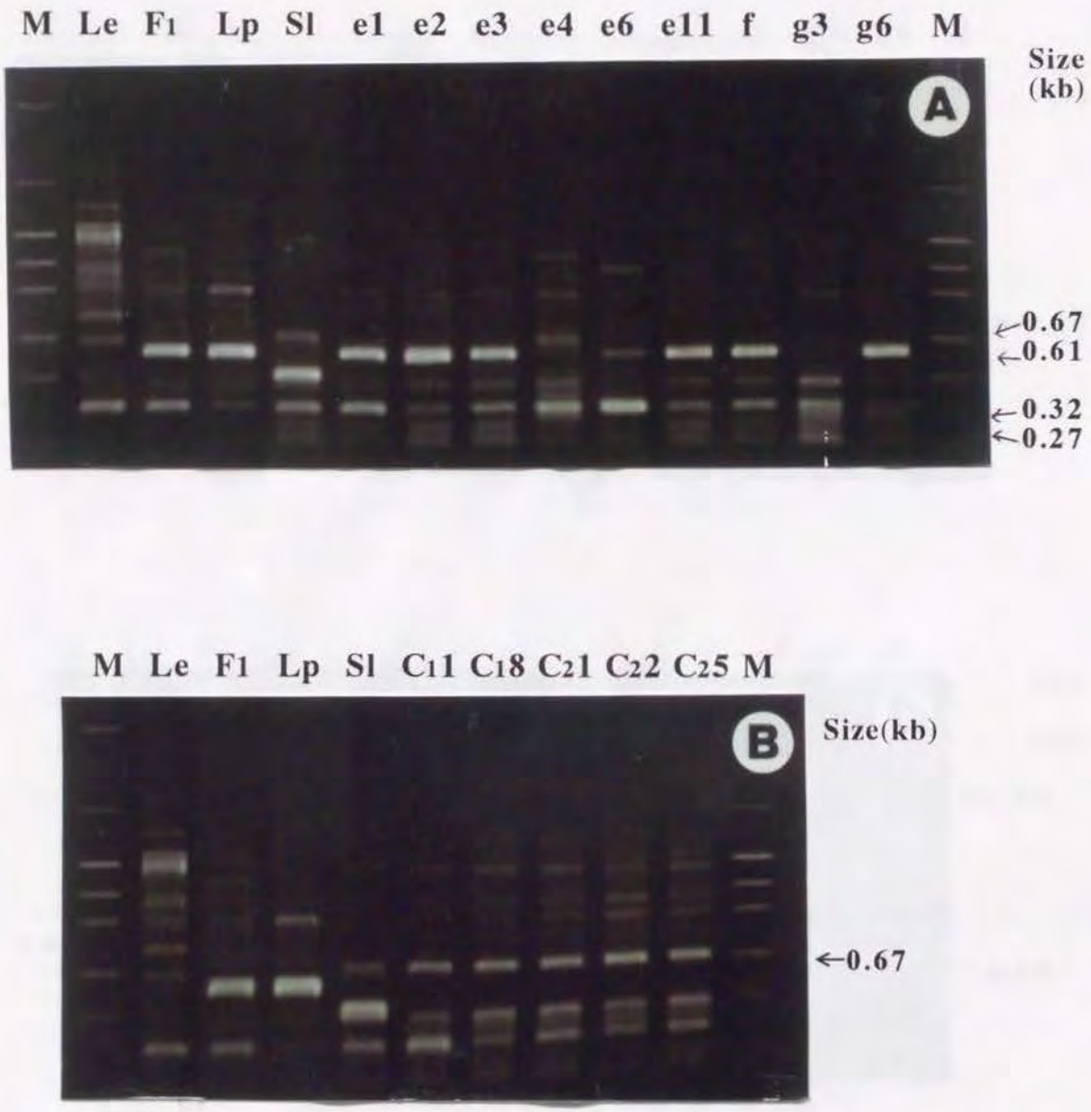


Fig. 4-3-1 RAPD profiles of *L. esculentum* (Le), *L. peruvianum* (Lp), sexual hybrid of Le and Lp (F_1), *S. lycopersicoides* (Sl) and somatic hybrids between F_1 and Sl. PHY marker (M). Arrows indicate species- specific fragments. **A:** PCR fragments amplified by OPA-11. e1-g6: somatic hybrids. **B:** PCR fragments amplified by OPA-11. C_11 -C_25: somatic hybrids.

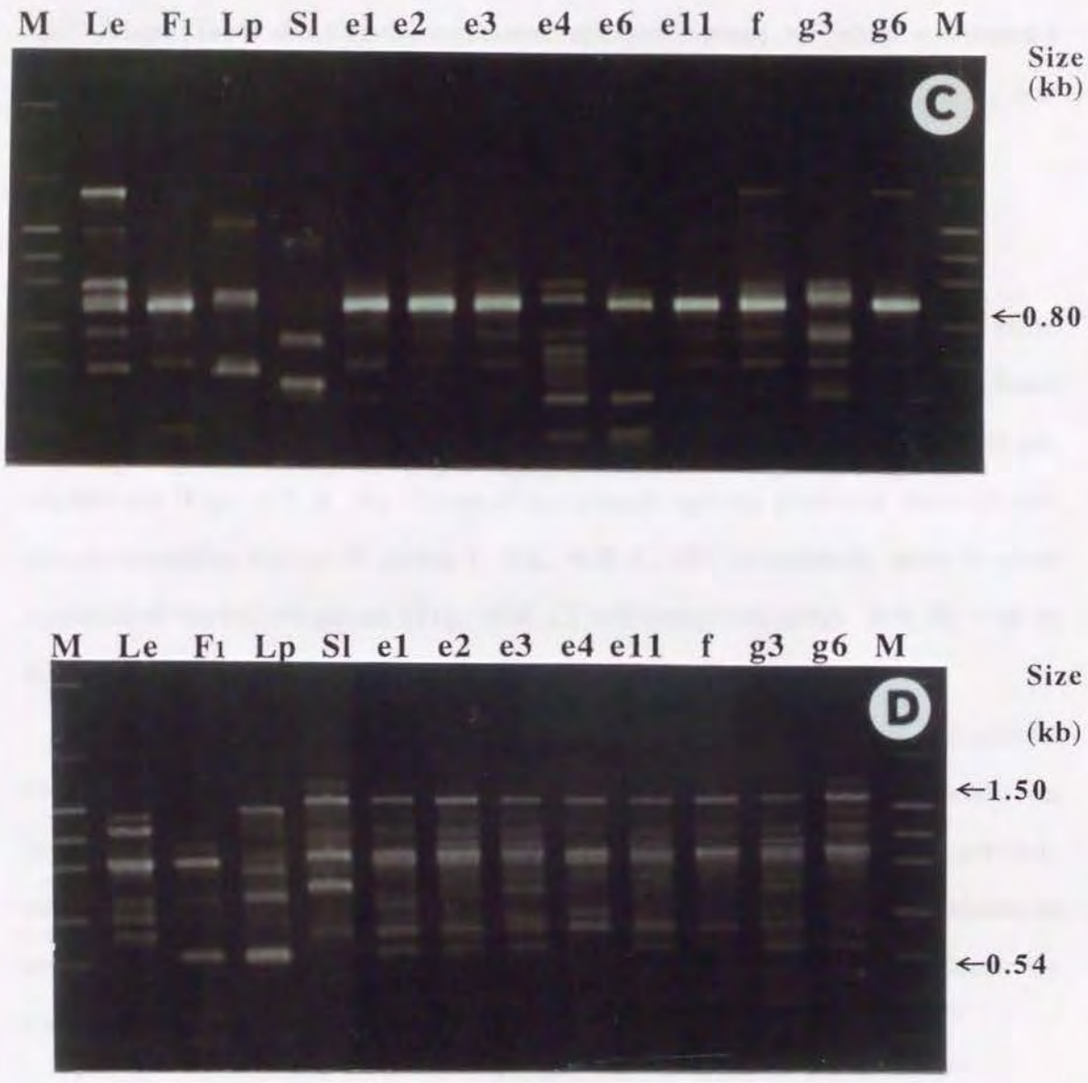


Fig. 4-3-2 RAPD profiles of *L. esculentum* (Le), *L. peruvianum* (Lp), sexual hybrid of Le and Lp (F_1), *S. lycopersicoides* (Sl) and somatic hybrids between F_1 and Sl. PHY marker (M). Arrows indicate species- specific fragments. **C:** PCR fragments amplified by OPA-16. e1-g6: somatic hybrids. **D:** PCR fragments amplified by OPK-13. e1-g6: somatic hybrids.

Thirteen plants out of the 20 somatic hybrids plants produced, except for 2 plants which were not hybridized with the probe, possessed the Le cpDNA and 5 plants had the Sl cpDNA type (**Table 4-3**). In these enzyme/probe combinations, no hybrid was found to contain a combination of some cpDNA fragments from both parents nor new, non-parental fragments.

4.3.6 Pollen viability and fruits production

Out of the 20 hybrid plants, 9 plants produced pollen in the greenhouse. Fertile pollen was observed and their viability varied with the accessions, i.e. 42.0 to 82.0 % (**Table 4-3**). Pollen sizes of the Sl parent and the F_1 parent were about 30 μm and 20 μm, respectively (**Fig. 4-5 A, B**). Those of the somatic hybrids measured about 28 μm, almost resembled that of Sl parent (**Fig. 4-5 C, D**). Additionally these 9 plants consisted of euploic tetraploids (**Fig. 4-5 C**) and hexaploids (**Fig. 4-5 D**) with no difference in the size of their pollens.

Eight of the 9 hybrids developed fruits and 7 out of them produced matured seeds upon self pollination (**Table 4-6** and **Fig. 4-6**). The level of seed production was poor and only plant e2 produced fruits which contained an average of 2 seeds per fruit, while fruites of the other 6 hybrids contained only one seed or some fruits produced no seed at all. The germination ability of the seeds was between 33. 3 to 46. 2% without any rescue treatments (**Table 4-6**).

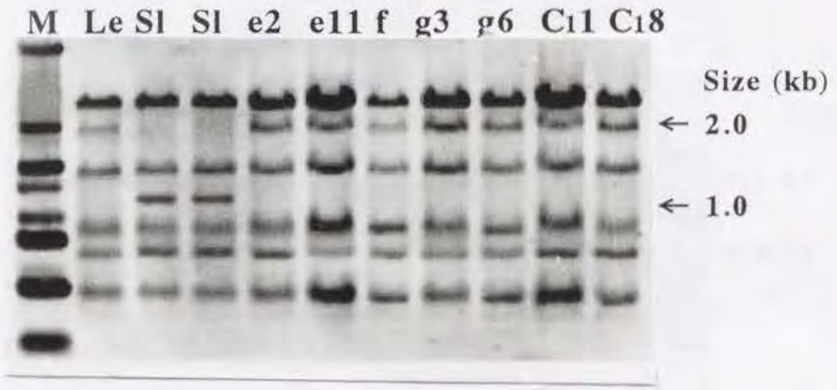


Fig. 4-4-1 Chloroplast inheritance by Southern blot analysis. Total DNA of the somatic hybrids and their parents digested with *Hae* III were hybridized with cpDNA probe (pTB a1) derived from tobacco. Le: *L. esculentum* cv. Early Pink, Sl: *S. lycopersicoides* LA2386. e2-$C_1$8 : somatic hybrids. M: size marker.

Fig. 4-4-2 Chloroplast inheritance by Southern blot analysis. Total DNA of the somatic hybrids and their parents were digested with *Hae* III (**A**) and *Kpn* I (**B**), and hybridized with cpDNA probe (pTB13) derived from tobacco. Le: *L. esculentum* cv. Early Pink, Sl: *S. lycopersicoides* LA2386, e2- $C_1$8 and e3-g3 : somatic hybrids.

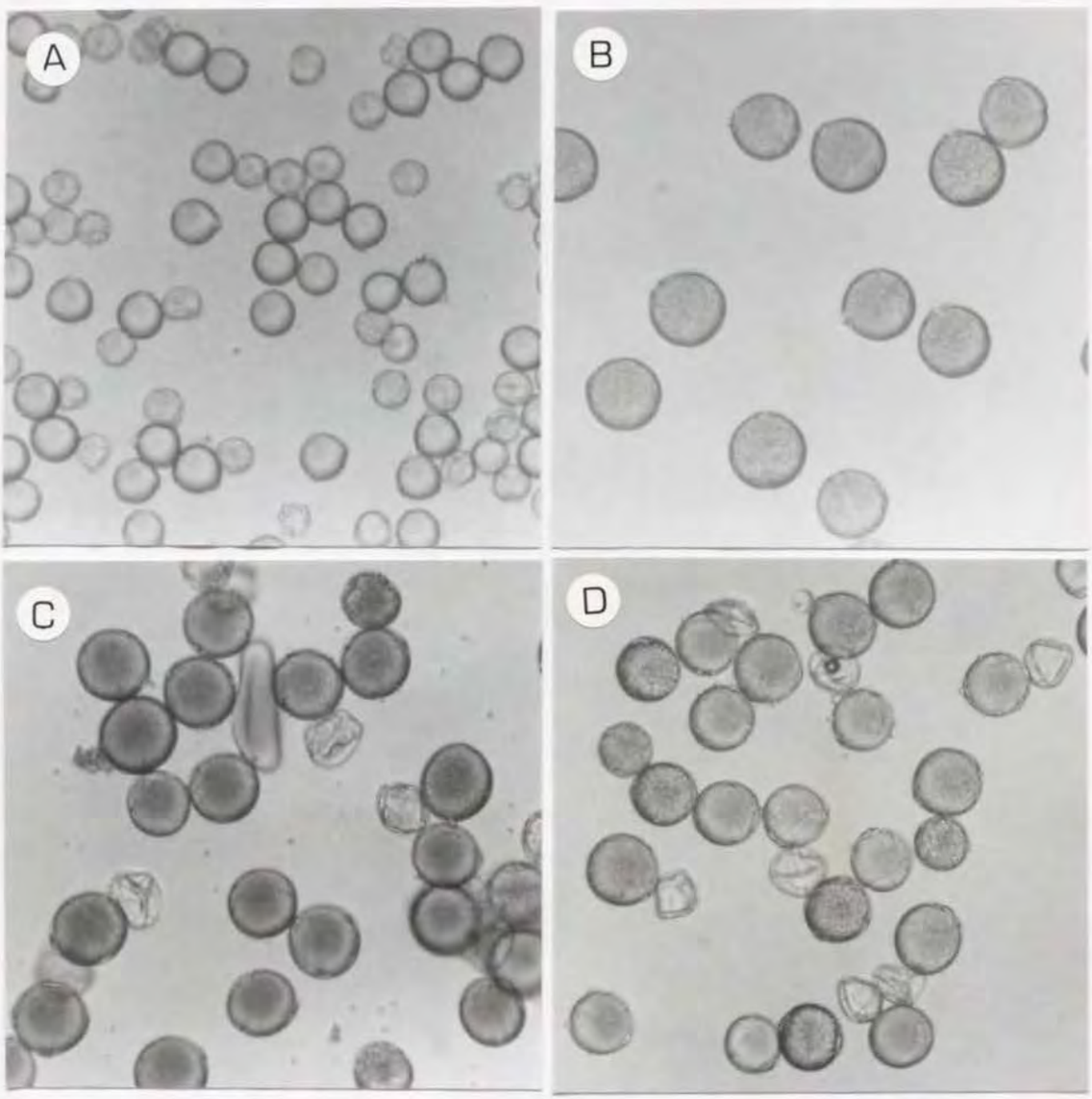


Fig. 4-5 Pollen stainability of somatic hybrids and their fusion parents. **A.** F1 (*L. esculentum* x *L. persuvianum*), **B.** *S. lycopersicoides,* **C.** Somatic hybrid (e3: chromosome number= 48), **D.** Somatic hybrid (e11: chromosome number= 72).

Table 4-6. The production of fruits and seeds of somatic hybrids.

Plant accession	Fruits set[1] (%)	No. of seeds/fruit	Seed germination(%)[2]
e1	0	0	0
e2	43.3	2.3	46.2
e3	58.8	0.43	46.2
e4	43.6	0.71	33.3
e6	19.2	0.53	40.0
e11	40.0	0.88	37.5
f	51.6	0.03	0
g6	66.7	0.04	0
$C_4$8	16.7	0	0

[1] The total number of fruits per the total number of flower.

[2]The total number of germinated seed per the total number of seed sown.

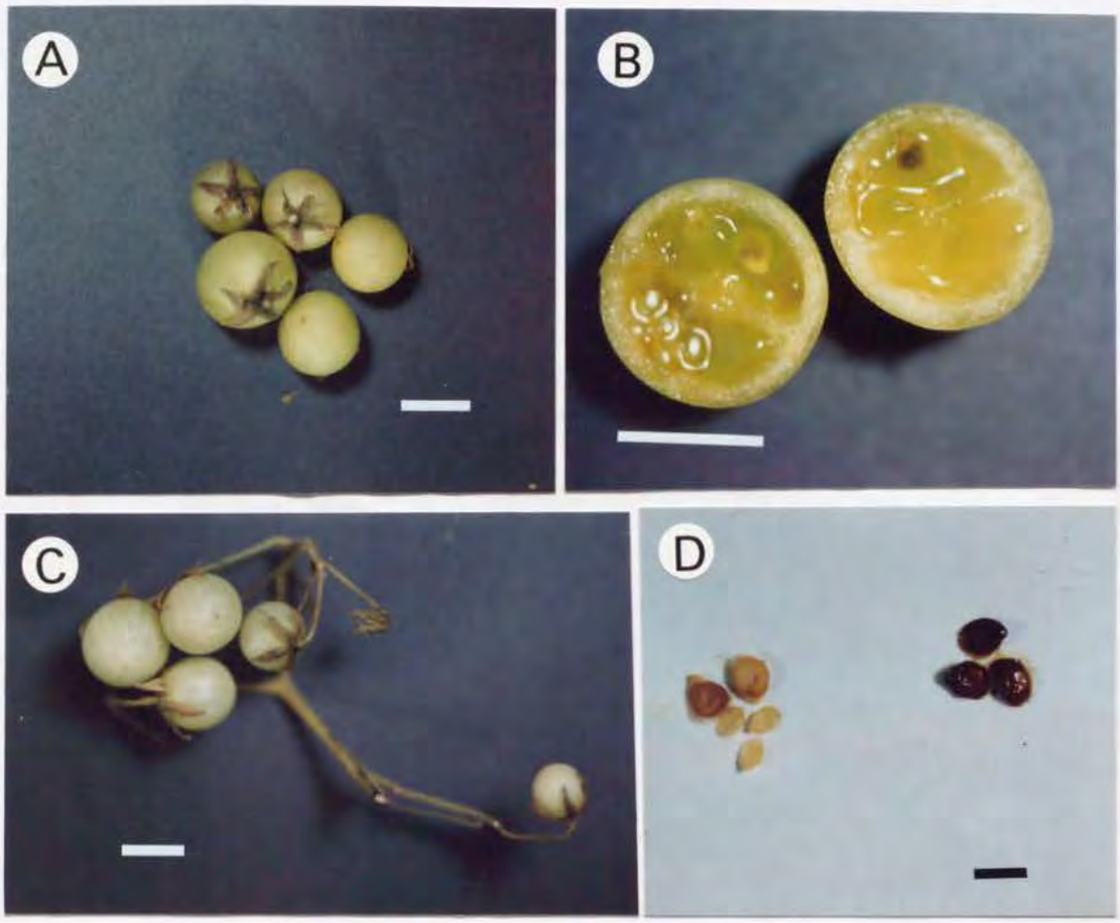


Fig. 4-6 Fruits and seeds of somatic hybrid between F_1 and *S. lycopersicoides*. **A:** Fruits derived from self pollination of somatic hybrid(f) (bar=1cm). **B**: Sectional plane of a fruit derived from self-pollination of a somatic hybrid (e11) (bar=1cm). **C**: Fruits derived from self pollination of somatic hybrid (e11) (bar=1cm). **D**: Open-pollinated seeds of *S. lycopersicoides* LA2386 (left side) and self-pollinated seeds of a somatic hybrid (e11) (right side) (bar=1mm).

4.4 Discussion

The hybridity of the putative somatic hybrids could be easily determined based on the morphological characters. Several species-specific morphological markers were scored in the current study. F_1 and Sl parental specific and their intermediate characters were confirmed in the putative somatic hybrids. However, the morphology of the somatic hybrids between Le and Sl primarily resembled that of Sl, the same observations were also reported by Handley *et al.* (1986) and Hossain *et al.* (1994). This result suggested that the morphological traits of Sl had strong tendency to be expressed in hybrids even in sexual hybrids obtained by Rick (1951).

All the 32 plants were considered to be true somatic hybrids on the basis of the morphological characterization. However, observation of the phenotypes alone could be less accurate because somaclonal variation induced during *in vitro* culture could account for the observed difference in phenotypes (See review, Larkin and Scowcroft 1981). In tomato, Gavazzi *et al.* (1987) reported that the frequency of somaclonal variation via cell culture was higher than chemically induced mutagenesis.

Allozyme polymorphisms have been proved as a highly useful tool for measuring the extent of variation within and between the species (See review, Doebley 1989) and often been used to confirm the hybrid nature of regenerated plants. Gekelor (1984) demonstrated the feasibility of allozyme screening in the fusion products obtained through somatic hybridization of *Solanum tuberosum* and *L. esculentum*. Three isozymes such as PGM, PGI and SKD were used to analyze the allozyme polymorphisms in the current study. Handley *et al.* (1986) applied PGM polymorphism to detect the hybridity of the fusion products between *L. esculentum* and *S. lycopersicoides*. Another two isozymes, PGI and SKD also gave feasible polymorphisms for hybridity identification according to Guri *et al.* (1991) and McCabe *et al.* (1993). Moreover, isozyme activity proves existence of a certain chromosomes. The map position of each isozyme gene was determined, for instance, PGI-1 was mapped to chromosome 12 (Vallejos and Tanksley

1983), PGM-2 was mapped to chromosome 4 (Tanksley 1979) and SKD was mapped to chromosome 1 (Tanksley and Rick 1980) in tomato. Therefore, if the hybridity of the somatic hybrids was identified according to allozyme polymorphisms, those plants probably possess chromosomes from both parents possibly found around the isozyme locus.

Based on the morphological characters and isozyme analysis, all the plants were initially considered as true somatic hybrids. However, twenty somatic hybrids were randomly selected for further analysis.

The construction of the RAPD profile is one of the DNA-based molecular markers used for hybridity identification in somatic hybrids in recent years. Williams *et al.* (1990) reported the application of PCR technique with 10 base random primer to generate RAPD markers. It is considered that RAPD overcomes some of the limitations of RFLPs. For instance, it promises to be inexpensive and requires a small amount of tissue. Moreover, the procedure is very simple and fast (Takemori *et al.* 1994, Rokka *et al.* 1994).

The features of RAPD allow the early-stage selection of somatic hybrids (Baird *et al.* 1992, Takemori *et al.* 1994, Rokka *et al.* 1994, Hossain *et al.* 1994). However, there has been no report with RAPD of the hybridity determination in somatic hybrids which involved three parental genomes. Species-specific bands in our fusion combination were obtained in the Le, Lp, and Sl parents using 3 random primers, indicating that the RAPD markers could be used to confirm the presence of the three parental genomes in the somatic hybrids. One random primer revealed the Le-, Lp-, and Sl-specific RAPD markers. In the current study, there were a few cases in which the size of some polymorphic bands were so close or signal of bands was so light that they were inapplicable to use. The amplification of some parental specific bands by only one random primer could be possible if many primers were screened in the prersent study.

The three parental genomes produced by the three primers were exhibited in

more than half of the somatic hybrids. However, it seems that some RAPD fragments have not been amplified in some somatic hybrid plants. Further investigation using more primers would add stronger evidence into the analysis. The absence of some amplified RAPD markers in somatic hybrid plants has been reported (Xu *et al.* 1993, Takemori *et al.* 1994, Hossain *et al.* 1994, Kobayashi *et al.* 1996). It has been suggested that this phenomenon was related to somaclonal variation as a result of the inactivation of organellar genomes (Sidorov *et al.* 1981) and variation in chromosome number (Karp 1991). Variability in the number of chromosomes might be associated with chromosome elimination (more than half the number of somatic hybrids tested were aneuploid in the tetraploid level) as a consequence of lagging chromosomes at telophase and incomplete chromosome pairing at metaphase. Probably another reasons could be added to explain the phenomenon.

The determination of cpDNA composition of the somatic hybrids is one of the important objects in somatic hybridization program because of cpDNA segregation in the somatic hybrids was not possible in sexual hybrids. There are two known cpDNA transmission phenomena; (1) random transmission and (2) non-random transmission. It has been observed that somatic hybrids derived from symmetric protoplast fusion follows a random transmission of cpDNA, while somatic hybrid from asymmetric fusion follows the non-random manner. Bonnema *et al.* (1992) observed non-random delivery of cpDNA in the asymmetric somatic hybrids between tomato and *L. pennellii,* but a random transmission in the symmetric hybrids of the same combination. In somatic hybrid between tomato and *L. peruvianum,* the symmetric fusion provided the random transmission of cpDNA (San *et al.* 1990). However, Levi *et al.* (1988) reported non-random transmission of cpDNA in the somatic hybrids between tomato and *S. lycopersicoides* which was obtained by symmetric fusion. The cpDNA in almost all hybrids was preferentially inherited from tomato. This suggests the predominance of tomato protoplasts in the heterokaryon. Jordan *et al.* (1993) also reported non-random

transmission of cpDNA in somatic hybrids between tomato and *L. hirsutum*, wherein all somatic hybrids inherited the cpDNA of *L. hirsutum*. In the present study, all symmetric hybrid plants, a total of 18 being analyzed, showed either the Le pattern or the Sl pattern in a random manner although the number of hybrids with Le cpDNA was more than the number with Sl cpDNA. The result agrees well with the other symmetric fusion reports (Levi *et al.* 1988, Li and Sink 1992). When both parents in the fusion originated from mesophyll cells like our work, equal distribution of plastids among the somatic hybrids were observed. On the other hand, we observed no association between the cpDNA type and the ploidy level and could not regard Le protoplast as predominant over Sl in the somatic hybrids. Consequently, it might be considered that transmission of cpDNA to somatic hybrids was controlled by not only the fusion system but also the original of the fusion materials.

Recombination and/or rearrangement of the chloroplast genome was not observed in a number of somatic hybrids (Ratushnyak *et al.* 1993, Han San *et al.* 1990, Bonnema *et al.* 1992). Our current finding also supports the previous reports. However, we could not expect the possibility of cpDNA recombination in our hybrids because only 2 cpDNA probes of tobacco namely: pTBa1 and pTB13 were used. A few reports are available on cpDNA recombination (O'Connell and Hanson 1985, Derks *et al.* 1991, Wolters *et al.* 1995), though the frequency is low.

In the present study, 9 somatic hybrid plants were obtained that produced fertile pollen. Gavrilenko *et al.* (1992) suggested that the sterility in somatic hybrids was due to aneuploidy, while fertjlity in euploid hybrids was due to its regular meiotic activities. Kirkham (1981) and Parokonny *et al.* (1997) reported that the homoeology between the chromosomes of *L. esculentum* and *L. peruvianum* was close and their chromosomes showed normal bivalent pairing, even in the hexaploid hybrids (*L. esculentum* x *L. peruvianum* doubled with colchicin). Therefore, it was likely that chromosome pairing in our tetraploid and hexaploid somatic hybrids in the present study were comparatively

normal and this could be one of the reasons which have contributed to the production of pollen fertile somatic hybrids. On the other hand, Rick (1951) and Menzel (1962) reported that despite the near complete chromosome pairing of F_1 hybrids between *L. esculentum* and *S. lycopersicoides* at meiotic pachytene, the pollen of the hybrids were mostly sterile. Escalante *et al.* (1997) indicated that there were remarkable differences in the chromosome sizes between *L. esculentum* and *S. lycopersicoides* in the mitosis by using GISH analysis. It is considered that triparental amphidiploid-like somatic hybrid plants were developed in the fertile somatic hybrids in the present study.

Somatic hybrids involving three parental species such as *Lycopersicon* and its relation species has been reported (Guri *et al*. 1991, McCabe *et al.* 1993, Gavrilenko *et al.* 1992). Hybrids obtained through symmetric and asymmetric somatic hybridization between F_1(*L. esculentum* x *L. pennellii*) and *S. lycopersicoides* retained only low viable pollens (Guri *et al* . 1991, McCabe *et al.* 1993). In contrast, somatic hybrids obtained in the present study has a comparatively high fertility and self pollinated fruits contained normal viable seeds.