GENERAL CONCLUSION

Somatic hybridization is one of the promising tools to overcome crossability problems and it has possibility to introduce useful genes of wild species to commercial plants. Although several genes which are preserved in two species, *L. peruvianum* var. *humifusum* (LA2153) and *S. lycopersicoides* (LA2386) are interesting for tomato breeders, these species are also involved in crossability problems. Therefore, somatic hybridization was carried out between F1 (*L. esculentum* x *L. peruvianum*) and *S. lycopersicoides* by electrofusion. As a result, the plants which consist of new organelles and genomes in combination were expected to be obtainable.

Firstly, the regeneration system from F1 (*L. esculentum* x *L. peruvianum*) mesophyll protoplasts was optimized. Especially, enzyme combinations for protoplast isolation, basic media and combinations of plant growth regulators for protoplast and plant regeneration cultures were investigated (CHAPTER 2). Secondly, many putative somatic hybrids between F1 (*L. esculentum* x *L. peruvianum*) and *S. lycopersicoides* were obtained by symmetric electrofusion of mesophyll protoplasts (CHAPTER 3). The procedures were able to produce plenty of calli which readily regenerated into plants. According to the previous report (Hossain *et al.* 1994), adventitious shoots of *S. lycopersicoides* protoplasts were regenerated within 3 months of initial plating. The term up to the shoot bud regeneration from fused protoplasts was approximately 20 days which were considered to be shorter than those from F1 protoplasts.

Eventually, putative somatic hybrid plants were analyzed by several methods (CHAPTER 4). The hybrid nature of those plants was evaluated according to the morphological characteristics and isozyme analysis. Consequently, all regenerated plants were probable to be somatic hybrids. However, the hybridity of the regenerated plants including three parental genomes was not confirmed by PGI, PGM and SKD, because those isozyme patterns could not show any differences between *L. esculentum* and *L.*

peruvianum. Bernatzky and Tanksley (1986) suggested such a limitation due to fewer polymorphisms. RAPD method is convenient and reliable for determination of somatic hybrids (Barid *et al.* 1992, Xu *et al.* 1993). 10-mer random primers have a number of amplification sites on genomic DNA, which would produce many polymorphisms among inter- or intraspecies. The nuclear genomes in the 20 somatic hybrid plants randomly selected contained three parental specific RAPD markers. Moreover, cytological study added another evidence that regenerated plants were somatic hybrids. Half of the plants analyzed were aneuploid at tetraploid level and another half were euploid at hexaploid, tetraploid or octoploid level.

In conclusion, all of the 20 randomly selected somatic hybrids were inferred as true somatic hybrids, even if they did not retain the whole triparental genomes as revealed by the molecular analysis. If so, why the heterokaryons were effectively selected? Although the unfused and self-fused protoplasts of *S. lycopersicoides* were inhibited to divide in MTM2 medium (Hossain *et al.* 1994), the unfused and self-fused protoplasts of F_1 plant should not be eliminated in this selection system. Nevertheless, none of the 20 hybrids analyzed resembled the F_1 parent. Some lines belonging to the *peruvianum*-complex have higher regeneration capacity, and this desirable trait has been introduced into tomato from *L. peruvianum* (Koornneef *et al.* 1987). *L. peruvianum* var. *humifusum* LA2153 also possesses the same traits (Chen and Imanishi 1991). The present study confirmed that the protoplasts of its F_1 plants had rapid shoot regeneration capacity in *in vitro* culture in CHAPTER 2. Additionally, it is inferred that the colonies derived from unfused and self-fused F_1 protoplasts were not selectively collected since the somatic hybrid colonies were easily detected to have higher hybrid vigor than the F_1 protoplasts-derived ones. Handley *et al.* (1986) also suggested that somatic hybrid cells between tomato and *S. lycopersicoides* grew at least twice as rapidly as *S. lycopersicoides* and even more than the tomato. It is probable that the hybrids exhibited a heterotic response in culture and exceeded the parents.

Somatic hybrid nature was determined using Southern hybridization. The chloroplast genotype was analyzed using tobacco chloroplast DNA probes and species-specific RFLPs. Out of 15 somatic hybrid plants, 10 plants inherited tomato chloroplast DNA and 5 plants *S. lycopersicoides* one, which supports random transmission of chloroplasts into somatic hybrids. Chloroplast in tomato is normally controlled by maternal inheritance. It means that sexual hybrids which have been successfully obtained using tomato as pistillate parent possess only tomato chloroplasts. Hence, somatic hybrids with *S. lycopersicoides* chloroplasts in the present study could be especially precious material plants for tomato breeding. Mitochondria DNA (mt DNA) type in these plants has not been analysed yet. While, in contrast with chloroplast DNA, rearrangement events in mt DNA were reported in numerous somatic hybrids. Therefore, somatic hybrids in the present study would also be expected as variable plants for tomato breeding in terms of organelle genotypes.

Thus, the present study demonstrated that the somatic hybrids between *L. esculentum* x *L. peruvianum* and *S. lycopersicoides* were able to be obtained easily and effectively.

A large number of studies have been attempted to produce interspecific and intergeneric hybrids between *L. esculentum* and the related wild species, which aimed to introduce the valuable genes of the wild species into cultivated tomatoes, *L. esculentum*. The highest breeding barriers in terms of cross-incompatibility have severely restricted access by tomato geneticists and breeders to *L. peruvianum*, especially *L. peruvianum* var. *humifusum*, and *S. lycopersicoides*. Therefore, it is very interesting and exciting that the somatic hybridization of the present study has incorporated the genomes derived from both the wild species and the cultivated tomato into a novel somatic hybrid plant at the same time. In addition, those plants have sufficient pollen fertility. These triparental genome plants created will be expected to have their progeny by backcrossing to tomato or self-pollination in future work.

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CHAPTER 4

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GENERAL CONCLUSION

Baird, E., S. Cooper-Bland, R. Wangh, M. DeMaione and W. Powell (1992) Molecular characterization of inter- and intra-specific somatic hybrids of potato using randomly amplified polymorphic DNA (RAPD) markers.Mol. Gen. Genet. 233: 469-475.

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APPENDIX A-1

CPW salts (mg/l)	
$MgSO_4$ $7H_2O$	246
KH_2PO_4	27.2
$CaCl_2$ $2H_2O$	1480
KI	0.16
$CuSO_4$ $5H_2O$	0.025
pH	5.6-5.8

APPENDIX A-2

Organic components of B5 medium (mg/l)	
Myo-inositol	100
Nicotinic acid	1
Thiamine HCl	1
Sucrose	20000

APPENDIX A-3

MS culture medium formulation

(1) Major salts (mg/l)	
KNO_3	1900
NH_4NO_3	1650
$MgSO_4\ 7H_2O$	370
KH_2PO_4	70
$CaCl_2\ 2H_2O$	440
(2) Iron and minor elements (mg/l)	
$MnSO_4 4H_2O$	4
H_3BO_3	6
$ZnSO_4 7H_2O$	9
$FeSO_4 7H_2O$	28
Na_2EDTA	37
KI	0.83
$CuSO_4\ 5H_2O$	0.025
$NaMoO_4\ 2H_2O$	0.25
$CoCl_2\ 6H_2O$	0.025
(3) Organic components (mg/l)	
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glysine	2
Inositol	100
Sucrose	30000
(4) pH-value	5.8

APPENDIX A-4

8E culture medium formulation

(1) Major salts (mg/l)	
KNO_3	1900
$MgSO_4\ 7H_2O$	370
KH_2PO_4	70
$CaCl_2\ 2H_2O$	440
(2) Iron and minor elements (mg/l)	
$MnSO_4 4H_2O$	22
H_3BO_3	6
$ZnSO_4 7H_2O$	9
$FeSO_4 7H_2O$	28
Na_2EDTA	37
KI	0.83
$CuSO_4\ 5H_2O$	0.025
$NaMoO_4\ 2H_2O$	0.25
$CoCl_2\ 6H_2O$	0.025
(3) Organic components (mg/l)	
Thiamine HCl	2
Nicotinic acid	0.5
Casein hydlysate	20
Glysine	2
Inositol	1000
Sucrose	10000
Glucose	5000
Mannitol	75000
(4) pH-value	5. 8

APPENDIX A-5

TM-2 culture medium

(1) Major salts (mg/l)		Pyridoxine HCl	1
KNO_3	500	Folic acid	0.5
$MgSO_4\ 7H_2O$	370	Biotin	0.05
KH_2PO_4	170	D-Ca-pantothenate	0.5
$CaCl_2\ 2H_2O$	440	Choline chlolide	0.1
(2) Iron and minor elements (mg/l)		Glycine	0.5
$MnSO_4 4H_2O$	22	L-Cysteine	1
H_3BO_3	6	Malic acid	10
$ZnSO_4 7H_2O$	9	Ascorbic acid	0.5
$FeSO_4 7H_2O$	14	Riboflavin	0.25
Na_2EDTA	18.5	Casein hydlysate	150
KI	0.83	Adenine sulfate	40
$CuSO_4\ 5H_2O$	0.025	L-Glutamine	100
$NaMoO_4\ 2H_2O$	0.25	Myo-inositol	4600
$CoCl_2\ 6H_2O$	0.025	Xylitol	3800
(3) Organic components (mg/l)		Sorbitol	4560
Thiamine HCl	10	MES	97.6
Nicotinic acid	2.5	(4) pH-value	5.7

APPENDIX A-6

TM-3 culture medium

(1) Major salts (mg/l)		(3) Organic components (mg/l)	
KNO_3	1500	Thiamine HCl	0.5
$MgSO_4\ 7H_2O$	370	Nicotinic acid	5
KH_2PO_4	170	Folic acid	0.5
$CaC_2\ 2H_2O$	440	Pyridoxine HCl	0.5
(2) Iron and minor elements (mg/l)		Biotin	0.05
$MnSO_4 4H_2O$	22	Choline chlolide	0.1
H_3BO_3	6	Riboflavin	0.25
$ZnSO_4 7H_2O$	9	Casein hydlysate	100
$FeSO_4 7H_2O$	14	Adenine sulfate	40
Na_2EDTA	18.5	L-Glutamine	100
KI	0.83	Myo-inositol	100
$CuSO_4\ 5H_2O$	0.025	MES	97.6
$NaMoO_4\ 2H_2O$	0.25	Sucrose	50000
$CoCl_2\ 6H_2O$	0.02	(4) pH-value	5.75

APPENDIX B

W5 solution (g/l)	
NaCl	9.0
$CaCl_2$ $2H_2O$	18.375
KCl	0.37
Glucose	0.9
pH 5.6	

(Menzel and Wolfe 1984)

APPENDIX C-1

Isozyme and buffer combinations

Isozyme	Electrode buffer		Gel buffer	
PGI	H_3BO_3	1.85%	Tris	1.64%
	NaOH	0.34%	Citrate	0.36%
	pH 8.5		pH 8.5	
PGM	H_3BO_3	1.85%	Tris	1.64%
	NaOH	0.34%	Citrate	0.36%
	pH 8.5		pH 8.5	
SKDH	Tris	0.92%	Tris	0.09%
	Citrate	0.11%	Citrate	0.07%
	pH 8.6		pH 7.2	

APPENDIX C-2

Buffer solution for enzyme staining.			
B-B buffer:	Tris - HCl	0.076%	pH 8.0
Tris- buffer:	Tris - HCl	1.21%	pH 7.5

APPENDIX C-3

Solution for isozyme staining

PGI	
$MgCl_2 4H_2O$	240 mg
D-fuructose-6-phosphate	20 mg
NADP+	13.3 mg
MTT	10 mg
PMS	2 mg
Glucose-6-phosphate dehydrogenase	15 units

Dissolved in 100 ml of B-B buffer.

PGM	
$MgCl_2 6H_2O$	200 mg
Glucose-1-phosphate	140 mg
NADP+	13.3 mg
MTT	10 mg
PMS	2 mg
Glucose-6-phosphate dehydrogenase	15 units

Dissolved in 100 ml of B-B buffer.

SKDH	
Shikimic acid	100 mg
NADP+	15 mg
MTT	20 mg
PMS	4 mg

Dissolved in 100 ml of Tris- buffer.

APPENDIX C-4

DNA extraction buffer

Tris- HCl (pH 8.0)	100 mM
EDTA (pH 8.0)	50 mM
Na Cl	500 mM
2-Mercaptoethanol	10 mM

APPENDIX C-5

Solutions for vacuum blotting

Depurination solution :	0.25 M HCl	
Denaturation solution:	1.5 M NaCl	87.6 g/l NaCl
	0.5 M NaOH	20.0 g/l NaOH
Neutralising solution:	1.0 M Tris	121.1 g/l Tris
	1.5 M NaCl	87.0 g/l NaCl
	pH 7.5	
Transfer solution:	20 x SSC	175 g/l NaCl
		88.2 g/l Trisodium citrate

APPENDIX C-6

LB medium (Luria-Bertani Medium)

For 1 liter:

Distilled water	950 ml
Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

Sterilized by autoclaving for 20 min at 120°C.

APPENDIX C-7

TE (10,1) buffer pH8.0

10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)

TE (50,10) buffer pH8.0

50 mM Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)

APPENDIX C-8

Alkaline lysis buffers for preparation of plasmid DNA

Solution I:	50 mM glucose	
	25 mM Tris HCl (pH8.0)	
	10 mM EDTA (pH8.0)	
Solution II:	0.2 N NaOH	
	1% SDS	
Solution III:	5 M potassium acetate	60 ml
	Glacial acetic acid	11.5 ml
	H_2O	28.5 ml

APPENDIX C-9

Solution for hybridization

20 x SSC	NaCl	175 g/l
	Trisodium citrate	88.2 g/l

Hybridization solution

Blocking reagent	2.0 % (w/v)
N-lauroylsarcosine	0.1 % (w/v)
SDS	0.02 %
Formaid	50 % (v/v)
5 x SSC	

APPENDIX C-10

Solutions for immunological detection

Buffer 1: Malic acid 0.1 M
NaCl 0.15 M
pH 7.5 (adjust with 10 N NaOH)

Washing buffer 1:
Buffer 1 + 0.3 % (v/v) Tween 20

Buffer 2: Buffer 1 + 1 % Blocking reagent

Buffer 3: Tris-HCl (pH 9.5) 0.1 M
NaCl 0.1 M
$MgCl_2$ $6H_2O$ 50 mM