Studies on Utilization of the Wild Tomato Species, *Lycopersicon peruvianum* (L.) Mill. and *L. chilense* Dun. for Tomato Breeding

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Contents

1. Chapter I
Introduction

1.1. *Economic importance of tomato*

The center of origin of the wild tomato is considered to be Andean zone, whereas it is considered that the tomato was domesticated in Mexico, and that the name of tomato was derived from the "tomatl" in the Nahua tongue of Mexico (Kalloo 1991). Presently, the tomato is one of the vegetables with the highest production in the world (Table I-1), and its production is increasing all over the world, primarily, in Asia (Table I-2). The production area in Europe, North and Latin America tends to stop increasing, or to decrease, but the production is sustained by the increase of yield per hectare, probably using high yielding varieties. In Japan, although both the area and production of the tomato is not large, the total wholesale price of the tomato is the highest among the vegetables {about 212 billion yen (about two billion US dollars) in 1995, Statistics and information Department 1998}. This fact implies that the tomato is indispensable not only to the diet in the US and European countries but also to the diet in Japan.

Tomatoes are served as various raw and processed food materials, such as salads, drinks, paste, puree, ketchup, whole peeled tomato, etc. The tomato fruit contains abundant and well-balanced nutrition consisting of minerals (potassium, magnesium, calcium, iron, zinc, etc.), vitamins (A, B_1, B_2, C, E, etc.), dietary fiber (pectin), citric acid, etc. In addition, the red pigment of the licopene, which tomato fruit contains in plenty, has recently attracted interest, because the licopene has high antioxdant ability against oxgen radicals that probably cause cancer, aging,

Table I-1. Production of vegetables in the world in 1998

Item of vegetable	Production (Mt)
Vegetables and Melons, Total	604,684,646
Vegetables Fresh Nes	179,397,864
Tomatoes	90,468,429
Watermelons	48,967,666
Cabbages	47,631,966
Onions, Dry	39,912,183
Cucumbers and Gherkins	26,641,377
Eggplants	20,163,680
Carrots	18,355,845
Cantaloupes and other Melons	18,125,624
Chillies and Peppers, Green	16,712,445
Lettuce	15,698,871
Pumpkins, Squash, Gourds	14,647,300
Cauliflower	13,685,686
Garlic	8,733,964
Green Corn (Maize)	8,431,821
Spinach	7,188,274
Peas, Green	6,897,971
Beans, Green	4,312,420
Okra	3,875,059
Onions and Shallots, Green	3,761,182
Asparagus	3,549,148
Mushrooms	2,183,448
String Beans	1,541,967
Leeks and Oth.Alliac.Veg	1,523,150
Artichokes	1,255,810
Broad Beans, Green	1,021,496

Source: FAO internet database.

Table I-2. Area and production of tomato in the world

Region	Area harvested (ha)			Yield (Kg / ha)			Production (metric ton)		
	1980	1990	1998	1980	1990	1998	1980	1990	1998
Africa	322,868	411,190	544,777	15,030	19,259	20,053	4,852,818	7,918,900	10,924,172
Asia	777,872	1,103,424	1,570,741	18,843	22,458	25,683	14,657,341	24,781,164	40,341,883
Europe	No data	700,054	660,772	No data	26,696	29,487	No data	18,688,309	19,484,321
North America	169,823	212,277	175,690	42,492	54,392	59,753	7,216,190	11,546,070	10,497,915
Latin America and Caribbean	133,603	157,339	151,463	23,053	29,154	37,960	5,168,897	7,364,754	8,805,433
Oceania	10,243	11,456	10,146	26,972	39,851	40,874	276,275	456,533	414,705
Total in the world	2,439,329	2,868,443	3,245,552	21,603	26,503	27,875	52,696,051	76,022,116	90,468,429
Japan	19,300	14,200	14,000	52,539	54,021	57,143	1,014,000	767,100	800,000

Source: FAO internet database. Data shown for Europe in 1990 are the data in 1992 .

arteriosclerosis, etc. Thus, the tomato would contribute to our enjoyable diet and good health all over the world.

1.2. *Taxonomy and crossability relationship among species in the genus Lycopersicon*

At present, the genus *Lycopersicon* in the family *Solanaceae* is classified into nine species (Fig. I-1 and I-2). The key to classification is the morphological trait such as fruit, seed, sympodium and bract as shown by Rick *et al.* (1990). Muller (1940) divided the genus *Lycopersicon* into two subgenera, *Eulycopersicon* (true *lycopersicon*) and *Eriopersicon* (woolly *persicon*). *Eulycopersicon* is a glabrous and red- fruited type, which comprises *L. esculentum*, *L. pimpinellifolium*, and *L. cheesmanii*, whereas *Eriopersicon* is a pubescent and green-fruited type, which comprises *L. chmielewskii*, *L. parviflorum*, *L. pennellii*, *L. hirsutum*, *L. peruvianum and L. chilense*. On the other hand, Rick (1979) also divided the genus into two subgenera, '*esculentum*-complex' (EC) and '*peruvianum*-complex' (PC). The EC contains seven species, *L. esculentum*, *L. pimpinellifolium*, *L. cheesmanii*, *L. chmielewskii*, *L. parviflorum*, *L. pennellii*, *L. hirsutum*, whereas the PC contains two species, *L. peruvianum* and *L. chilense*. The EC species are cross-compatible with the cultivated tomato, while The PC species are not with the cultivated tomato. However, the hybrid between the PC species and the cultivated tomato can be obtained through embryo rescue technique such as ovule culture (Imanishi 1988).

The genus *Lycopersicon* has both self-compatible (SC) and self-incompatible (SI) species. There are five self-compatible species, i. e., *L. esculentum*, *L. cheesmanii*,

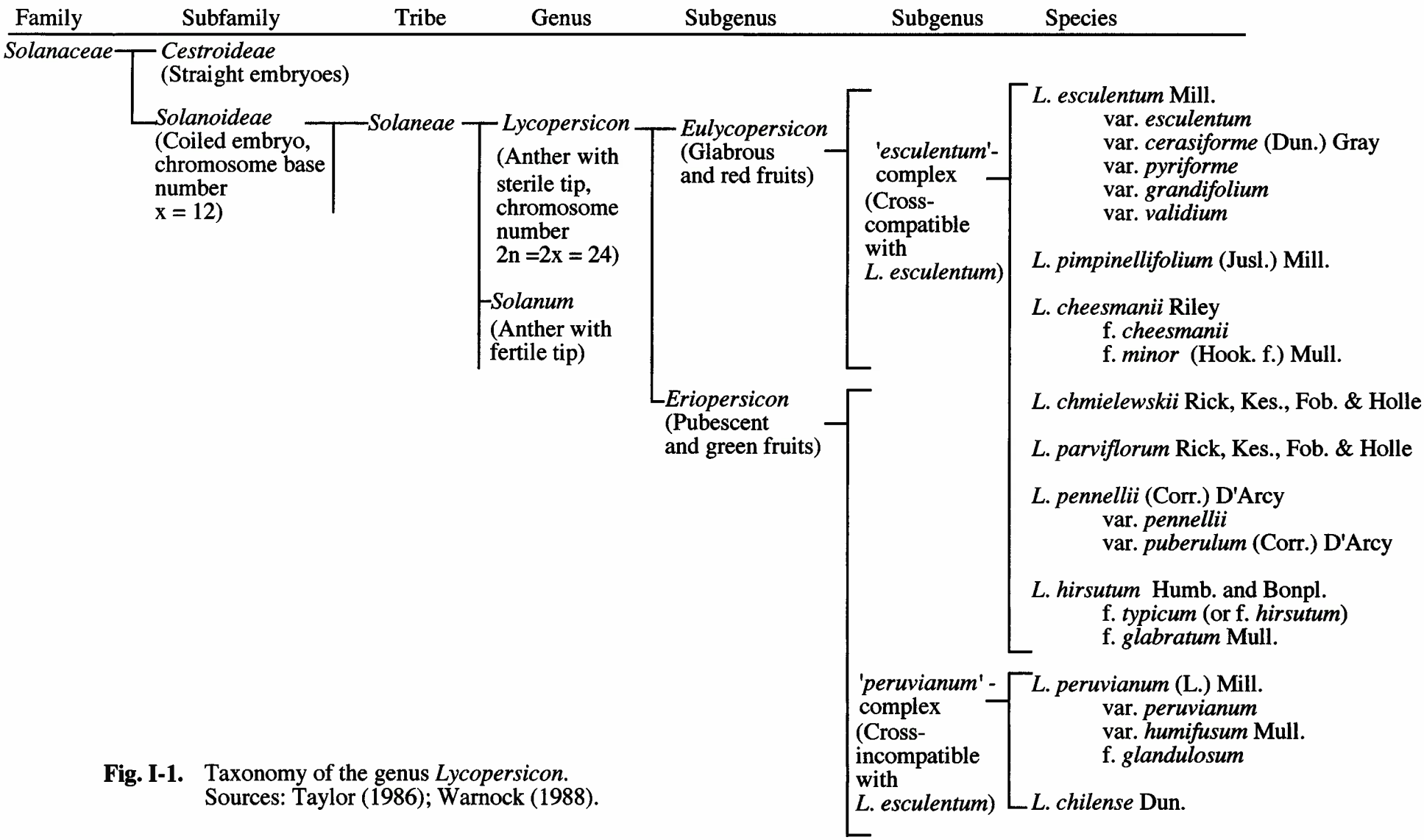

Fig. I-1. Taxonomy of the genus *Lycopersicon*. Sources: Taylor (1986); Warnock (1988).

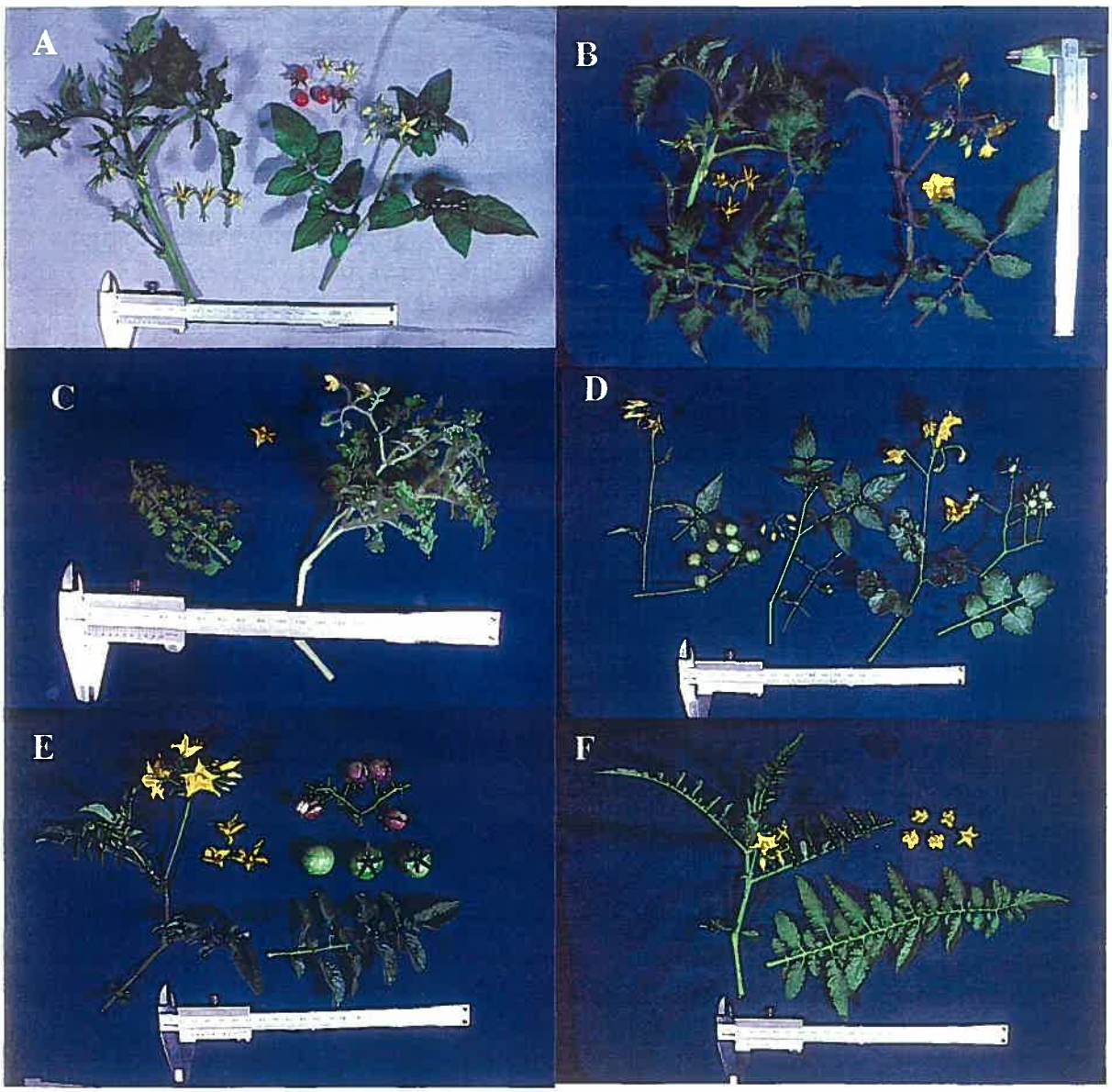

Fig. I-2. Nine species of *Lycopersicon*. A, left, *L. esculentum*, right, *L. pimpinellifolium;* B, left, *L. esculentum*, right, *L. hirsutum;* C, *L. cheesmanii;* D,left, *L. chmielewskii*, middle, *L. parviflorum*, right, *L. pennellii;* E, *L. peruvianum;* F, *L. chilense.*

L. pimpinellifolium, L. chmielewskii and L. parviflorum, while SI species is *L. chilense*. In addition, the species with both SC and SI accessions are *L. pennellii, L. hirsutum and L. peruvianum*. The *L. peruvianum* has only one accession LA2157 as the SC. Generally, unilateral incompatibility is observed in the interspecific cross between SC and SI species / accessions. That is, unilateral incompatibility is observed only when the stigma of SI species / accessions is pollinated with the pollens of SC species / accessions.

The tomato has a relatively small genome of 710Mbp per haploid nucleus (Galbraith *et al.* 1983) in comparison with genome sizes of several plant species for genetic studies such as *Arabidopsis thaliana* (100Mbp), *Oryza sativa* (430Mbp), *Zea mays* (2,716Mbp) and *Allium cepa* (15,797Mbp). In consistent with the small genome size, the tomato behaves as a diploid with a minimum of internal duplication, and displays a very low tolerance of chromosomal unbalance (Rick and Yoder 1988). All the *Lycopersicon* species have the same chromosome number (2n = 2x = 24), and many studies indicated that the chromosomes in haploids of the tomato did not pair in the prophase of meiosis (Kirillova 1991).

1.3. Genetic improvement of the cultivated tomato

The cultivated tomato is poor as new breeding materials due to limited genetic variation in itself. On the other hand, the wild relatives of tomato have many desirable traits such as fruit qualities and resistances against pests, diseases and environmental stresses (Table I-3), and for further genetic improvement of the cultivated tomato, it is necessary to introduce their useful traits into the cultivated tomato. However, some wild species,

Table I-3. Notable traits of the wild tomato species for genetic improvement of the tomato

Species	Traits
L. pimpinellifolium	Resistance to *Fusarium oxysporum* (Fusarium wilt), *Cladosporium fulvum* (leaf mold), *Pseudomonas syringae* pv. tomato (Bacterial speck), *Xanthomonas campestris* pv.*vesicatoria* (bacterial spot) and moth. High soluble solid.
L. cheesmanii	Salt tolerance. Moth resistance. Sucrose accumulation in fruits.
L. hirsutum	Resistance to *Cladosporium fulvum* (leaf mold), tobacco mosaic virus (TMV), *Clavibacter michiganensis* (bacterial canker), *Alternaria solani* (early blight), *Pyrenochaeta lycopersici* (corky root), moth (caterpillars), white fly, beetle, fly, mite and aphid.
	Low temperature tolerance. Sucrose accumulation and high β-carotene content in fruits.
L. pennellii	Resistance to tomato spotted wilt virus (TSWV), curly top virus (CTV), moth, beetle, aphid and white fly. Sucrose accumulation in fruits. Drought and salt tolerance.
L. parviflorum	Sucrose accumulation in fruits.
L. chmielewskii	High soluble solid and sucrose accumulation in fruits.
L. peruvianum	Resistance to *Fusarium oxysporum* (Fusarium wilt, Fusarium crown and root rot), *Cladosporium fulvum* (leaf mold), *Pseudomonas syringae* pv. *tomato* (Bacterial speck), *Meloidogyne* spp. (root knot nematode), *Clavibacter michiganensis* (bacterial canker), *Pyrenochaeta lycopersici* (corky root), *Botrytis cinerea* (gray mold), TMV, TSWV, tomato yellow leaf curl virus (TYLCV), mite and moth. Sucrose accumulation in fruits. Regeneration ability for *in vitro* culture. Sweet aroma in leaves and fruits. High vitamin C content in fruits. High glutamic acid content in fruits.
L. chilense	Resistance to *Pseudomonas syringae* pv. *tomato* (Bacterial speck), *Clavibacter michiganensis* (bacterial canker), *Leveillula taurica* (powdery mildew), TYLCV and CTV. Drought and low temperature tolerance. Regeneration ability for *in vitro* culture, Sucrose accumulation in fruits.

Sources: Egashira *et al.* (unpublished); Farrar Jr. and Kennedy (1991); Gilbert and McGuire (1956); Kamal *et al.* (unpublished); Kalloo (1991); Koornneef et al. (1987); Lukyanenko (1991); Manning and Maw (1975); Mochizuki *et al.* (1996); Stevens and Rick (1986); Takashina et al. (1998).

particularly the PC species have the cross incompatibility with the cultivated tomato as described above. Then, the study on the breeding method and genetic analysis is essential to efficiently introduce useful traits from the PC into the cultivated tomato.

In order to produce new varieties to meet the further demands in the future, the works on collection, evaluation, preservation, documentation and distribution of tomato germplasm are also important. The International Board for Plant Genetic Resources (IBPGR) was established in 1974 to create an international network of centers dealing with genetic resources, and has taken initiative to organize missions for collectiing tomato germplasm (Garanko 1991, Kalloo 1991). The National Plant Germplasm System, USDA and the Tomato Genetic Resource Center at the University of California, Davis have been collecting and maintaining excellent stocks of tomato genetic resources, including morphological, physiological and isozymic types, linkage marker stocks, chromosomal variants, and wild species accessions (Rick and Yoder 1988). For the genetic study of the tomato, high-density linkage maps that comprised morphological, isozymic, RFLP and AFLP markers are also available (Tanksley *et al.* 1992, Haanstra *et al.* 1999).

In this study, the PC species were mainly used as experimental materials. Because the PC accessions have not been sufficient to exploit the breeding of new tomato varieties due to severe reproductive barriers with the cultivated tomato although the PC possesses very high genetic diversity (Rick 1986, Miller and Tanksley 1990, Bretó 1993, Fig. I-3) and many practically attractive traits (Table I-3).

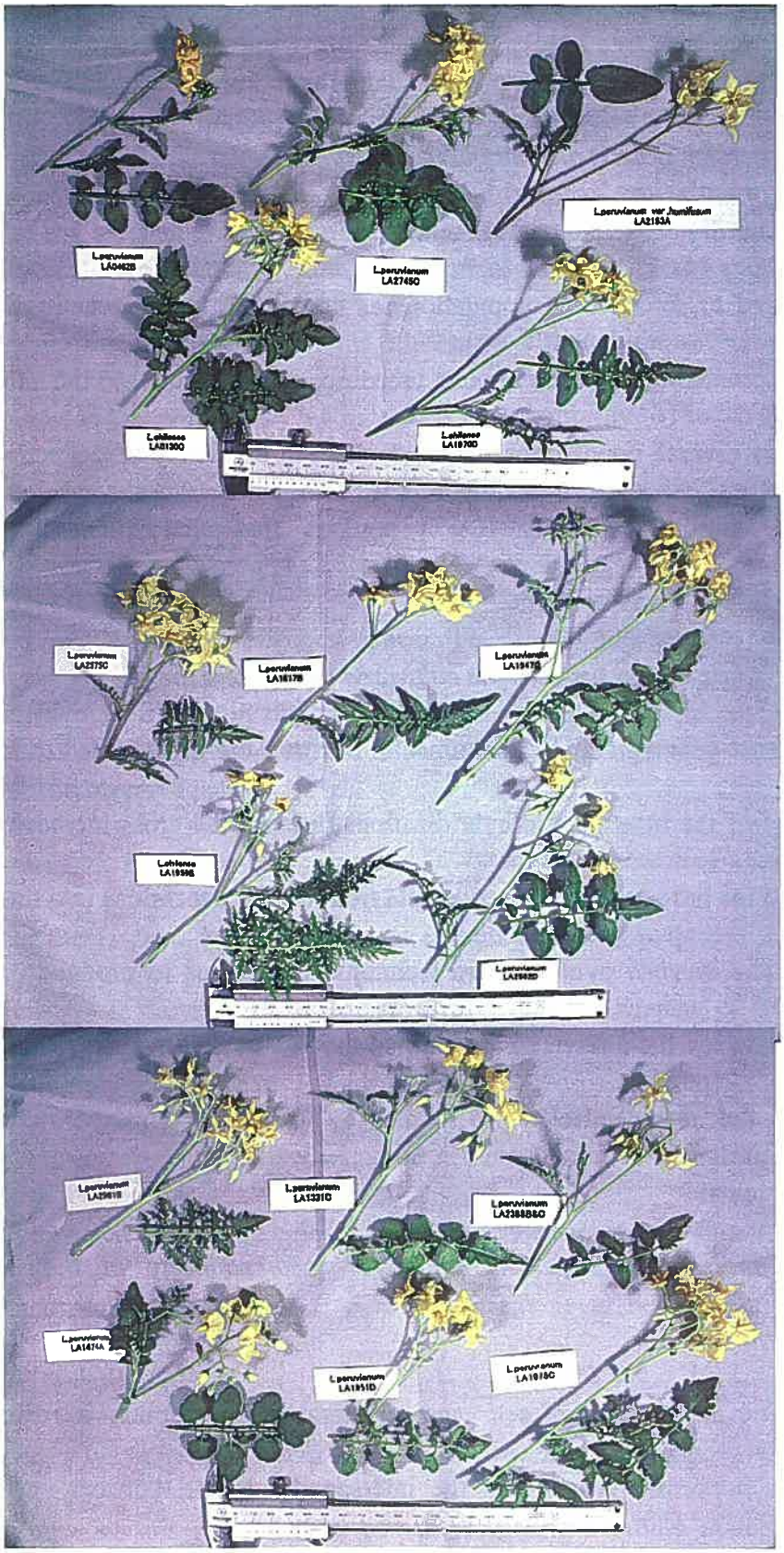

Fig. I-3. Genetic variation of the flower and leaf in the '*peruvianum*-complex' of *Lycopersicon*.

1.4. Objectives of the present study

Five objectives are involved in the present studies as follows:

1) Investigation for genetic diversity of the PC species

The genetic diversity and mutual relationship of the PC and the EC species were investigated to screen such populations as containing larger genetic diversity for selecting promising materials in plant breeding, and to understand the habitats of the genetically diverse populations for increasing the efficiency of collecting valuable genetic resources by using RAPD markers that could facilitate the rapid identification of many markers.

2) Efficient hybridization between the cultivated tomato and the PC species

The parental-genotypic and environmental factors were investigated to increase the efficiency of the interspecific hybridization. Because the cross incompatibility has hampered the introduction of useful traits from the PC accessions into the cultivated tomato. The *in vitro* ovule culture technique enables us to produce interspecific hybrids between the cultivated tomato and the PC. In such works, the efficiency of obtaining the interspecific hybrid seemed to depend on tomato varieties used as female parents.

3) Genetic analysis of self- and unilateral incompatibility in the progeny of interspecific hybrids

The genetic mechanisms of self- and unilateral incompatibility were investigated using the backcross progeny of the interspecific hybrid between the cultivated tomato and *L. chilense*. The backgrounds of this study are as follows: The pollen

tube of the PC is able to grow into the pistil of the cultivated tomato, and the fertilization occurs successfully. On the contrary, the pollen tube of the cultivated tomato stops growing in the pistil of the PC and fails to fertilize the ovule. Due to such unilateral incompatibility, the cytoplasm of the PC species remained to be untouched for tomato breeding. On the other hand, although the self-incompatibility of *Lycopersicon* is controlled by a single-self-incompatibility (S)-locus gene, several researchers have reported that the self-incompatibility in the progeny of the interspecific hybrids between the cultivated tomato and some wild species may be controlled by two or more genes. Thus, the genetic mechanisms of self- and unilateral incompatibility in the progeny of interspecific hybrids in *Lycopersicon* have not been revealed yet.

4) *Utilization of the PC as a breeding source for sucrose-accumulating ability*

To utilize the *L. peruvianum* accessions as new breeding materials to supply an alternative sweet taste for the tomato fruit, the inheritance of the sucrose-accumulating ability was investigated using the backcross progeny of interspecific hybrids between the cultivated tomato and *L. peruvianum*. Although *L. peruvianum* has a sucrose-accumulating ability that the cultivated tomato does not possess, the sucrose-accumulating ability of *L. peruvianum* has never been integrated into the cultivated tomato.

5) *Utilization of the PC as a breeding source for resistance against gray mold disease*

The objective of this study was to screen promising breeding materials resistant against the gray mold among 44 wild accessions including the PC. Because the

tomato gray mold is a serious disease worldwide in the cultivated tomato, which infects leaves, stems, flowers and fruits. Resistant breeding materials have not been found yet, and it is difficult to raise new resistant varieties against the gray mold.

2. Chapter II.

Genetic Diversity of the '*peruvianum*-complex' (*Lycopersicon peruvianum* (L.) Mill. and *L. chilense* Dun.) Revealed by RAPD Analysis

2.1. Introduction

For further genetic improvement of the cultivated tomato, it is essential to introduce new useful attributes from wild accessions due to the limited genetic resources in the cultivated tomato. Then, it is necessary to promote the effective collection, classification, preservation and utilization for tomato breeding, of wild accessions.

It is reported that the '*peruvianum*-complex' (PC) species, *L. peruvianum* and *L chilense* have large genetic diversity (Rick 1986, Miller and Tanksley 1990, Bretó *et al.* 1993). Although the two species of the PC are promising as genetic resources for tomato breeding, the utilization of the PC is hampered due to their severe cross incompatibility with the cultivated tomato (Hogenboom 1972a). Recently it has been possible to obtain interspecific hybrids between the cultivated tomato and the PC species more readily through an ovule culture than it was before (Imanishi 1988, Chen and Imanishi 1991, Takashina *et al.* 1997, Doganlar *et al.* 1997, Sacks *et al.* 1997). The ovule culture technique has been increasingly applicable to wide interspecific hybridization between cultivated tomatoes and PC accessions when suitable cultivated tomatoes as seed parents and appropriate conditions of growth were supplied (Sacks *et al.* 1997, Takashina *et al.*

1997). Thus, the importance of the PC species in tomato breeding seems to increase much more than before. For further efficient utilization of the PC, it would be necessary to investigate genetic variation within the PC. However, there are very few reports about the characteristics of the genetic variation within the PC (Rick and Lamm 1955, Rick 1979, 1986).

Recently, molecular markers such as allozyme markers (Rick and Fobes 1975, Bretó *et al.* 1993), chloroplast DNA (Palmer and Zamir 1982), mitochondrial DNA (McClean and Hanson 1986) and RFLP markers (Miller and Tanksley 1990) have been used for the phylogenic study of *Lycopersicon.* Particularly, random amplified polymorphic DNA (RAPD) markers, which can be used simply and fast (Welsh and McClelland 1990, Williams *et al.* 1990), are widely used for determination of genotypes (Hashizume *et al.* 1993), gene mapping (Chagué *et al.*, 1996; Ohmori *et al.*, 1996; Foolad and Chen 1998; Takashina *et al.* 1998), and QTL analysis (Grandillo and Tanksley 1996). However, there are very few reports for the phylogenic study of *Lycopersicon* using RAPDs (Williams and St. Clair 1993, Villand *et al.* 1998). The main reason seems to be a low reproducibility caused by the sensitivity of RAPD technique, as it has been often discussed (Yang and Quiros 1993, Iqbal and Rayburn 1994). However, RAPD technique would be a powerful method that enable us to rapidly identify many markers and to estimate the relationship and genetic diversity among or within populations even with the limitation of time, costs, equipment,

etc.

Recently, the bootstrap method (Efron 1979, Felsenstein 1985) has been used to estimate the reproducibility of the branching pattern of the dendrogram produced by the cluster analysis in the phylogenic studies. In addition, a cluster analysis of the neighbor-joining (NJ) method (Saitou and Nei 1987) has also often been used. The NJ method is superior to other methods such as the UPGMA method in recovering the true branching pattern of the genetic relationship among populations (Saitou and Nei 1987).

In this study, RAPD analysis was performed in order 1) to investigate the genetic variation within the PC species, 2) to elucidate genetic relationships among the PC species and/or the '*esculentum*-complex' (EC) species, 3) to estimate geographical and genetic relationships among the PC accessions and 4) to evaluate the validity and the efficiency of RAPD analysis in the phylogenic study of *Lycopersicon.*

2.2 Materials and methods

2.2.1. Plant material

The plant material contained 50 accessions (Table II-1), which included two pure Japanese cultivated varieties: 'Sekaiichi' and 'Early Pink' and 48 wild accessions: 22 *L. peruvianum* and 12 *L. chilense* accessions, two accessions of *L. pennellii*, *L. hirsutum*, *L. chmielewskii*, *L. parviflorum*, *L. cheesmanii*, *L. pimpinellifolium* and

Table II-1. Plant materials used in this study

Lycopersicon species	Accession /cultivar	Location[1] for collection / origin	Country
L. peruvianum	LA0455	Arequipa	Peru
	LA0462	Tarapaca	Chile
	LA1331	Ica	Peru
	LA1474	Arequipa	Peru
	LA1517	Lima	Peru
	LA1692	Lima	Peru
	LA1910	Huancavelica	Peru
	LA1947	Arequipa	Peru
	LA1951	Arequipa	Peru
	LA1975	Lima	Peru
	LA2328	La Libertad	Peru
	LA2388	Cajamarca	Peru
	LA2562	Ancash	Peru
	LA2575	Ancash	Peru
	LA2724	Lima	Peru
	LA2745	Tarapaca	Chile
	LA2981	Moquegua	Peru
	LA3156	Moquegua	Peru
L. per. f. glandulosum	LA0366	Lima	Peru
	LA1551	Lima	Peru
L. per. var. *humifusum*	LA0389	Cajamarca	Peru
	LA2153	Cajamarca	Peru
L. chilense	LA0130	Moquegua	Peru
	LA0470	Antofagasta	Chile
	LA1917	Ica	Peru
	LA1932	Arequipa	Peru
	LA1938	Arequipa	Peru
	LA1959	Moquegua	Peru
	LA1970	Tacna	Peru
	LA1972	Tacna	Peru
	LA2406	Tarapaca	Chile
	LA2739	Tarapaca	Chile
	LA2747	Tarapaca	Chile
	LA2930	Antofagasta	Chile
L. pennellii	LA1734	Lima	Peru
	LA1940	Arequipa	Peru
L. hirsutum	LA1772	Lima	Peru
L. hir. f. *glabratum*	LA1252	Loja	Ecuador
L. chmielewskii	LA1316	Ayacucho	Peru
	LA2695	Cusco	Peru
L. parviflorum	LA1326	Apurimac	Peru
	LA2133	Azuay	Ecuador
L. cheesmanii	LA0166	Galapagos Island	Ecuador
L. cheesmanii f. *minor*	LA0436	Galapagos Island	Ecuador
L. pimpinellifolium	LA1246	Loja	Ecuador
	LA1670	Tacna	Peru
L. esculentum var. cerasiforme	LA1268	Lima	Peru
	LA1455	Nuevo Leon	Mexico
L. esculentum cv.	'Early Pink'	NIAR[2]	Japan
	'Sekaiichi'	NIAR	Japan

[1] Described in more detail by Rick and Chetelat (1995)

[2] National Institute of Agrobiological Resources, Japan.

L. esculentum var. *cerasiforme*. All the wild accessions were provided by the courtesy of Prof. Dr. C. M. Rick of the Tomato Genetic Resource Center, University of California, and the cultivated varieties were by the National Institute of Agrobiological Resources (NIAR), Japan. The *L. peruvianum* and *L. chilense* accessions used in this study were selected from geographically different habitats in wide areas as much as possible in terms of different watersheds and northern and southern limits of their distribution (Fig. II-1).

2.2.2. DNA isolation and RAPD analysis

Total genomic DNA was isolated by a simple modified CTAB method (Böhm *et al.* 1993) from approximately 0.1g of fresh leaf tissue of two greenhouse-grown plants in each accession. The PCR was performed using ten 10-mer primers (OPK-01 to 10, Operon Technologies Inc.) according to Takashina *et al.* (1998). Total DNA was amplified twice each plant, and reproducible products amplified in the replications were rated as the plant's own products. Subsequently, all the plant's own products in an accession were rated as the accession's own products. The plant's own products and the accession's own products were used for calculating the genetic distance among plants, accessions and species as described below. Only one plant was analyzed in *L. chilense* LA0130 and LA2930, and cv. 'Sekaiichi' and 'Early Pink'. Then, products of a single plant were rated as the accession's own products.

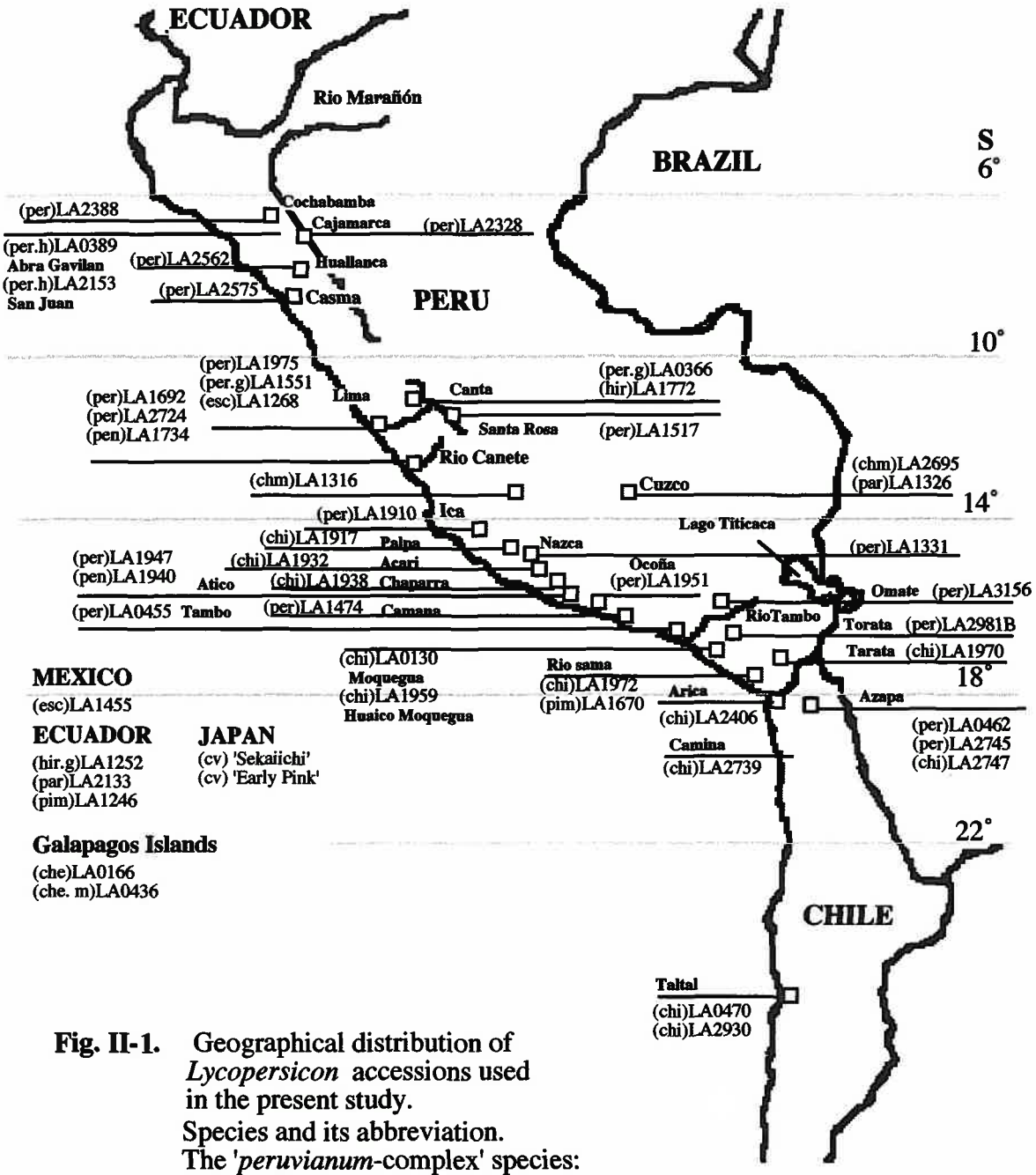

Fig. II-1. Geographical distribution of *Lycopersicon* accessions used in the present study.
Species and its abbreviation.
The '*peruvianum*-complex' species:
L. peruvianum (per), *L. peruvianum* var. *humifusum* (per.h), *L. peruvianum* f. *glandulosum* (per.g), *L. chilense* (chi).
The '*esculentum*-complex' species:
L. parviflorum (par), *L. pennellii* (pen), *L. hirsutum* (hir), *L. hirsutum* f. *glabratum* (hir.g), *L. chmielewskii* (chm), *L. cheesmanii* (che), *L. cheesmanii* f. *minor* (che.m), *L. pimpinelliifolium* (pim), *L. esculentum* var. *cerasiforme* (esc), *L. esculentum* cv. (cv).

2.2.3. Genetic distances and cluster analysis

Each accession's product was considered to be a unit character, with which the accessions were scored for the presence (1) or absence (0) of a product. Genetic similarity (S) between all pairs of accessions were calculated according to Nei and Li (1979): $S = 2c / (a + b + 2c)$, where c = the number of products shared by an accession (A) and another accession (B), a = the number of products possessed by A and not by B, and b = the number of products not possessed by A, but by B. The similarity indices were converted into dissimilarity D (= - ln (S)). The dissimilarity indices were regarded as the genetic distance, and a distance matrix among 50 accessions was constructed. A cluster analysis by the NJ method was conducted with the "TreeTree" software package (Saitou 1996) and an unrooted dendrogram was constructed. The cluster dendrogram was drawn by using the program of "DendroMaker" (version 4.1) developed by Prof. Dr. Tadashi Imanishi in the National Institute of Genetics, Japan. To evaluate the reproducibility of branching pattern, bootstrap probabilities were calculated with the program of "bootNJ" in the "TreeTree" using 1,000 bootstrap resampling data.

2.3. Results

2.3.1. Detection of RAPD markers and genetic distances between and within Lycopersicon species

A total of 438 PCR products were detected from all the accessions of nine species.

Of 438, three products were shared by all of the accessions, and 435 products were polymorphic among 50 accessions. Numbers of RAPDs by species and primer are shown in Table II-2. The total number of RAPDs produced by each primer varied from a minimum of 31 amplified by OPK-01 to a maximum of 55 amplified by OPK-08.

Average genetic distances between plants, accessions and species are shown in Table II-3. Average genetic distances of *L. peruvianum* and *L. chilense* among accessions and between plants in an accession were larger than that of any EC species. In addition, the average genetic distance of each PC species was almost as large as that (0.687) of the whole self-compatible EC species containing *L. chmielewskii, L. parviflorum, L. cheesmanii, L. pimpinellifolium* and *L. esculentum*. Interestingly, genetic distances between *L. esculentum* cv. and PC species were smaller than those between *L. esculentum* cv. and green-fruited EC species (*L. pennellii, L. hirsutum, L. chmielewskii* and *L. parviflorum*) although *L. esculentum* cv. is cross-compatible with the green-fruited EC species but not with the PC species.

2.3.2. Cluster analysis and characterization of the 'peruvianum-complex'

The dendrogram constructed by the NJ method is shown in Fig. II-2. The genus *Lycopersicon* was divided into the four major clusters of the PC, the self-compatible EC, *L. pennellii* and *L. hirsutum* at the internal branch with a bootstrap

Table II-2. Number of RAPDs generated using 10 random primers in *Lycopersicon*

Lycopersicon species	Number of accessions	Primer (OPK)										Number of RAPDs
		01	02	03	04	05	06	07	08	09	10	
'peruvianum-complex'	34	24	44	36	37	30	33	43	45	25	47	364
L. peruvianum	22	16	36	21	30	22	25	33	40	14	37	274
L. chilense	12	13	28	30	27	18	24	35	24	20	31	250
'esculentum-complex'	16	19	30	21	39	15	33	44	34	19	31	285
L. pennellii	2	0	10	5	7	4	1	9	6	4	5	51
L. hirsutum	2	5	5	7	6	5	10	6	12	7	14	77
L. chmielewskii	2	0	3	0	1	2	0	0	1	1	0	8
L. parviflorum	2	5	4	8	8	4	2	7	5	5	6	54
L. cheesmanii	2	4	7	1	9	1	7	10	9	2	8	58
L. pimpinellifolium	2	5	3	1	3	1	5	5	5	3	4	35
L. esculentum var. *cerasiforme*	2	3	3	0	1	3	3	7	4	1	2	27
L. esculentum cv.	2	1	0	1	0	0	0	0	0	0	3	5
Total	50	31	52	39	44	33	43	54	55	32	52	435

Table II-3. Average genetic distance between species, among accessions within a species, and between plants in an accession within a species in *Lycopersicon*

	per[1)]	chi	pen	hir	chm	par	che	pim	esc	cv
L. peruvianum	0.622 (0.192)[2)]									
L. chilense	0.878	0.677 (0.252)								
L. pennellii	1.036	1.078	0.335 (0.103)							
L. hirsutum	1.166	1.133	0.984	0.589 (0.095)						
L. chmielewskii	1.110	1.140	0.980	1.099	0.049 (0.045)					
L. parviflorum	0.952	1.050	0.894	1.114	0.672	0.382 (0.024)				
L. cheesmanii	1.129	1.233	0.988	1.357	0.788	0.840	0.417 (0.003)			
L. pimpinellifolium	1.088	1.114	0.980	1.260	0.826	0.790	0.427	0.264 (0.014)		
L. esc. var. *cer.*	1.064	1.095	1.064	1.279	0.866	0.855	0.418	0.243	0.189 (0.033)	
L. esculentum cv.	1.109	1.143	1.382	1.522	1.214	1.164	0.771	0.608	0.522	0.036 (no data)

[1)]See Fig. 1.

[2)]Figure in parenthesis is an average genetic distance between plants in an accession within a species.

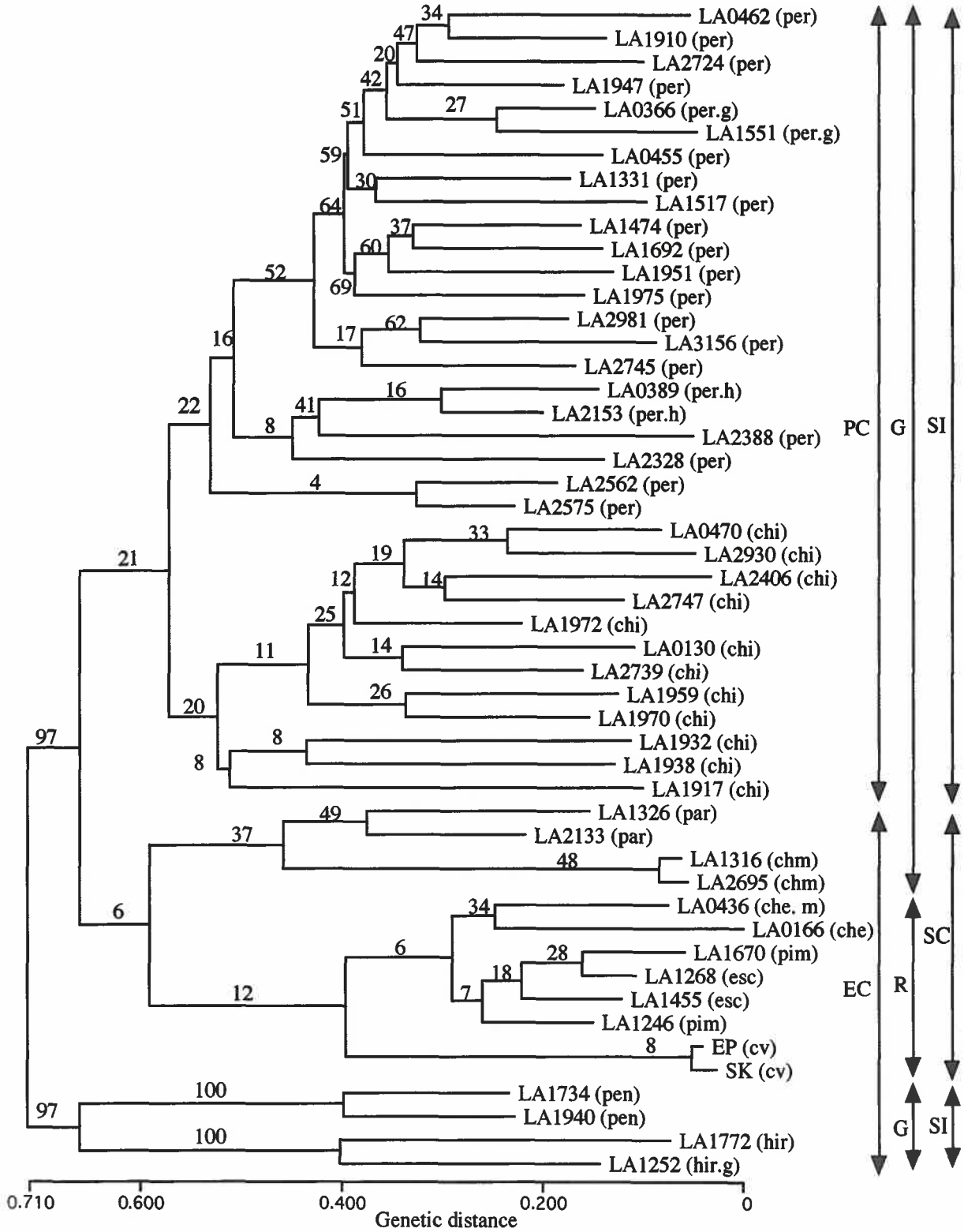

Fig. II-2. Cluster analysis of 50 accessions of *Lycopersicon* by the NJ method. Numbers above internal branches are bootstrap probabilities (%) based on 1,000 bootstrap resampling. SC, self-compatible; SI, self-incompatible; G, green-fruited species; R, red-fruited species; PC, '*peruvianum*-complex'; EC, '*esculentum*-complex'.

probability of 97%.

The sub-clusters of *L. peruvianum* and *L. chilense* branched out from the PC cluster at a bootstrap probability of 21%. In the sub-cluster of *L. peruvianum*, the six accessions (LA0389, LA2153, LA2388, LA2328, LA2562 and LA2575) which located around Rio Marañón in the north of Peru were genetically distant from the other middle and southern *L. peruvianum* accessions. LA0389 and LA2153, and LA2562 and LA2575 were geographically and genetically closer, respectively. On the other hand, the *L. peruvianum* accessions distributed in the middle and south of Peru formed one cluster at a bootstrap probability of 52% and did not show clear relationships between geographical and genetic distances. The average genetic distance (0.632) and the maximum genetic distance (0.8472) among the six Rio Marañón accessions were larger than those (0.531 and 0.767, respectively) among the middle and southern accessions. Thus, the Rio Marañón accessions showed the largest genetic diversity in *L. peruvianum*. In the sub-cluster of *L. chilense*, the *L. chilense* accessions in southern Peru, LA1932, LA1938 and LA1917, which were located in the most northern area of *L. chilense*, were genetically distant from the other *L. chilense* accessions. The average genetic distance (0.744) among the three northern accessions was larger than that (0.548) among the other *L. chilense* accessions.

The cluster of the self-compatible EC was divided into two sub-clusters, red-fruited species (*L. esculentum*, *L. pimpinellifolium* and *L. cheesmanii*) and

green-fruited species (*L. chmielewskii* and *L. parviflorum*) at a low bootstrap probability of 6 %. Furthermore, the red-fruited species were divided into the cultivars with large fruits and the species with small fruits (*L. esculentum* var. *cerasiforme*, *L. pimpinellifolium* and *L. cheesmanii*), and the latter three species formed one sub-cluster. *L. esculentum* var. *cerasiforme* and *L. pimpinellifolium* showed the closest genetic relationship among all the interspecific relationships in the *Lycopersicon* (Fig. II-2, Table II-3).

2.4. Discussion

Rick (1979) reported that *L. peruvianum* is a morphologically and allozymatically polymorphic species. Bretó *et al.* (1993) also reported that the genetic diversities of *L. chilense*, *L. peruvianum* and *L. pennellii* were the largest in the *Lycopersicon*. Miller and Tanksley (1990) examined the genetic variation in the *Lycopersicon* species except *L. chilense*, using average genetic distances between and within accessions. From the results, they concluded that *L. peruvianum* had the highest genetic variation and that self-incompatible species (*L. peruvianum, L. pennellii* and *L. hirsutum*) had clearly higher genetic diversities than the self-compatible species. Particularly, the genetic variation of *L. peruvianum* was nearly tenfold higher than that of the self-compatible species. These results were consistent with the present observation as shown in Table II-3.

In the cluster analysis, the PC species was clearly isolated from the EC

species. This indicates that the PC and the EC are not only reproductively isolated, but also clearly different in those genetic backgrounds. In other words, the PC would possess many traits that the EC is devoid of.

Rick (1986) described that the Marañón group was comprised of three reproductively isolated groups, the Chamaya-Cutiva race, the Chotano-Yamalúc race, and the Balasas race, whereas the accessions in the middle and south of Peru were intra- and inter-crossable with only the Chotano-Yamalúc race of the Marañón group. In this study, the group of Rio Marañón accessions was found to be distant from the other accessions and to have the largest genetic diversity in the *L. peruvianum*. These observations are consistent with Rick's results. The northern *L. chilense* accessions were genetically distant from the other *L. chilense* accessions in the present result. This suggests that *L. chilense* may also be comprised of at least two or more genetically different groups like *L. peruvianum*.

The cluster of self-compatible EC species was divided into two sub-clusters, red-fruited species and green-fruited species, as shown in the other cluster analyses using genomic RFLP (Miller and Tanksley 1990), chloroplast DNA (Palmer and Zamir 1982) and allozyme markers (Bretó *et al.* 1993). In the present study, self-incompatible EC species, *L. hirsutum* and *L. pennellii* formed each own cluster. Palmer and Zamir (1982) and Miller and Tanksley (1990) also reported that *L. pennellii* and *L. hirsutum* tended to form different clusters. These facts indicate *L. pennellii* and *L. hirsutum* also have clearly different genetic

backgrounds from other *Lycopersicon* species. *L. pennellii* had been placed in the *Solanum* genus (Correll 1958). However, Rick (1960) suggested that *Solanum pennellii* was a close relative of *Lycopersicon* due to cross compatibility with *L. esculentum* and D'Arcy (1982) transferred *S. pennellii* to the *Lycopersicon* genus.

The RAPD marker was not only able to classify all the tested accessions into the PC and the EC, but also into the same group as those classified on the basis of morphology, fruit color, self-incompatibility, etc. In addition, only ten 10-mer primers generated as many as 435 RAPDs in the *Lycopersicon*. Therefore, RAPD analysis was reconfirmed to be a powerful technique as well as time- and cost- saving techniques, and was considered to be useful to characterize the genetic diversity of populations and the genetic relationship among populations.

In conclusion, the PC species with such high and different genetic variation in comparison with the EC species would have potential to supply unknown but useful traits for future tomato breeding.

3. Chapter III.

Pistillate-Parental Differences and the Ability to Produce Interspecific Hybrids between *Lycopersicon esculentum* and '*peruvianum*-complex' Species (*L. peruvianum* and *L. chilense*)

3.1. Introduction

Wild tomato species are often used as genetic resources for improving practically important characteristics of cultivated tomatoes (*Lycopersicon esculentum* Mill.) (Stevens and Rick 1986, Kalloo 1991). The '*peruvianum*-complex' (PC) consisting of two wild species, *L. peruvianum* (L.) Mill. and *L. chilense* Dun., is also a valuable genetic resource, but its use is hampered by severe cross incompatibility with cultivated tomatoes (Hogenboom 1972a). The pistil of the PC barely accepts the pollen of the cultivated tomato. Although the reciprocal cross induces pollen tube elongation and fertilization, most of the hybrid embryos degenerate before maturing (McGuire and Rick 1954, Hogenboom 1972a, Barbano and Topoleski 1984, Chen and Adachi 1992).

To overcome such hybrid embryo degeneration, various attempts have been made, which included zygotic embryo rescue culture (Smith 1944), chronic gamma-ray irradiation to *L. peruvianum* pollen before pollination (Yamakawa 1971), plant regeneration procedure after callus induction from hybrid embryos or ovules (Thomas and Pratt 1981), and the use of bridge lines involving a part of the genome from a wild species (Poysa 1990). Compared with these measures, the *in vitro* ovule culture method proposed by Imanishi (1988), which includes a selection method of favorable ovules, appears to be superior, because it does not need any special equipment and plant

materials. This method readily provides interspecific hybrids between *L. esculentum* and the PC without any somaclonal variations (Takashina *et al.* 1997). It has been pointed out, however, that the effectiveness of this method might depend on the cultivated variety used as a pistillate parent (Imanishi 1988, Chen and Imanishi 1991, Sacks *et al.* 1997).

The objectives of this study are to elucidate the pistillate-parental differences in the number of ovules per fruit (OPF), the rate of number of germinated ovules / number of total ovules obtained (GPO) and the number of germinated ovules per fruit (GPF), and to identify morphological traits of sexual organs and fruits that can help find promising pistillate parent for enhancing GPF in the interspecific crossing between the cultivated tomato and the PC.

3.2. Materials and Methods

3.2.1. Plant materials

Seventeen cultivated varieties of tomato were used as female parents (Fig. III-1) and three PC accessions, *L. peruvianum* PI270435 (LP), *L. peruvianum* var. *humifusum* LA2153 (LPH) and *L. chilense* PI128652 (LC) as male parents in 1991. The LA2153 accession was kindly provided by Prof. Dr. C. M. Rick at the Tomato Genetic Resource Center, University of California, Davis, U. S. A., and all the cultivated tomato varieties and PI number accessions were provided by the National Institute of Agrobiological Resources (NIAR), Tsukuba, Japan.

Nine of the 17 varieties were used as the counterparts of the three PC

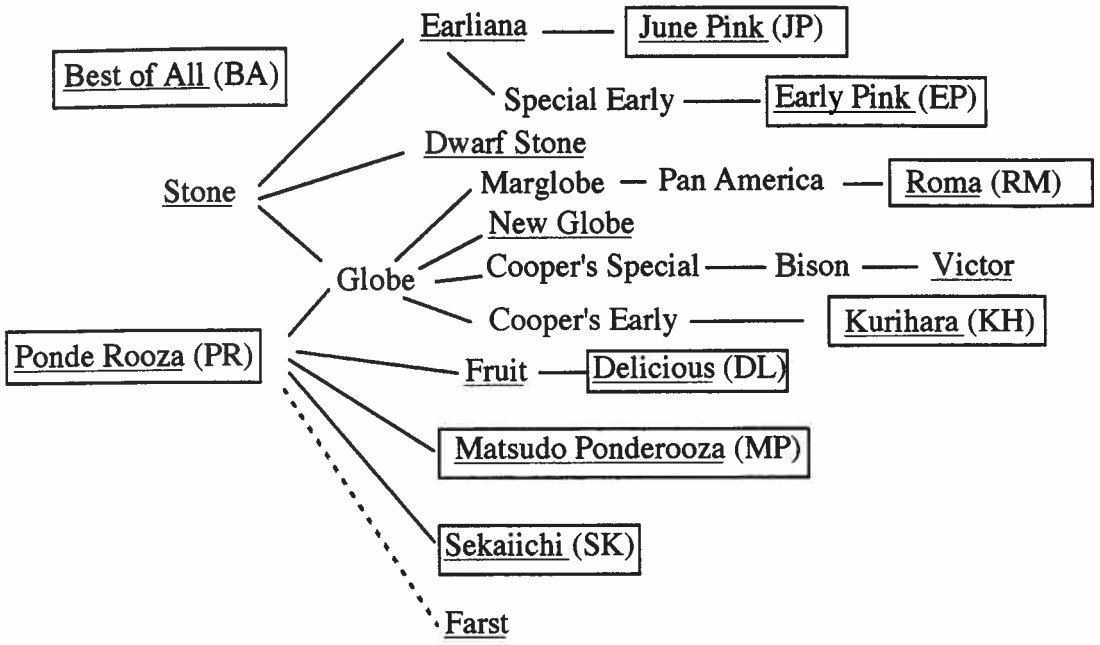

Fig. III- 1. Pedigree of Japanese tomato varieties. All the varieties shown here are true breeds. The 17 cultivated varieties used in the experiment in 1991 are underlined. The nine varieties used both in 1991 and 1993 are in boxes. Abbreviation of each varietal name is shown in parenthesis.

accessions in 1993. Five, two, and two varieties of them were selected due to their relatively large, intermediate variations for sexual-organ- and fruit- related morphological traits, and the largest GPF, respectively, based on a preliminary principal component analysis conducted for the results of 1991.

3.2.2. Crossing and in vitro ovule culture

Crossing and *in vitro* ovule culture (Fig. III-2) were conducted in 1991 and 1993 according to the method of Imanishi (1988). Emasculation and pollination were performed on the same day when the anthers of pistillate parents became yellow-green in color and the petals were still whitish, about three days before anthesis. Pistils of the first to the fourth inflorescence flowers were fertilized with fresh pollens collected from

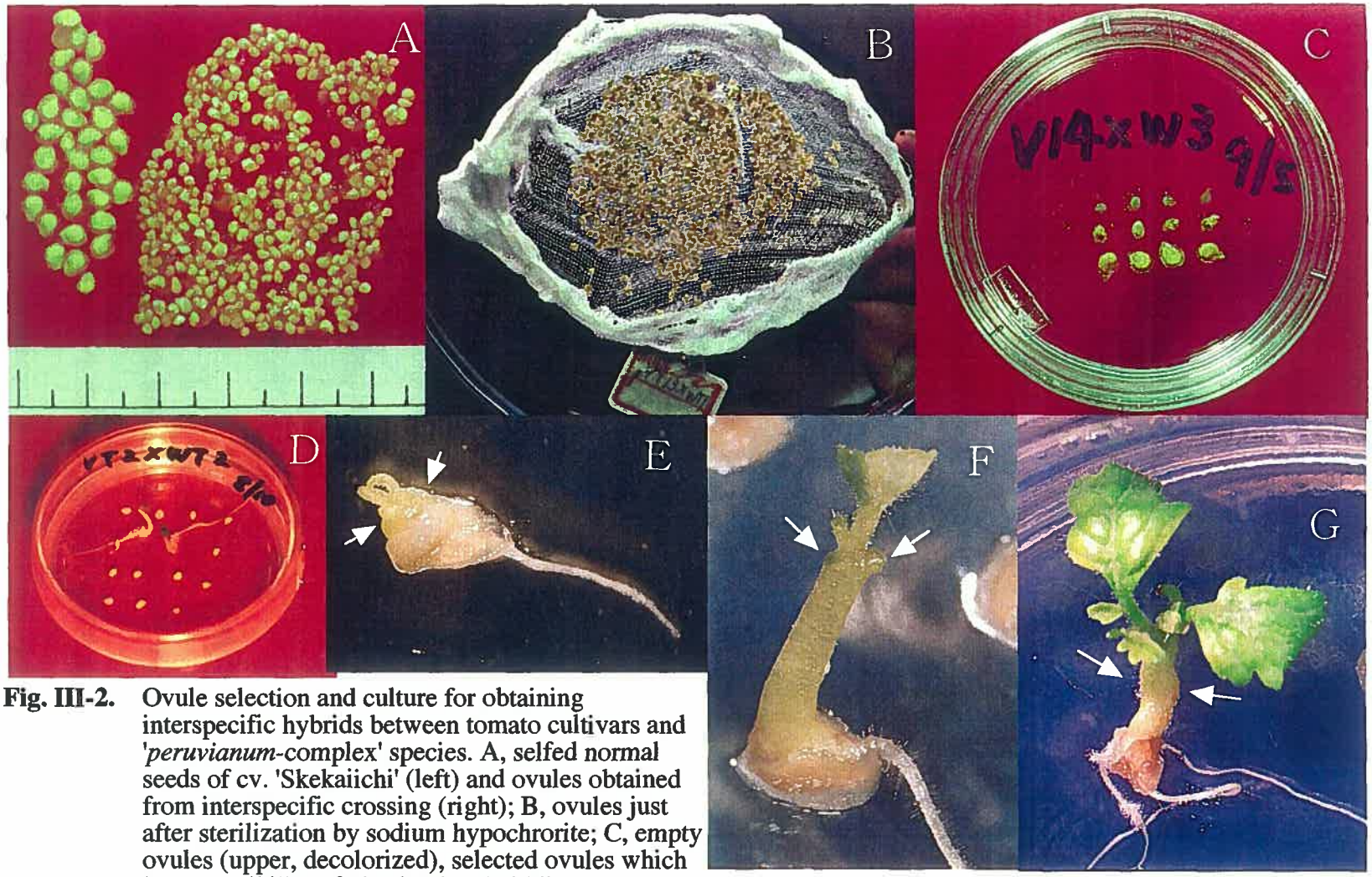

Fig. III-2. Ovule selection and culture for obtaining interspecific hybrids between tomato cultivars and '*peruvianum*-complex' species. A, selfed normal seeds of cv. 'Skekaiichi' (left) and ovules obtained from interspecific crossing (right); B, ovules just after sterilization by sodium hypochrorite; C, empty ovules (upper, decolorized), selected ovules which have possibility of girmination (middle, non-decolorized) and normal seeds of a cultivar (lower); D, germinatin of selected ovules; E-G, germinated ovuels which have no normal cotyledons.

the wild accessions just before pollination. The number of set fruits on an inflorescence was limited to five. About 40-50 days after pollination, reddish fruits were harvested. The ovules were collected and washed free of their jelly-like substances. After rating OPF, the ovules were sterilized by soaking in 3%(v/v) sodium hypochlorite (0.15% available chlorine in final concentration) for 7 minutes, and then rinsed thoroughly. The sterilization of ovules made it easy to estimate the viability due to bleaching inviable ovules. Putative viable ovules were selected according to the criterion of Imanishi (1988) as follows: Ovules with normal brown seed coats were regarded as viable, while transparent or whitish ovules were rated as inviable. Viable ovules also showed large axes comparatively longer (1.9 - 2.4 mm) than those of inviable ones (1.2 - 1.8 mm). Putative viable ovules were cultured on a half-strength hormone-free MS medium (Murashige and Skoog 1962), supplemented with 1.5 % sucrose and 0.8 % agar. The pH of the medium was adjusted to 5.8. The cultures were incubated at 25°C under a 16-hour photoperiod of white fluorescent light of about 2.28 $\mu Em^{-2}sec^{-1}$. Three weeks after culture initiation, the number of germinated ovules, GPO, and GPF were determined. The relationship among OPF, GPO and GPF is formulated: GPF = OPF × GPO.

3.2.3. Identification of hybridity

The hybridity of the putative hybrid plants obtained was identified on the basis of morphological characteristics such as leaf shape and growth habit (prostrate stem type). This is because hybrid plants between cultivated tomatoes and the PC are clearly different from cultivated tomatoes and are similar to the wild parents (Nagata and

Imanishi 1984, Takashina *et al.* 1997).

3.2.4. Statistical analysis

Analysis of variance for the rate of fruit set (RFS), OPF, GPO and GPF was performed using the ANOVA procedure of SAS® (SAS Institute 1988). Main effects of year (Year), varietal genotype (VGT), PC genotype (PGT) and their interactions were tested. RFS and GPO data, and GPF data were transformed by the function of arcsine $\sqrt{x}$, and $\sqrt{x}$, respectively (Snedecor and Cochran 1967). Multiple comparison of means was carried out by the REGWQ option of SAS.

Correlation coefficients were calculated to investigate the relationship of RFS, OPF, GPO and GPF with the seven different morphological traits of sexual organs (stigma width, anther length, anther width, style length, style width, ovary length, and ovary width) and three fruit-related traits (fruit length, fruit width, and fruit weight) for the nine varieties in both 1991 and 1993. The calculation was performed by using the CORR procedure of SAS. More than seven flowers were used for the sexual organ traits data, while for the fruit traits data, all fruits obtained were used.

3.3. Results

3.3.1. Interspecific crossing and in vitro ovule culture

In ovule culture, there were some germinated ovules that only rooted, or stopped growing before true leaves developed. However, most of the germinated ovules grew into mature plants, all of which were identified to be as hybrids by morphological

characteristics. Without exception, the hybrid plants did not have complete cotyledons.

RFS, OPF, GPO and GPF observed after the interspecific crossing are shown in Table III-1 and the results of variance analysis for them are shown in Table III-2. For RFS, the effects of Year and VGT factors were statistically significant. The RFS in 1993 was significantly lower than in 1991. 'Delicious' (DL), 'Kurihara' (KH), and 'Sekaiichi' (SK) were significantly higher than 'June Pink' (JP). The RFS of JP was very low, less than 0.5 in both the years. For OPF, the VGT was highly significant, and the Year and the Year x VGT interaction were also significant. Multiple comparisons of OPFs among varieties were then performed by year. In summary, the orders of pedigree groups in OPF {namely the PR and its derivatives group, DL, KH, 'Matsudo Ponderooza' (MP), 'Ponde Rooza' (PR) and SK > the 'Earliana' derivatives group, JP and 'Early Pink' (EP) > the others, 'Best of All' (BA) and 'Roma' (RM)} were stable over the two years, although between-year-changes of OPFs in BA, DL and PR were a little larger. The difference between the group of DL, KH, MP, PR and SK and the other group of BA and RM was statistically significant. For GPO, only the Year was significant. The GPO, contrary to RFS, was higher in 1993 than in 1991. For GPF, the Year and the VGT were significant, and the GPF in 1993 was significantly higher than in 1991 with the same tendency as GPO. Since the significance of the VGT on GPF was very marginal, multiple comparisons could not reveal significant differences among varieties. The higher GPF, however, tended to be observed in DL, EP, PR and SK, especially in the following four cross-combinations over the two-year period: EP × LP (0.14-0.15), SK × LP (0.1-0.17), SK × LPH (0.37-0.40), and PR × LC (0.20-0.40). In contrast, the GPFs of

Table III-1. Results of interspecific crossing and *in vitro* ovule culture between nine tomato varieties (*L. esculentum*) and three '*peruvianum* - complex' (*L. peruvianum* and *L. chilense*) accessions

Female parent	Male parent																							
	L. peruvianum PI270435							*L.per* .var. *humifusum* LA2153						*L. chilense* PI128652						Average among male parents			
Variety	Year	NFS	RFS	NG	OPF	GPO	GPF	NFS	RFS	NG	OPF	GPO	GPF	NFS	RFS	NG	OPF	GPO	GPF	RFS	OPF	GPO	GPF
'Best of All'	'91	11	0.85	0	35	0	0	8	0.89	0	38	0	0	7	0.70	0	23	0	0	0.81	32	0	0
	'93	22	0.43	0	100	0	0	26	0.51	0	60	0	0	24	0.56	0	77	0	0	0.50	79	0	0
'Delicious'	'91	8	0.80	3	192	0.0020	0.3750	3	1.00	3	202	0.0050	1.0000	8	0.89	0	131	0	0	0.90	175	0.0023	0.4583
	'93	15	0.52	0	102	0	0	23	0.53	1	106	0.0004	0.0435	28	0.78	8	112	0.0025	0.28571	0.61	107	0.0010	0.1097
'Early Pink'	'91	7	0.78	1	109	0.0013	0.1429	5	0.33	0	86	0	0	3	1.00	0	68	0	0	0.70	87	0.0004	0.0476
	'93	26	0.60	4	86	0.0018	0.1538	21	0.38	1	64	0.0007	0.0476	31	0.72	11	88	0.0040	0.35484	0.57	79	0.0022	0.1854
'June Pink'	'91	3	0.43	0	88	0	0	2	0.25	0	84	0	0	4	0.80	0	75	0	0	0.49	82	0	0
	'93	22	0.28	1	101	0.0004	0.0455	18	0.19	0	78	0	0	19	0.40	4	65	0.0033	0.21053	0.29	81	0.0012	0.0853
'Kurihara'	'91	6	0.75	0	119	0	0	3	1.00	0	131	0	0	5	1.00	0	94	0	0	0.92	114	0	0
	'93	18	0.46	8	126	0.0035	0.4444	7	0.54	2	103	0.0028	0.2857	20	0.53	5	107	0.0023	0.2500	0.51	112	0.0029	0.3267
'Matsudo	'91	5	0.50	0	90	0	0	7	0.70	0	130	0	0	3	0.50	0	152	0	0	0.57	124	0	0
Ponderooza'	'93	15	0.38	1	136	0.0005	0.0667	15	0.38	2	111	0.0012	0.1333	15	0.44	1	127	0.0005	0.06667	0.40	125	0.0007	0.0889
'Ponde Rooza'	'91	14	1.00	0	185	0	0	8	0.80	0	162	0	0	10	0.67	2	114	0.0018	0.2000	0.82	154	0.0006	0.0667
	'93	20	0.45	1	69	0.0007	0.0500	22	0.50	2	91	0.0010	0.0909	30	0.58	12	124	0.0032	0.4000	0.51	95	0.0017	0.1803
'Roma'	'91	6	0.75	0	67	0	0	4	0.80	1	61	0.0041	0.2500	3	1.00	0	36	0	0	0.85	54	0.0014	0.0833
	'93	23	0.51	2	37	0.0023	0.0870	19	0.41	0	33	0	0	21	0.51	0	41	0	0	0.48	37	0.0008	0.0290
'Sekaiichi'	'91	10	0.91	1	153	0.0007	0.1	10	1.00	4	117	0.0034	0.4000	6	1.00	0	136	0	0	0.97	135	0.0014	0.1667
	'93	30	0.63	5	116	0.0014	0.1667	19	0.40	7	99	0.0037	0.3684	24	0.52	7	134	0.0022	0.29167	0.51	116	0.0024	0.2756

NFS, number of fruit set; RFS, rate of number of fruits set / number of flowers pollinated; NG, number of germinated ovules (F_1 plants); OPF, number of ovules per fruit; GPO, rate of number of germinated ovules / number of total ovules obtained; GPF, number of germinated ovules per fruit.

Table III-2. Variance analysis for RFS, OPF, GPO and GPF

		RFS		OPF		GPO		GPF	
Source	d.f.	MS	P	MS	P	MS	P	MS	P
Year	1	2.0823	0.0001	2687.33	0.0259	4.46×10^{-3}	0.0053	0.3465	0.0110
Varietal genotype (VGT)	8	0.1436	0.0117	6909.25	0.0001	8.05×10^{-4}	0.4649	0.1124	0.0444
Peruvianum -complex genotype (PGT)	2	0.1023	0.0996	650.07	0.2621	4.42×10^{-5}	0.8986	0.0052	0.8833
Year x VGT	8	0.0440	0.3848	1740.25	0.0099	6.71×10^{-4}	0.2479	0.0917	0.0871
Year x PGT	2	0.0136	0.7067	1390.83	0.0718	1.02×10^{-3}	0.1197	0.1261	0.0779
VGT x PGT	16	0.0485	0.3200	251.75	0.8684	3.97×10^{-4}	0.5138	0.0430	0.4802
Error	16	0.0383		446.00		4.19×10^{-4}		0.0419	

RFS, OPF, GPO and GPF, see Table 1. MS, mean square.

RFS and GPO data and GPF data were transformed by the function of arcsine$\sqrt{x}$ and$\sqrt{x}$, respectively.

BA were all zero in both of the years. The PC genotype (PGT) and its interactions (Year x PGT and VGT x PGT) had no significant effects on RFS, OPF, GPO or GPF.

3.3.2. Correlation analysis

Correlation coefficients of RFS, OPF, GPO and GPF with sexual-organ- and fruit- related traits are shown in Table III-3. OPF had a significant positive correlation with sexual-organ- and fruit- width (stigma width, style width, ovary width, and fruit width) and fruit weight. In addition, OPF also had a significant negative correlation with style length. OPF had no significant correlation with GPO. GPO had a significant weak negative correlation with anther length. GPF had no significant correlation with any sexual-organ- and fruit-related traits. However, it had significant positive correlation coefficients with both OPF and GPO. The size values for sexual organs and fruits correlated with OPF and GPO in the varieties with a GPF of more than 0.1 are shown in Table III-4 for reference.

3.4. Discussion

The present results revealed the three following points. First, enhancing both OPF and GPO was necessary for high GPF, due to the low correlation between OPF and GPO. Second, the OPF and GPO were significantly affected by the VGT and Year factors, respectively. Third, varieties such as PR and SK with wider sexual organs and fruits would be good pistillate-parental partners owing to the higher OPF and GPF. This work may be the first to have described the hybrid-production-ability-related components

Table III-3. Phenotypic correlation coefficients of RFS, OPF, GPO and GPF with 7 sexual organ- and 3 fruit- related traits in 9 tomato varieties for two years

	Stigma wid.	Anther len.	Anther wid.	Style len.	Style wid.	Ovary len.	Ovary wid.	Fruit len.	Fruit wid.	Fruit weight.	RFS	OPF	GPO
RFS	0.0667	0.3047	-0.2062	0.2836	-0.0717	0.6365**	-0.166	0.3399	0.1044	0.1524			
OPF	0.7951***	-0.2173	0.3875	-0.4736*	0.6942**	0.0170	0.6297**	0.0624	0.7642***	0.7341***	0.269		
GPO	-0.0260	-0.4817*	-0.2886	-0.3668	-0.0672	-0.4378	-0.1329	0.0127	0.0374	0.0091	-0.0247	0.2654	
GPF	0.2116	-0.3642	-0.1578	-0.3621	0.1264	-0.2634	0.0540	-0.0163	0.2103	0.1930	0.1324	0.5396*	0.8863***

*, **, and ***, significant at P=0.05, P=0.01 and P=0.001, respectively. RFS, OPF, GPO and GPF, see Table 1. Wid, Width. Len, Length.

Table III-4. Ranges of seven sexual-organ- and fruit-related morphological traits significantly correlated with OPF and GPO in the pistillate parents with GPFs of more than 0.1

Trait	Range	Average
Stigma wid.	0.88 - 1.24 mm	1.04 mm
Anther len.	9.05 - 10.45 mm	9.94 mm
Style len.	7.04 - 7.78 mm	7.37 mm
Style wid.	0.63 - 0.89 mm	0.77 mm
Ovary wid.	2.33 - 2.95 mm	2.63 mm
Fruit wid.	66.20 - 75.33 mm	69.60 mm
Fruit wt.	122.0 - 176.7 g	143.91 g

OPF, GPO and GPF, see Table III-1.
wid., width. len., length.

affecting GPF and its relation with reproductive organ morphology, which may be useful for finding favorable pistillate parents.

The seedlings of interspecific hybrids which lacked complete cotyledons were observed also by Sacks *et al.* (1997) in the cross combination of *L. esculentum* × *L. peruvianum*, and Chmielewski (1966) in *L. hirsutum* f. *glabratum* × *L. esculentum*. Chmielewski considered that the cotyledon deformation might be caused by some physical stress resulting from the difference in seed and embryo sizes between the species. At least, as in *L. esculentum* x the PC, such an aberration could be used as an alternative marker for differentiating hybrid seedlings from selfed seedlings after interspecific crossing.

The pistillate-parental differences of GPF in interspecific crossing between the tomato varieties and the PC were observed by Imanishi (1988), Chen and Imanishi (1991), and Sacks *et al.* (1997). These results are consistent with them. In considering

the formula GPF = OPF × GPO, the significant positive correlation between GPF and OPF and between GPF and GPO as well as the low correlation between OPF and GPO, it can be concluded that the combination of a high OPF and a high GPO would be required in order to enhance GPF,

Sacks *et al.* (1997) suggested the necessity of choosing favorable cultivated varieties as pistillate-parental partners to consistently produce interspecific hybrids under different environmental conditions. In addition, the present variance analysis revealed that the varietal genotype significantly affected not GPO, but OPF and GPF; that is, the pistillate-parental differences of GPF may depend on OPF. In the present experiments, relatively high and stable OPFs were observed in PR and its derivatives. This indicates that those varieties may have gene(s) which enhance OPF, or a high capacity for fertilization. Considering the present variance analysis and correlation analysis of OPF together, it may be important to pay attention to choosing varieties that have especially wider sexual organs and fruits for high OPF and GPF.

Contrastingly, GPO appeared to depend on environmental factors rather than on parental genetic factors, since GPO was significantly affected not by the VGT and PGT, but by the Year. GPO generally became larger in 1993 than in 1991. The weather during the period from initial crossing (late June) to final fruit harvesting (late August) in 1993 was unusually cool with average temperature of 21.3°C, which was 2.0°C lower than those in an average year, and cloudy with total sunshine hours of 136.7 hours per month, which was 71% of those in an average year. As shown in the present results, Kuriyama *et al.* (1970) and Sacks *et al.* (1997) also reported that a lower temperature

was better for the higher production of '*L. esculentum* x *L. peruvianum*' hybrids. It is likely that environmental control or choosing suitable places with an appropriate climate is important for a high GPO.

Interestingly, GPO had a significant weak negative correlation with anther length. All the pistillate parental varieties whose anther length was longer than 10.45 mm could not produce any hybrids with any wild accessions (the latter data was not shown). Conversely, except for BA, all the varieties whose anther length was equal to or less than 10.45 mm, produced one or more hybrids. These facts imply that both GPO and sexual organ lengths are synchronously variable after being affected by environmental conditions. However, now it is not sure whether GPO is directly affected by environmental conditions or by anther length itself.

In this study, only the efficiency of obtaining hybrids was discussed. Takashina *et al.* (1997) and Doganlar *et al.* (1997) pointed out that the efficiency was reduced in the BC_1 generation. It is interesting to investigate whether or not the varietal differences are observed in backcross generations.

In conclusion, in order to enhance GPF, attentions to choosing such varieties as having wider reproductive organs for high OPF and appropriate environmental conditions for high GPO may be needed. This results would be helpful in finding promising pistillate parents from leading varieties suitable for a specific country, and enhancing the efficiency of obtaining interspecific hybrids between the cultivated variety and the PC for further tomato breeding.

4. Chapter IV.

Genetic Analysis of Self- and Unilateral Incompatibility in the Progeny of *Lycopersicon esculentum* Mill. × *L. chilense* Dun.

4.1. Introduction

The wild tomato species possess valuable horticultural traits such as resistance to biotic and abiotic stresses and fruit quality attributes. In order to utilize such traits for genetic improvement of the cultivated tomato (*L. esculentum* Mill.), interspecific crosses have often been carried out between the cultivated tomato and the wild species. However, unilateral incompatibility (UI) and self-incompatibility (SI) are hampering the breeding operations in producing interspecific hybrids and their progenies.

In the UI of many plant species, pollen-tube growth of self-compatible (SC) species stops in the style of SI species, whereas in the reciprocal cross, the pollen tube of SI species normally grows and successfully fertilizes the female gamete of SC species. Such a relationship between SI and UI is called the SI × SC rule. Due to such a SI × SC rule, the cytoplasm of SI species cannot be used to develop new varieties. Since such UI phenomenon often occurs in connection with the SI phenomenon, the SI × SC rule has been considered to be caused by the effect of self-incompatibility gene (S-gene) on the UI (Lewis and Crowe 1958). Also in the *Lycopersicon*, the SI × SC rule almost holds true. However, on the basis of the exceptions from the SI × SC rule, some researchers have supported a hypothesis that UI is controlled by a genetic system unrelated to the S-gene (Martin1967, Hogenboom1972bc, Liedl *et al.* 1996). Thus, in the *Lycopersicon*, the relationship between the UI and the S gene has not been

established yet.

On the other hand, the gametophytic SI of *Lycopersicon* is controlled by a single-S-locus gene (Lamm 1950, McGuire and Rick 1954) that is located near the centromere of the chromosome 1 (Tanksley and Loaiza-Figueroa 1985). However, it has often been reported that the mode of inheritance of SI in the progeny of interspecific hybrids cannot always be explained according to the hypothesis of single-S-gene control, and that both the S-gene and modifying gene(s) may be involved in the SI phenotype (Martin 1968, Bernatzky *et al.* 1995).

Recently, from the viewpoint of molecular biology, many interesting findings were reported on the S-gene product. Mau *et al.* (1986) showed a high level of homology between amino-terminal sequences of the two S-gene products (S-glycoproteins) obtained from the styles of *L. peruvianum* and *Nicotiana alata*. McClure *et al.* (1989, 1990) clarified that the S-glycoprotein of *Nicotiana alata* is RNase, which is then called S-RNase, and suggested that the S-RNase causes degradation of pollen rRNA and consequently arrests pollen-tube growth. In fact, some researchers have demonstrated that the S-RNase activity is essential to expression of the SI (Kowyama *et al.* 1994, Lee *et al.* 1994, Royo *et al.* 1994). Also in Rosaceae (Sassa *et al.* 1992, Tao *et al.* 1997) and Scrophulariaceae (Xue *et al.* 1996), their SIs have been reported to be controlled by S-RNases.

The objective of this study is to clarify the genetic mechanisms of UI and SI in the progeny of interspecific hybrids between *L. esculentum* and *L. chilense*.

4.2 Materials and Methods

4.2.1. Plant materials

SC *L. esculentum* cv. 'Sekaiichi' (for short, SK), SI F_1 plant between SK and self-incompatible *L. chilense* PI128652 (for short, F_1), and 68 BC_1 plants obtained from the cross of SK × F_1 were used to investigate the segregation and pollen-tube-growth inhibition for SI and UI. The UI is observed in the cross of *L. chilense* × SK and the cross of F_1 × SK. Of 68 BC_1 plants, 45 plants selected randomly were used for stylar protein analysis. SK plants were grown from seeds, and F_1 and BC_1 plants, which were obtained through interspecific crosses and ovule culture (Imanishi 1988), were maintained by vegetative propagation.

4.2.2. Determination of SI and UI

Flower buds with yellow petals and anthers were collected one day before anthesis for observation of SI and UI reactions in the style. After emasculation, the peduncles of flower buds were put onto 0.8% agar medium (0.8-*g* agar + 100-*ml* water) in a plastic box ventilated with membrane filter (0.22 μm pore size). The stigma was pollinated with self- or SK pollens collected freshly for the determination of SI and UI. Buds pollinated were incubated at 25 °C for 48 h. Pollen-tube growth in the pistil was observed under a fluorescent microscope after staining with aniline blue dye as described by Maheswaran *et al.* (1986). Some BC_1 plants used in this study could not produce fruits or seeds even after self or SK pollen tubes penetrated the base of the styles. Then, the term of 'incompatibility' was used only for the pollen-tube-growth

inhibition in the style, and 'compatibility' was used for normal pollen-tube growth in the style even without fruit and seed set. SI and UI were determined on the basis of the behavior of pollen tubes (Fig. IV-1) and the number of pollen tubes (less than five) penetrating the style base. The data were recorded after observing more than five styles.

4.2.3. Protein-gel electrophoresis and detection of RNase-activity

Extraction of stylar protein, SDS-PAGE, silver staining, and RNase activity staining were performed according to Kowyama *et al.* (1994).

4.3. Results and Discussion

4.3.1. Fluorescent microscopic observation of pollen tubes showing SI and UI

Inhibition of pollen-tube growth in the SI and the UI style was clearly different in the position where pollen tubes stopped growing and in the shape of the pollen-tube tip (Fig. IV-1). That is, the pollen-tube-growth inhibition in the UI was observed near the stigma, i.e., in a narrow range of about 19 - 25% upper part of the style from the stigma, while SI inhibition was observed in a broad range of about 20 - 90 % of the style from near the stigma to near the style base. The observation that the SI and UI pollen tubes stopped growing in different positions of the styles was consistent with the report of Liedl *et al.* (1996) where *L. esculentum* and *L. pennellii* were used. Moreover, most of the pollen-tube tips in the UI burst out like horns, whereas pollen-tube tips in the SI burst and swelled. Such different shapes of the SI and the UI pollen-tube tips were observed also by De Nettancourt *et al.* (1974). These observations suggest that different

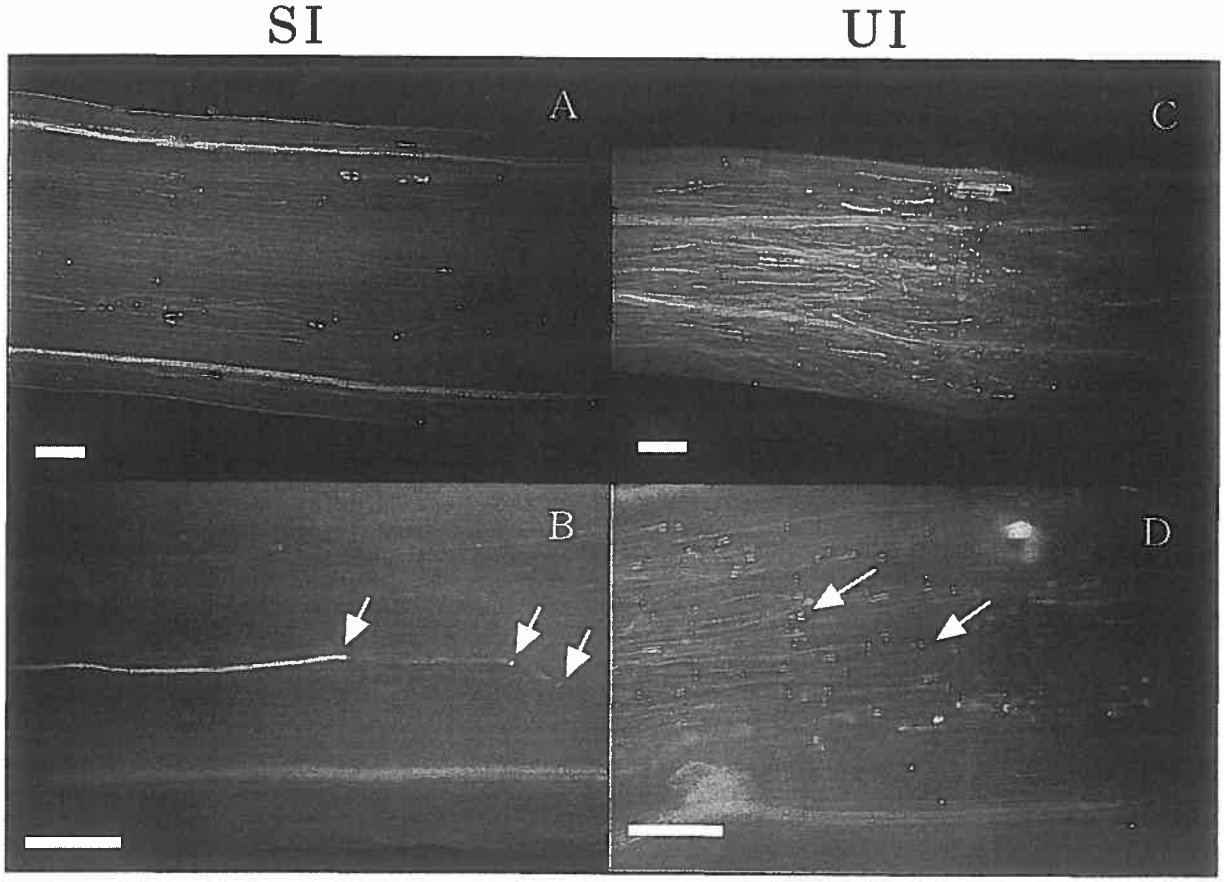

Fig. VI-1. Pollen-tube behavior of self- and unilateral incompatibility in the styles of an F_1 plant between *L. esculentum* cv. 'Sekaiichi' x *L. chilense* PI128652 and BC_1 plants from cv. 'Sekaiichi' x F_1. A white bar in the figure indicates 0.1mm. A, typical self-incompatible pollen-tubes observed in the style of a BC_1 plant; B, self-incompatible pollen-tubes in the selfed F_1 style.Arrows indicate swollen pollen-tube tips; C, typical unilateral incompatible pollen-tubes observed in the F_1 style pollinated with cv. 'Sekaiichi' pollens; D, unilateral incompatible pollen-tubes in the style of a BC_1 plant pollinated with cv. 'Sekaiichi'. Arrows indicate burst pollen-tube tips.

recognition systems may be involved in the SI and UI reactions in the *Lycopersicon*.

4.3.2. Segregation for SI and UI in BC_1 generation

As shown in Table IV-1, segregation of 37 : 31 for UI and bilateral compatibility (C) in the BC_1 generation showed a good fitness to the expected ratio of 1 : 1 for single-allele segregation (P = 0.467). On the other hand, the segregation of 48 : 20 for SI and SC showed a good fitness to the expected ratio of 3 : 1 for two-allele segregation (P = 0.401). In addition, the segregation of UI and SI was not statistically independent (P < 0.001). These results suggest that UI and SI are controlled by one gene and two genes, respectively, and that one of the two SI genes is associated with UI.

Table IV-1. Segregation for self- and unilateral incompatibility in the BC_1 generation from *L. esculentum* x F_1 (*L. esculentum* x *L. chilense*)

	SI[2)]	SC	Total
UI[1)]	35	2	37
C	13	18	31
Total	48	20	68

[1)]UI and C, unilateral incompatibility and compatibility with *L. esculentum* pollens, respectively;

[2)]SI and SC, self-incompatibility and compatibility, respectively.

Goodness of fit for UI : C = 1 : 1, χ^2=0.529 (P=0.467).

Goodness of fit for SI : SC = 3 : 1, χ^2=0.706 (P=0.401).

Test of independence between UI and SI, χ^2=20.064(P<0.001).

Generally, SI of the Solanaceae family is controlled by a single S-locus gene. However, there are some reports that SI may be controlled by modifying gene(s) in

addition to the S-gene in the progeny of interspecific hybrids of *Lycopersicon* (Martin 1968, Bernatzky *et al.* 1995). Martin (1967) indicated that SI and UI in the progeny of *L. esculentum* and *L. hirsutum* were controlled by two major genes, and suggested that SI and UI were strongly correlated in the pollen tube growth inhibition. These reports are consistent with the present study.

4.3.3. Analysis of the style protein and its relationship with SI and UI

The S-glycoprotein (S-RNase) in *Lycopersicon* is about 30kD in molecular size (Mau *et al.* 1986). Also in the present experiments, three to five-protein bands with RNase activity and molecular sizes of about 30kD were found in SDS-PAGE of stylar extract (Fig. IV-2). Of these protein bands, two protein bands (B and C) were shared by all the materials used in this study. In addition, as shown in the photograph of RNase active staining, all the materials also shared another RNase band (D). The band D seems to comprise one or two faint band(s) in the photograph of silver staining. The reasons why the band(s) D is(are) faint in the photograph of silver staining though clearly visible in the photograph of RNase activity staining would be considered to be a very small amount of the protein D with a relatively high level of RNase activity and short time for development in the silver staining in order to obtain a clear background. At least, the B, C and D protein bands would be unrelated to SI and UI because those bands were shared by all the materials. The other protein band A was observed only in the F_1 and the BC_1 plants that showed UI with *L. esculentum* pollens (Fig. IV-2 and Table IV-2). Considering the present result that UI was controlled by one gene, it is concluded that

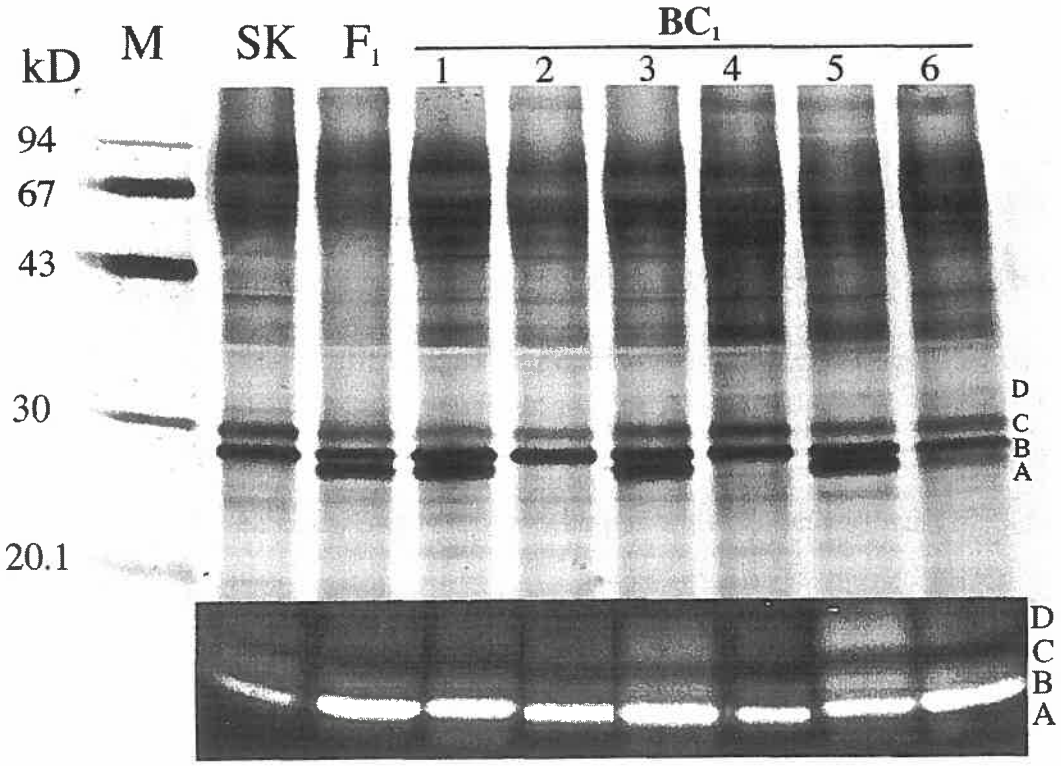

Fig. IV-2. SDS-PAGE of style protein in the progeny of *L. esculentum* x *L. chilense*. Upper, silver staining; Lower, RNase active staining. M, marker; SK, *L. esculentum* cv. 'Sekaiichi'; F_1, SK x *L. chilense* PI128652; $BC_1$1-6, BC_1 plants from SK x F_1. $F_1$and BC_1 1, 3, 5 and 6 indicates unilateral incompatibility with SK pollens, while SK andBC_1 2 and 4 indicate compatible with SK pollens. A, B, C and D band proteins have RNase actibity. A is a unique band to the plants showing unilateral incompatibility with SK pollens.

Table IV-2. Segregation frequency of BC_1 plants for the protein-band A, self- and unilateral incompatibility

band A		SI	SC	Total
+[1)]	UI	26	2	28
+	C	0	0	0
-	UI	0	0	0
-	C	7	10	17
Total		33	12	45

[1)]+ and -, presence and absence of the protein-band A, respectively.

Test of independence between band A and SI, χ^2=11.925 (P<0.001)

the band A protein may control UI. As shown in Table IV-2, of the 28 BC_1 plants with the protein band A, two plants were self-compatible, and of 17 plants without the protein band A, seven plants were self-incompatible. As a result of the independence test between the protein band A and SI, the relationship was highly significant ($P<0.001$). Then, the band A would probably not only contribute to UI, but also to SI. These results suggest that the band A protein may be S-RNase.

According to some reports, UI may be controlled by the S-gene. Lewis and Crowe (1958) firstly proposed the hypothesis that the S-gene has both SI and UI function simultaneously. Bernacchi and Tanksley (1997) also mapped a major QTL controlling UI to the S locus using the backcross generation of *L. esculentum* × *L. hirsutum*.

Since the SI of *Lycopersicon* is gametophytic, the SC pollen would be able to fertilize the SI female gamete as well as the SC female gamete in selfing of F_1 plants between SI and SC plants. Therefore, the F_1 plants between SI and SC plants are expected to be SC. In fact, F_1 hybrids between SC and SI accessions in a species are SC as shown in F_1s between an SI accession (Nazca) and an SC accession (Atico) in *L. pennellii* (Hardon 1967) and in F_1s between an SC accession LA2157 and an SI accession LA2163 in *L. peruvianum* (Rick 1986, Kowyama 1994). However, the F_1 between SC and SI species is SI as shown in the F_1s from the cross-combinations of SC *L. esculentum* with SI *L. peruvianum* (McGuire and Rick 1954), SI *L. chilense* (Martin 1961), SI *L. hirsutum* (Canta) (Martin 1967) and SI *L. pennellii* (Nazca and Sisicaya) (Hardon 1967) as male counterparts. Interestingly, the SC F_1s as mentioned above had

parents not showing UI in the reciprocal crosses, while the SI F_1s as mentioned above had parents that showed UI. These facts seem to indicate that the expression of SI in F_1s is associated with UI gene(s). Abdalla and Hermsen (1972) also explained the reason why the hybrids between *L. esculentum* and *L. pennellii* were SI (Hardon 1967) in the same way.

If the S-gene and UI gene(s) were genetically independent, SC plants would be expected to segregate in the F_2 population from sib crosses of SI F_1 hybrids described above. However, McGuire and Rick (1954) and Hardon (1967) showed that all the F_2 plants obtained from sib crosses of SI F_1s were SI. As the reason, De Nettancourt *et al.* (1974) considered that the UI gene is the same as the S-gene, or is closely linked to the S-gene on the chromosome.

Contrastingly, some researchers explained that UI is controlled by gene(s) unrelated to the S-gene from the exception of the SI × SC rule in *Lycopersicon* (Rick 1960, Hogenboom 1972bc) and from the difference of UI and SI reactions in the timing and location of expression in the style (Liedl *et al.* 1996). Interestingly, in *Nicotiana*, Murfett *et al.* (1996) showed that different (S-RNase-dependent and independent) interspecific pollen-rejection mechanisms control the expression of UI in the different genetic background among species, and suggested that loss of SI function in the style could result from absence or defectiveness of S-RNase itself and from defectiveness of a non-S-RNase factor which controls the SI expression. These facts imply that multiple genetic systems related and unrelated to the S-gene may control the unilateral pollen-rejection also in *Lycopersicon*.

4.3.4. Genetic factor(s) of SI within the ovary

In the present study, of 18 BC_1 plants with SC and bilateral cross-compatibility determined by the *in vitro* pollen-tube penetration through the style (Table IV-1), five BC_1 plants produced fruits and viable seeds by self-pollination in the experimental field. Such a low frequency of the plants that bore selfed fruits may be attributed to incompatible factor(s) within the ovary. Hogenboom (1972bc) described that the UI between *L. peruvianum* ovary and *L. esculentum* pollen seems to be governed by one or more genes that are independent of the S-gene. In this study, considering the segregation ratio (5 / 18) of the BC_1 plants that bore selfed fruits, the number of genes controlling the incompatibility in the ovary might be one or two.

4.3.5. Conclusion

This is the first report to have shown that RNase may control UI in the interspecific crosses in *Lycopersicon*. In the further study, the amino acid sequencing of the band A protein would directly clarify whether the S-gene controls UI or not.

5. Chapter V.

Genetic Analysis of Sucrose-Accumulating Ability in *Lycopersicon peruvianum*

5.1. Introduction

The cultivated tomato *Lycopersicon esculentum* (LEL) species, which belongs to the red-fruited group of the genus *Lycopersicon*, produces fruits that accumulate mainly hexoses, namely glucose and fructose, and little or no sucrose (Davies 1966, Manning and Maw 1975, Mochizuki *et al.* 1986, Garvey and Hewitt 1991, Mochizuki *et al.* 1996). On the contrary, *L. chmielewskii* (LCW), *L. hirsutum* (LHT) and *L. peruvianum* (LPV), which belong to the green-fruited group, accumulate mainly sucrose in fruits (Davies 1966, Manning and Maw 1975, Mochizuki *et al.* 1986, Yelle *et al.* 1988, Miron and Schaffer 1991, Stommel 1992, Mochizuki *et al.* 1996). In the former, sucrose translocated from leaves to fruits is hydrolyzed to hexose by acid invertase and sucrose synthase. In the latter, sucrose accumulates, because sucrose is not hydrolyzed due to the low activity of acid invertase and sucrose synthase (Manning and Maw 1975, Yelle *et al.* 1988, Miron and Schaffer 1991). Therefore, it is assumed that it may be possible to develop new tomato varieties with a sweeter taste including a higher concentration of sucrose, if the sucrose-accumulating ability of the wild species could be introduced into LEL.

The LCW species, which is compatible with LEL (Rick 1979, Mutschler and Liedl 1994), has been studied as a useful genetic resource to improve the quality of soluble solids in the cultivated tomato (Rick 1974). These studies inspired many

researchers to introduce sucrose-accumulating genes from LCW into cultivated tomatoes (Yelle *et al.* 1991). In the progeny of interspecific LEL × LCW crosses, it was found that the sucrose-accumulating trait is controlled by a monogenic recessive gene, i. e. an acid invertase gene, and that this gene is located near the RFLP marker TG102 in the centromeric region of the chromosome 3 (Chetelat *et al.* 1993, 1995). Similarly, the sucrose-accumulating ability of LHT and *L. pennellii* (LPN) was introduced into cultivated tomatoes, and found to be controlled by a monogenic recessive gene (Stommel and Haynes 1993, Hadas *et al.* 1995) and by multiple recessive genes (Mochizuki *et al.* 1996), respectively. On the other hand, LPV displays the largest genetic diversity among the *Lycopersicon* species (Rick 1986, Miller and Tanksley 1990, Bretó *et al.* 1993), and is considered to be promising for providing alternative sucrose-accumulating breeding materials to LCW, LHT and LPN. However, no researchers have reported the mode of inheritance of the sucrose-accumulating ability of LPV, presumably due to the strong cross incompatibility between LEL and LPV (Hogenboom 1972a, Rick 1979).

The acid invertase gene of LEL was sequenced (Klann *et al.* 1992, Ohyama *et al.* 1992, Elliot *et al.* 1993, Sato *et al.* 1993), as well as parts of the acid invertase genes of LCW and LHT (Hadas *et al.* 1995, Harada *et al.* 1995). Based on these results, PCR primers were designed in order to detect the differences between the acid invertase genes of LEL and LCW, or LEL and LHT (Chetelat *et al.* 1995, Hadas *et al.* 1995, Harada *et al.* 1995). Imanishi *et al.* (1996) successfully selected wild (sucrose-

accumulating) types for the acid invertase genotype from the BC_2F_2 generation by a PCR assay with the primers designed by Harada *et al.* (1995). This BC_2F_2 is a self-compatible population obtained from the interspecific crosses of LEL cv. 'Early Pink' (for short, EP) with LPV var. *humifusum* LA2153 (for short, LA2153) by using the ovule culture method (Imanishi 1988) to overcome their strong cross-incompatibility.

The present study was carried out to obtain information for utilizing the sucrose-accumulating ability from LPV for tomato breeding. The objectives of the study were as follows: 1) to analyze the mode of inheritance of the sucrose-accumulating ability of LPV using the progeny of LEL × LA2153 populations and 2) to reconfirm the effectiveness of the PCR primers of Harada *et al.* (1995) in selecting sucrose-accumulating plants in the BC_2F_2 of the LEL × LA2153 which was used by Imanishi *et al.* (1996) and its progeny.

5.2. Materials and Methods

5.2.1. Plant materials

The seeds of the wild accessions described below were kindly provided by Prof. Dr. C. M. Rick of the Tomato Genetic Resource Center, University of California. The F_2 plants from sib crosses of cv. 'Kyoryoku Ogata Toko' (KOT) × LA2153 F_1s (namely, F_2-A) and the F_2 plants from crosses between cv. 'Matsudo Ponderooza' (MP) × LA2153 F_1 and cv. 'Early Pink' (EP) × LA2153 F_1 (namely, F_2-B) were used to determine the mode of inheritance of the PCR bands generated by the primers by amplifying a part of an acid

invertase (Harada *et al.* 1995), and to map the PCR band of LA2153. The mode of inheritance of the PCR bands was also studied, using BC_2F_3 plants derived from a self-crossed heterozygous type in the BC_2F_2 generation of EP × LA2153, BC_2F_4 from reciprocal crosses between KOT and a heterozygous type of BC_2F_3, and a self-crossed wild type of the BC_2F_3 generation. The relationship between the PCR-band genotypes {wild (LA2153), heterozygous, and cultivar types} and the sugar content was examined, using BC_2F_2, BC_2F_3, BC_2F_4, and BC_2F_5 plants from the selfed progeny of BC_2F_1 of EP × LA2153 and the control, EP.

F_2 plants were grown from the seeds in pots in a greenhouse. BC_2F_2, BC_2F_3, BC_2F_4, BC_2F_5 and EP were also grown from seeds to seedlings in pots in a greenhouse. The seedlings were transplanted and grown in a field for fruit harvesting after determination of their acid invertase genotypes.

5.2.2. PCR assay for the acid invertase gene and RFLP analysis

Total DNA for the PCR was isolated from approximately 0.1-0.2g of fresh young leaf tissue by a modification of the CTAB method developed by Murray and Thompson (1980). The PCR primers AIT-1 (5'-CGGTGAAAAACATTCAATGAG-3') and AIT-2 (5'-TCCACAATTGAGTGATCCAC-3') are known to amplify a part of the acid invertase genes of LEL and LCW, and to enable the discrimination between the invertase genotypes based on band-size differences (Harada *et al.* 1995). These were used to estimate the acid invertase genotypes of LEL and LA2153 as described by

Imanishi *et al.* (1996). DNA amplification and electrophoresis of the PCR products were performed according to the method used by Takashina *et al.* (1998). For RFLP analysis, 10-15μg of total DNA was digested with *Eco*RI, *Eco*RV, or *Hin*dIII. The tomato RFLP probe TG102 on the chromosome 3 (Tanksley *et al.* 1992) was kindly provided by Prof. Dr. S. Tanksley. The RFLP analysis procedure has been described in detail by Escalante *et al.* (1998).

5.2.3. Sugar determination

Ripened fruits were harvested separately from each plant. A small amount of fruit tissue in which the rind had been peeled off was lyophilized and ground. The 3-g samples in 7 ml of 80% ethanol were treated at 70 °C for 30 min., 3 times. The homogenate was centrifuged at 2400 *g* for 15 min. after each extraction. The supernatant was diluted to 50 ml with distilled water. One out of the 50 ml was used for the determination of the glucose, fructose and sucrose concentrations (mg/g FW) by a spectrophotometric test according to the method of Nishizawa and Shishido (1998).

5.3. Results and Discussion

5.3.1. PCR assay and segregation of the PCR band types in the progeny of L. esculentum × L. peruvianum var. humifusum LA2153

The PCR bands of LEL, LA2153, and their BC_2F_3 are shown in Fig. V-1. LEL and LA2153 had a single band, which corresponded to the 180bp and 188bp bands,

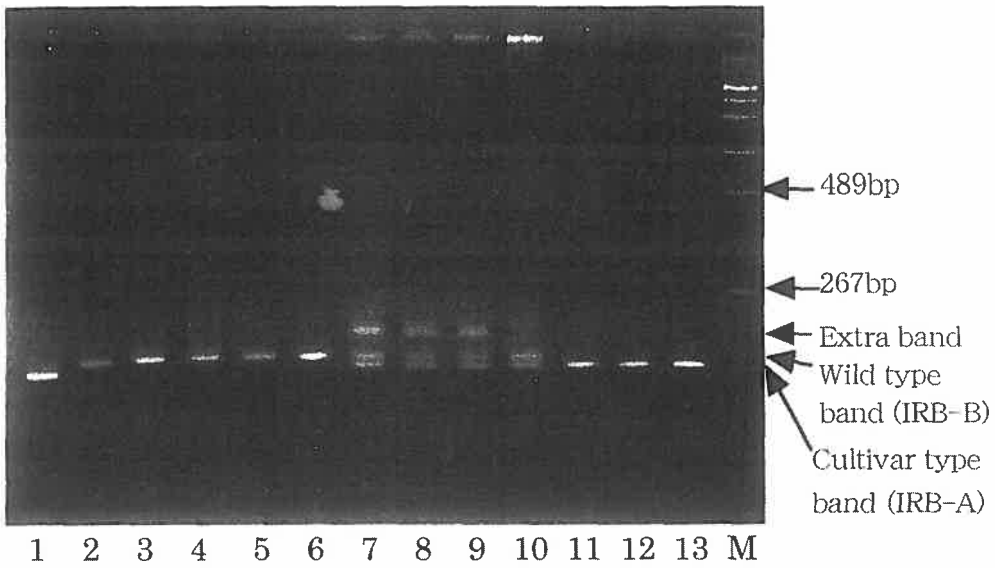

Fig. V-1. PCR bands generated by the AIT-1 and AIT-2 primers in *Lycopersicon esculentum*, *L. peruvianum* var. *humifusum* LA2153, and their BC_2F_3 plants. 1, *L. esculentum*; 2, *L. peruvianum* var. *humifusum* LA2153; 3-6, wild (LA2153) type in BC_2F_3; 7-10, heterozygous type in BC_2F_3; 11-13, cultivar (*L. esculentum*) type in BC_2F_3; M, pHY marker.

respectively, as described by Imanishi *et al.* (1996). The two bands are to be designated here as IRB (invertase-gene-sequence-related band)-A and IRB-B bands, respectively. However, most of the heterozygous types had an extra large-sized band as well as IRB-A and IRB-B derived from their parents. The extra band was also observed by Harada *et al.* (1995) and Imanishi *et al.* (1996). Harada *et al.* (1995) assumed that annealing of the *L. esculentum* fragment with the *L. peruvianum* fragment and formation of a stem-loop structure might have produced the extra band.

The segregation of PCR band types generated by the AIT-1 and AIT-2 primers in the LEL × LA2153 population is shown in Table V-1. F_2 segregation was totally within the range of the expected ratio, although a significantly distorted segregation was

Table V-1. Inheritance of the PCR band generated by the AIT-1 and AIT-2 primers in F_2, BC_2F_3 and BC_2F_4 derived from the crosses between *L. esculentum and L. peruvianum* var. *humifusum* LA2153

Generation	Cross	PCR band type			Expected ratio	χ^2	P
		W	H	C			
F_2	A[1]	3	19	3	1:2:1	6.760	0.025<P<0.05
	B[2]	10	14	4	1:2:1	2.571	0.25<P<0.50
	Total	13	33	7	1:2:1	4.547	0.10<P<0.25
BC_2F_3	Selfed H[3]	47	89	36	1:2:1	1.616	0.25<P<0.50
BC_2F_4	CxH[4]	0	6	14	0:1:1	3.200	0.05<P<0.10
	HxC[5]	0	25	23	0:1:1	0.083	0.75<P<0.90
	Total	0	31	37	0:1:1	0.529	0.25<P<0.50
	Selfed W[6]	24	0	0	1:0:0	0.000	P=1.000

W: wild type, H: heterozygous type, C: cultivar type.

[1] Sib cross of F_1 (cv.'Kyoryoku Ogata Toko' x LA2153).

[2] (cv. 'Matsudo Ponderooza' x LA2153) x (cv. 'Early Pink' x LA2153).

[3] In the BC_2F_2 generation of cv. 'Early Pink' x LA2153

[4] Cv. 'Kyoryoku Ogata Toko' x BC_2 F_3 (in the cross combination of cv. 'Early Pink' x LA2153)

[5] BC_2F_3 (in the cross combination of cv. 'Early Pink' x LA2153) x cv. 'Kyoryoku Ogata Toko'

[6] In the BC_2F_3 generation (in the cross combination of cv. 'Early Pink' x LA2153)

observed in the cross combination of A. The segregation of both BC_2F_3 and BC_2F_4 showed a good fitness to the expected ratios. Progeny from a selfed wild-type plant in BC_2F_3 was fixed with only the wild type. These results indicated that the mode of inheritance of the PCR band type generated by the AIT-1 and AIT-2 primers in the LEL × LA2153 population is monogenic, regardless of cultivar parents and segregating generations.

5.3.2. Sugar determination

Fruit sugar contents are shown for each of PCR band types in BC_2F_2, BC_2F_3, BC_2F_4, BC_2F_5 and EP in Table V-2. The sucrose content of the wild-type fruit was higher than the glucose or fructose content throughout the generations, although these wild-type fruits accumulated all of the three kinds of sugar. On the other hand, fruits of the heterozygous and cultivar types accumulated a substantial amount of glucose and fructose, but only a small amount of sucrose. This observation indicates that there is a close relation between the wild type PCR band and sucrose accumulation, and between the heterozygous or cultivar types and only slight sucrose accumulation. It also shows that the sucrose-accumulating ability of LA2153 is mainly controlled by a monogenic recessive gene, similar to the sucrose accumulation of LCW and LHT (Chetelat *et al.* 1993, Stommel and Haynes 1993, Hadas *et al.* 1995), and that this gene would be an acid invertase gene. The percentage of sucrose to total sugars in the wild-type fruits of the BC_2 generations from the cross combination of LEL with LA2153 was 44%, while

Table V-2. Relationship between PCR band types and sugar contents in fruits of BC_2F_2, BC_2F_3, BC_2F_4 and BC_2F_5 derived from the selfed BC_2F_1 progeny of cv. 'Early Pink' x *L. peruvianum* var. *humifusum* LA2153

Generation	Year	PCR band type	Sugar content (mg/g F.W.) Glucose	Fructose	Sucrose	Sucrose % (in total sugar)
BC_2F_2	1995	W	10.5	12.1	21.1	47.8
		H	16.3	17.7	0.6	1.4
		C	15.7	16.9	2.0	5.5
BC_2F_3	1996	W	9.1	11.2	13.6	40.0
		H	11.4	12.1	1.4	6.0
		C	11.4	11.7	0.6	2.9
BC_2F_4	1998	W	13.2	16.8	19.4	38.6
BC_2F_5	1998	W	11.9	15.5	19.7	32.6
		H	19.7	21.8	0.7	1.7
		C	16.9	20.3	1.0	2.6
EP	1996	C	9.3	13.2	0.0	0.0

W: wild type, H: heterozygous type, C: cultivar type, EP: cv. 'Early Pink'.

the percentages in the wild-type fruits of the BC_1 generations from the combinations of LEL and LHT (Hadas *et al.* 1995) and LEL and LCW (Chetelat *et al.* 1993) were 23% and 60%, respectively. The sucrose-accumulating ability of LA2153 might be intermediate between LHT and LCW. Therefore, LA2153 would be an alternative breeding sources.

5.3.3. Linkage analysis

Linkage analysis between the PCR band and the RFLP marker TG102 on the chromosome 3 using 42 F_2 plants was carried out to compare the location of the LA2153 invertase gene with that of the LCW invertase gene (Chetelat *et al.* 1993) (Fig. V-2 and Table V-3). The RFLP between LEL and LA2153 was obtained after digestion with the restriction enzyme *Eco*RV. The F_2 plants showed the wild, heterozygous and cultivar type- PCR bands which were identical with the TG102 genotypes. The close linkage between the PCR band and TG102 suggested that the PCR band type expresses an invertase genotype, and that LA2153 accumulates sucrose by the same genetic mechanism as in LCW.

5.3.4. Conclusion

In the progeny of LEL × LA2153, wild-type plants with only the IRB-B and cultivar-type plants with only the IRB-A showed the sucrose-accumulating and non-sucrose-accumulating ability, respectively. Moreover, using these PCR markers, the

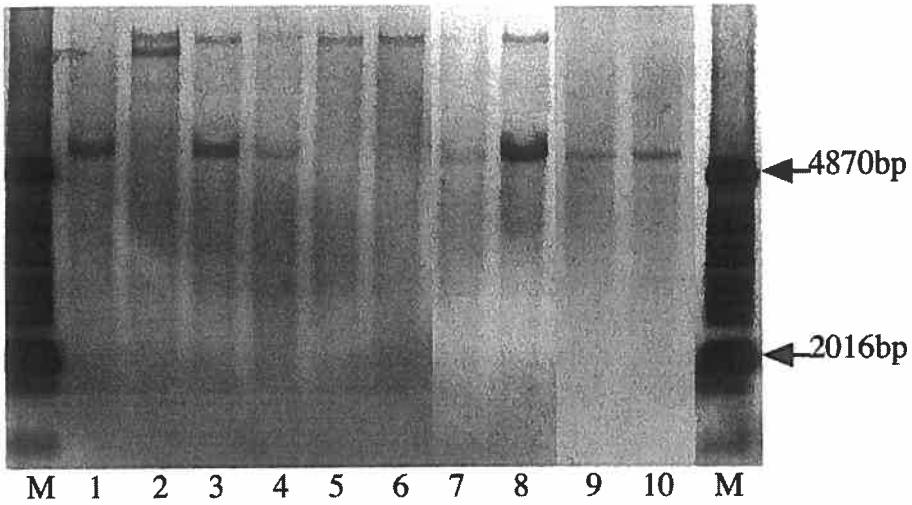

Fig. V-2. RFLP analysis with a TG102 probe and the total DNA digested with *Eco*RV in *L. esculentum*, *L. peruvianum* var. *humifusum* LA2153, their F_1 and F_2 plants. M, pHY marker; 1, *L. esculentum* cv. 'Early Pink'; 2, *L. peruvianum* var. *humifusum* LA2153; 3, 'Early Pink' x LA2153 F_1; 4, cv 'Matsudo Ponderooza' x LA2153 F_1; 5-6, wild (LA2153) type F_2; 7-8, heterozygous type F_2; 9-10, cultivar (*L. esculentum*) type F_2.

Table V-3. Co-segregation between the PCR band type and the TG102 genotype in the F2 generation derived from the crosses between hybrids of *L. esculentum* and *L. peruvianum* var. *humifusum* LA2153

PCR-band type	TG102 genotype			
	W	H	C	Total
W	13	0	0	13
H	0	22	0	22
C	0	0	7	7
Total	13	22	7	42

W:wild type, H: heterozygous type, C: cultivar type, TG102: RFLP marker

selection of sucrose-accumulators is possible for the progeny of this cross combination.

Based on the results obtained, it can be concluded that the sucrose-accumulating ability of LA2153 is controlled by a recessive acid invertase gene located on the chromosome 3, and that the PCR primers designed by Harada *et al.* (1995) are useful for selecting wild (sucrose-accumulating) type plants from the progeny of LEL × LA2153.

6. Chapter VI.

Screening of Wild Accessions Resistant to Gray Mold (*Botrytis cinerea* Pers.) in *Lycopersicon*

6.1. Introduction

Tomato gray mold (*Botrytis cinerea* Pers.) is a common disease worldwide (Watterson 1986), and often causes serious production loss by infection of all aboveground parts of leaves, stems, flowers and fruits. The fungus can easily invade dead leaves and remaining petals on fruits after anthesis, and can propagate its mycelia and conidia. Water-soaked lesions develop on fruits and finally the fruits become rotten overall. Recently, there have been serious problems of stem rotting caused by *B. cinerea*, because it infects stem through petioles or wounds after pruning and harvesting, heavily damages the entire plants, and leads to substantial yield loss (O'Neil 1994, O'Neil *et al.* 1997).

Optimum conditions for infection and spreading of *B. cinerea* onto tomato plants are about 20°C temperature with a high level of humidity. Therefore, the disease often occurs in greenhouses and plastic tunnels in moderately hot seasons. Presently, ventilation in greenhouses and plastic tunnels, and use of fungicides are practiced in order to control the disease. There are no resistant cultivars available at present.

From these reasons, breeding for tomato cultivars resistant to gray

mold is considered to be an urgent task. However, there are a very few reports about resistant materials in tomato cultivars and wild accessions (Urbasch 1986). So far, very few materials have been found to be highly resistant to the disease. The objective of this study was to identify resistant breeding materials to gray mold in wild accessions of the tomato.

6.2. Materials and Methods

6.2.1. Plant materials

Six tomato cultivars (*L. esculentum*) and 44 wild accessions, which comprised two of *L. esculentum* var. *cerasiforme*, seven of *L. chilense*, two of *L. chmielewskii*, three of *L. hirsutum*, 20 of *L. peruvianum*, three of *L. parviflorum*, three of *L. pennellii*, three of *L. pimpinellifolium*, and one accession of *Solanum lycopersicoides*, were used to evaluate the resistance to tomato gray mold. In a preliminary experiment, leaflets in young compound leaves from the first to the fourth with more than 3 cm length were found to be more susceptible than those in mature compound leaves. In many cases, lesion areas in such infected young leaflets were not usable to evaluate the resistance, because the lesion was quickly expanded to the whole area of young small leaflets. Then, leaflets of the sixth compound leaves were used in the leaflet bioassay. For the stem bioassay, 5 cm-stem-segments 10 cm down from the shoot apex were used due to the uniformity of age and width.

6.2.2. Proliferation of conidia and preparation of conidial suspension

The pathogen used in this study was isolated from tomato fruits infected naturally. The conidia were proliferated according to the modified method of Ko *et al.* (1981) as follows: Following the incubation for three days on potato-sucrose agar medium {20% (w/v) boiled potato extract, 2% (w/v) sucrose, 1.5% (w/v) agar} in dark conditions at 20°C, the mycelia were illuminated by a black light for two days, and incubated in the dark conditions another two-days for abundant formation of conidia. Conidial suspension was prepared at the density of 1×10^6 conidia / ml using a hemocytometer in suspension solution (0.1M KH_2PO_4, 2.5% glucose, a few drops of tween 20, distilled water, pH5) based on the results of Akutsu *et al.* (1987). As the conidial density becomes higher, infection response generally becomes larger to some extent. However, Masuta (1984) reported that the high conidial density of more than 1×10^6 conidia / ml inhibited the germination of conidia due to an autologous germination-inhibitory substance with low molecular weight and heat tolerance (121°C, 10 min.). Therefore, the density of 1×10^6 conidia / ml for the conidial suspension was adopted in this study.

6.2.3. Inoculation method

For the leaflet bioassay, a tip of seven sewing needles (one needle, 5.5cm length and 0.9mm width) bundled by a rubber band was dipped into the conidial suspension, and then was pushed on to the center of a leaflet. At the same time, the seven needle-holes of the pierced leaves were covered with about 5μl conidia suspension. Inoculated leaves were placed on a moistened filter paper in a 9 cm-diameter petridish. After sealed with Parafilm®, the petridishes were incubated at 20°C under a 16-h photoperiod condition. Lesion areas were measured 3 days after inoculation. The experiments were repeated more than five times. The total numbers of leaflets inoculated were five to 20 per accession.

For the stem bioassay, about 1 cm end of 5 cm-stem-segment was put into moistened vermiculite in a plastic box, and conidial suspension was dropped on to the cut surface of the upper end using a Pasteur pipette. The plastic box was covered and sealed, and incubated at 20°C under a 16-h photoperiod condition. The lesion length was measured three days after the inoculation. The experiments were repeated three times and the number of stem-segments inoculated were seven to nine per accession.

6.2.4. Statistical analysis

Both data of the leaflet and stem bioassay were used for analysis of variance and multiple comparison tests by GLM procedure and its REGWQ (Ryan-Einot-Gabriel-Welsch) option of SAS® (SAS institute 1988).

6.3. Results and Discussion

In the results of the leaflet bioassay (Fig. VI-1), *L. pennellii* LA2439 and LA1940 were significantly more susceptible than any other accessions. Considering that another accession, *L. pennellii* LA1734 was also susceptible, *L. pennellii* as a whole seemed to be a more susceptible species to gray mold than other species. Higher resistance was observed in *L. peruvianum* LA2745, LA3156, LA2981, *L. hirsutum* LA2314, and *L. pimpinellifolium* LA1246. Urbasch (1986) also reported that *L. hirsutum* accessions had resistance to gray mold, and that fact coincided with the present result for LA2314. In some accessions, lesions expanded overall in their leaves six days after inoculation, and in most of accessions lesions spread out the whole leaves ten days after inoculation. Contrastingly, only the leaves of *L. peruvianum* LA2745 remained green in color, and their lesions rarely expanded from the points infected with needles even two weeks after inoculation (Fig. VI-2). The results were reconfirmed by repeated experiments. Therefore, LA2745 is considered to be an accession

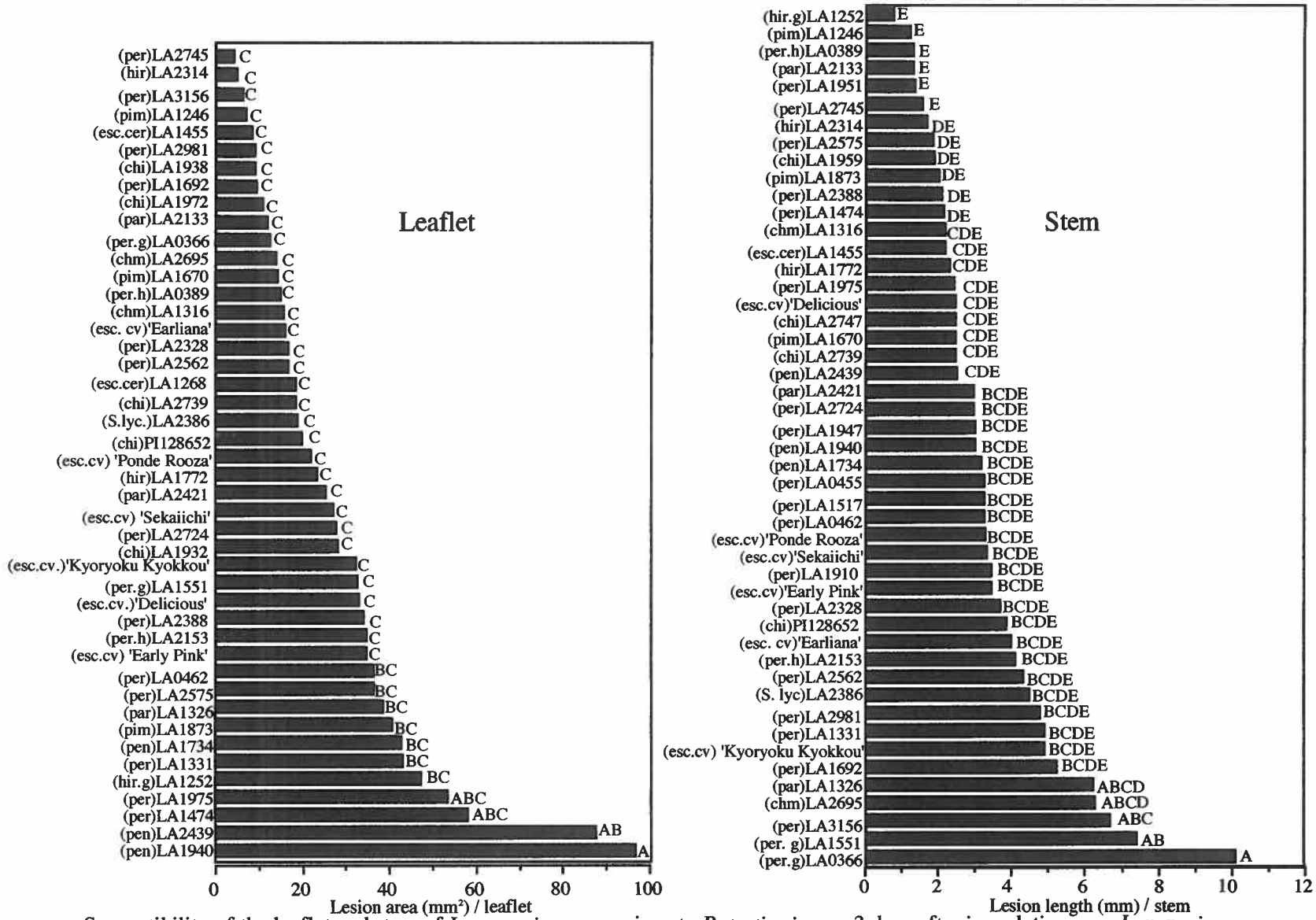

Fig. VI-1. Susceptibility of the leaflet and stem of *Lycopersicon* accessions to *Botrytis cinerea* 3 days after inoculation. per, *L. peruvianum;* per.g, *L. peruvianum* f. *glandulosum;* per. h, *L. peruvianum* var. *humifusum;* chi, *L. chilense;* hir, *L. hirsutum;* hir.g, *L. hirsutum* f. *glabratum;* pim, *L. pimpinellifolium;* esc.cv, *L. esculentum* cv.; esc.cer, *L. esculentum* var. *cerasiforme;* par, *L. parviflorum;* chm, *L. chmielewskii,* pen, *L. pennellii*; S. lyc, *Solanum lycopersicoides*. Same letters next to the graph bars show that difference of values between accessions are not significant at 5% level according to REGWQ multiple comparison procedure.

Fig. VI-2. Susceptibility of the leaf to *Botrytis cinerea* in *Lycoersicon* accessions. In A, left, *L. penellii* LA1940 (susceptible); middle, *L. parviflorum* LA2133 (moderate resistant); right, *L. peruvianum* LA2745 (highly resistant) six days after inoculation. In B, no lesion spread from inoculated points (center of the leaf) of a LA2745 leaf 12 days after inoculation. In C (magnified B), the lesion was completely inhibited from spreading.

with a high level of resistance to *Botrytis cinerea* in the leaflet.

From the stem bioassay, *L. hirsutum* f. *glabratum* LA1252, *L. pimpinellifolium* LA1246, *L. peruvianum* var. *humifusum* LA0389, *L. parviflorum* LA2133, *L. peruvianum* LA1951, and LA2745 were significantly more resistant than *L. peruvianum* LA3156, LA1551, *L. peruvianum* f. *glandulosum* LA0366, *L. chmielewskii* LA2695 and *L. parviflorum* LA1326. The correlation coefficient between susceptibilities of the leaflet and the stem was very small on the lesion area ($r=-0.127^{ns}$). However, *L. peruvianum* LA2745, *L. hirsutum* LA2314, and *L. pimpinellifolium* LA1246 showed a high level of resistance both in the leaflet and in the stem. Although *Solanum lycopersicoides*, a wild relative species of tomato, was reported to possess resistance to the gray mold disease (Gradziel and Robinson 1989), *S. lycopersicoides* accession LA2386 used in the present study failed to show high resistance either in the leaflet or in the stem.

At present, hybrids between cv. 'Sekaiichi' (for short, SK) and LA2745, and between SK and LA1246 have been raised. The leaflet of the hybrids of LA2745 indicated as high resistance as LA2745, while the leaflet of the hybrids of LA1246 was not so high in resistance. Then, at least, it was suggested that the resistance of LA2745 may be controlled not by the environmental factor(s), but by the dominant genetic factor(s).

It is reported that *L. peruvianum* has the largest genetic diversity among *Lycopersicon* species (Rick 1986, Miller and Tanksley 1990, Bretó *et al.* 1993). Actually, *L. peruvianum* accessions have been used as breeding materials against various disease such as tobacco (tomato) mosaic virus (Alexander 1963, Saccardo and Monti 1987, Yamakawa *et al.* 1987), *Fusarium* crown and root rot (Yamakawa *et al.* 1987), leaf mold (Yamakawa *et al.* 1988), root knot nematode (Gilbert and McGuire 1956), etc. Although it was difficult to use *L. peruvianum* as breeding materials due to severe cross incompatibility with cultivated tomatoes (Hogenboom 1972a), hybrids and their backcross progeny have been efficiently obtained in recent years through ovule culture after interspecific crossing (Imanishi1988, Chen and Imanishi1991, Doganlar *et al.* 1997, Sacks *et al.* 1997, Takashina *et al.* 1997). Therefore, *L. peruvianum* as an important genetic resources for tomato breeding would be more promising in future.

7. Summary

The tomato is one of the vegetables with the highest production in the world, and its production is increasing all over the world, mainly, in Asia. In Japan, the total wholesale price of the tomato is the highest as compared with all other vegetable items. This fact implies that the tomato is also indispensable in the Japanese diet. The tomato fruit that contains abundant and well-balanced nutrition is served as a raw vegetable and as various processed-food materials in the world.

At present, the genus *Lycopersicon* in the family Solanaceae is classified into nine species, and divided into two subgenera, the '*esculentum*-complex' (EC) and the '*peruvianum*-complex' (PC). The EC comprises seven species, *L. esculentum, L. pimpinellifolium, L. cheesmanii, L. chmielewskii, L. parviflorum, L. pennellii* and *L. hirsutum*, which are cross-compatible with the cultivated tomato, whereas the PC includes two species, *L. peruvianum* and *L. chilense*, which are cross-incompatible with the cultivated tomato.

The cultivated tomato is required to seek new breeding materials due to its limited genetic variation. On the other hand, wild relatives of the tomato harbor many genes for desirable traits, such as fruit qualities and resistances against pests, diseases and environmental stresses for further genetic improvement of the cultivated tomato. Although the PC is considered to possess particularly high genetic diversity, the PC is estranged from the cultivated tomato by severe cross incompatibility. Then, the study on breeding methods and genetic analyses is essential to efficiently introduce useful traits

of the PC species into the cultivated tomato.

In view of the above-mentioned goal, five study topics were taken up as follows:

1) Genetic Diversity of the 'peruvianum-complex' (Lycopersicon peruvianum (L.) Mill. and L. chilense Dun.) Revealed by RAPD Analysis.

To screen such populations as have larger genetic diversities is an effective approach to select promising materials for plant breeding. Understanding of the habitats of the genetically diverse populations would also increase the efficiency of collecting valuable genetic resources. Although the PC is thought to possess large genetic diversity, there are very few reports about the characteristics of the genetic variation within the PC. Therefore, the characteristics of the genetic diversity of the PC species in comparison with the EC species and whether the geographical variation is involved in the PC or not were investigated by using RAPD markers.

A total of 435 RAPDs were obtained from 50 accessions of all the nine *Lycopersicon* species using only 10 random primers. Genetic distances between accessions were estimated using similarity coefficients of Nei and Li (1979) by comparing pairwise PCR products between the accessions. The average genetic distance among accessions of *L. peruvianum* or *L. chilense* was larger than that of the EC species. All the accessions tested were divided into four clusters consisting of the PC species, the self-compatible EC species, *L. pennellii* and *L. hirsutum* by the cluster analysis using the neighbor-joining method. In addition, the dendrogram suggested that the *L. peruvianum* accessions in northern Peru and the *L. chilense*

accessions in southern Peru may have large genetic variation in each species. From these results, it was concluded that the PC with such high genetic variation and different genetic background in comparison with the EC species would have potential to supply unknown but useful traits for future tomato breeding. Moreover, all the tested accessions clustered as similar as the classification on the basis of morphology, fruit color, self-incompatibility and various molecular markers. RAPD analysis may be a simple and efficient method and an alternative measure to other molecular markers to characterize species or accessions for the phylogenetic study of *Lycopersicon*.

2) *Pistillate-Parental Differences and the Ability to Produce Interspecific Hybrids between Lycopersicon esculentum and 'peruvianum-complex' Species (L. peruvianum and L. chilense).*

To overcome the barriers for interspecific crosses between the cultivated tomato and the PC, the abilities of cultivated tomatoes to produce interspecific hybrids was investigated. As a criterion of the hybrid production ability, the number of germinated ovules per fruit (GPF) was used. The GPF was expressed by the formula, GPF = OPF × GPO, where OPF is the number of ovules per fruit, and GPO is the number of germinated ovules / number of total ovules obtained.

The interspecific crossing between nine cultivated varieties and three PC accessions revealed that cvs. 'Sekaiichi', 'Ponde Rooza' and 'Early Pink' had considerably high and stable GPF over years, but cv. 'Best of All' produced no

hybrids. The analyses of variance for GPF, OPF and GPO, and the analysis of correlation for seven sexual-organ- and three fruit-morphological traits showed that the selection of the pistillate parents with wider reproductive organs for high OPF given favorable environmental conditions for high GPO was important in order to enhance GPF in the interspecific crossing between the cultivated tomato and the PC.

3) *Genetic Analysis of Self- and Unilateral Incompatibility in the Progeny of Lycopersicon esculentum Mill. × L. chilense Dun.*

The pollen tube of the PC is able to grow into the pistil of the cultivated tomato, and attains the fertilization successfully. On the contrary, the pollen tube of the cultivated tomato stops growing in the pistil of the PC and fails to fertilize the ovule. Due to such unilateral incompatibility (UI), the cytoplasm of the PC species remained to be untouched for tomato breeding. On the other hand, the SI of *Lycopersicon* is controlled by a single-S-locus gene, while several researchers have reported that the self-incompatibility (SI) in the progeny of the interspecific hybrids between the cultivated tomato and some wild species may be controlled by two or more genes. Thus, the genetic mechanisms of the UI and the SI in the genus *Lycopersicon* have not been revealed yet.

L. esculentum cv. 'Sekaiichi' (SK), F_1 of SK × *L. chilense* PI128652 and 68 BC_1 plants were analyzed in order to elucidate genetic mechanisms of the UI and the SI in the progeny of interspecific hybrids.

Fluorescent microscopic observation of pollen-tube-growth inhibition in the

styles of F_1 and BC_1 plants after self- or SK-pollen pollination revealed the differences of SI and UI reactions in the position where pollen-tube growth stopped and in the shape of pollen-tube tips. The UI pollen tubes were stopped near the stigma in a narrow range, while the SI pollen tubes were stopped in a wide range in the style. The tips of the UI pollen tubes burst out, whereas the tips of SI pollen tubes burst or swelled.

From the segregation of UI and SI in the BC_1 generation, it was suggested that UI and SI in the progeny of *L. esculentum* × *L. chilense* were controlled by one allele and two alleles, respectively, and that the UI was associated with one of the two SI alleles. From the analysis of stylar proteins using SDS-PAGE, it was found that one of major protein bands with RNase activities and about 30kD molecular size was completely linked to the UI reaction. These results suggest that the UI would be controlled by an S-RNase, which is the product of the self-incompatibility gene. Although reported earlier in *Nicotian,* this was shown for the first time in *Lycopersicon* .

4) Genetic Analysis of Sucrose-Accumulating Ability in Lycopersicon peruvianum.

L. peruvianum has a sucrose-accumulating ability that the cultivated tomato does not possess. Its sucrose-accumulating ability has never been incorporated into the cultivated tomato. Therefore, in order to utilize the *L. peruvianum* as an alternative resource to supply sweet taste for the tomato fruit, the mode of inheritance was determined for the sucrose-accumulating ability using the progeny of interspecific

crossing between *L. esculentum* and *L. peruvianum* var. *humifusum* LA2153.

From the segregation of PCR band patterns (wild, heterozygous and cultivar types) resolved by the PCR primers AIT-1 and AIT-2 to amplify a part of the acid invertase gene, it was found that the PCR band pattern was controlled by an allele in all of the generations tested. In addition, the investigation of the relationship between the PCR band patterns and their sugar contents revealed that heterozygous and cultivar types accumulated mainly glucose and fructose, while wild-type plants accumulated more sucrose than glucose and fructose. The linkage analysis between the PCR marker and the RFLP marker TG102 also revealed that the PCR marker was located on the chromosome 3. From these results, it was concluded that the sucrose-accumulating trait is controlled primarily by a recessive gene closely linked to TG102 on the chromosome 3, and that these PCR primers are useful for selecting sucrose-accumulators from the progeny of the cross combination of *L. esculentum* and LA2153.

5) *Screening of Wild Accessions Resistant to Gray Mold (Botrytis cinerea* Pers.*) in Lycopersicon.*

The tomato gray mold (*Botrytis cinerea* Pers.) is a serious disease worldwide, which infects the leaves, stems, flowers, and fruits. It is difficult to raise new resistant varieties against the gray mold because highly resistant breeding materials have not been found yet. To find breeding materials resistant against gray mold, six tomato cultivars, 43 wild tomato accessions and a *Solanum lycopersicoides* accession were

screened. Although no correlation between the resistance of the leaf and the stem was observed ($r=-0.127^{ns}$), *L. peruvianum* LA2745, *L. hirsutum* LA2314, and *L. pimpinellifolium* LA1246 showed a high level of resistance both in the leaf and the stem bioassay. Particularly, in the leaves of LA2745, no lesion was observed even more than two weeks after inoculation. The F_1s between a cultivated tomato and LA2745 also showed as high resistance as LA2745. From these results, LA2745 could be considered to be a promising breeding material for producing resistant varieties against gray mold.

The present studies proposed general methods to use the PC species for producing new tomato varieties. In the chapter 2 and 3, effective screening of the promising breeding materials and efficient interspecific hybridization were discussed to introduce useful traits from the PC species into the cultivated varieties through the *in vitro* ovule culture technique. In the chapter 5 and 6, introduction of the sucrose-accumulating ability from *L. peruvianum* into the cultivated tomato and screening of promising resistant accessions against gray mold disease was practically demonstrated. In addition, in the chapter 4, problems of the UI and SI were taken up, which hamper the breeding operations when producing interspecific hybrids and its progeny.

To meet the extensive demands for new tomato varieties in the future, such studies for efficient utilization of valuable traits from the wild relatives would become more important as well as measures to protect, preserve and convey as many invaluable wild accessions and local varieties as possible for the future.

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9. Summary (in Japanese)

和文摘要

トマト野生種 *L. peruvianum* および *L. chilense* の育種的利用に関する研究

中・南米のメキシコとアンデス地方に起原を持つトマトは，世界で最も生産量の多い野菜の１つであり，その生産量はアジアを中心に世界的に増加の傾向にある．日本での栽培面積は欧米に比べてはるかに少ないが，我が国で栽培される野菜の中で総卸売価格が最も高い野菜(1995年の卸売価格合計で 2121 億円)となっている．このことは，我が国においてもトマトが非常に需要の大きな野菜であることを示している．トマトは生食としてサラダやジュース，加工してトマトソース，ピューレ，ケチャップなど，さまざまな用途に利用され，私たちの食卓を賑わせているのみならず，ミネラルやビタミン（A, B_1, B_2, B_3, B_6, C および E），食物繊維，リコピンなどの栄養を豊富にバランスよく含んでおり，私たちの健康を支える一翼を担っている．

ナス科トマト属(*Lycopersicon*)の種は現在９つに分類されている．また，トマト属は栽培種（*L. esculentum*）との交雑和合性の有無により大きく２つの亜属に分類されている．すなわち，交雑和合性を持たない'*peruvianum*-complex'（*L. peruvianum* および *L. chilense*）(以下 PC と略)と交雑和合性を持つ'*esculentum*-complex'（*L. esculentum*, *L. pimpinellifolium*, *L. cheesmanii*, *L. parviflorum*, *L. chmielewskii*, *L. hirsutum* および *L. pennellii*）(EC と略)の２つである．

栽培種はもともと遺伝的変異が小さいため，品種改良を行うための素材に乏しい．一方，野生種のなかには栽培種にはない耐病虫性，耐ストレス性，高果実品質（糖，酸，ビタミンなど）などの優良形質を持つ系統がある．特に PC は遺伝的変異の大きな野生種のグループであり，品種改良を進めていく上で貴重な遺伝資源であると考えられている．しかしながら栽培種と PC とは交雑が困難であるために，せっかくの有用形質を十分に活用できていない状況にある．そこで，PC の持つ有用形質を効率良く栽培品種に導入し活用するためには，育種方法の開発および有用な諸形質の遺伝学的研究を進める必要があると考えられる．このような状況に鑑み，以下に述べる５つの研究を行なった．

１． RAPD 分析によって明らかにされた'*peruvianum*-complex' (*Lycopersicon peruvianum*(L.) Mill. and *L. chilense* Dun.) の遺伝的多様性．

新たな育種素材を探索する際には，遺伝的変異の大きな集団から探索するのは効率の良いアプローチであると考えられる．また，遺伝資源を収集する際に遺伝的変異の大きな地域を把握することは，より多様な遺伝資源を効率よく収集したり，有用形質を持つ系統を効率よくスクリーニングすることに役立つと考えられる．PC は遺伝的変異の大きな集団であると考えられて

いるが，その変異がECと比べてどのような特徴を持つのか，またPC集団内の地理的変異などPCの変異の特性についてはあまり調べられていない．

そこで，PCの34系統を中心にトマト属9種から計50品種・系統 を供試し，10種類のランダムプライマー（OPK-1-10）を用いてRAPD分析を行なった．'*peruvianum*-complex'については，その自生地が地理的にできるだけ広い範囲にわたるように選んだ．RAPD 分析の結果，435 種類の RAPD マーカーを検出することができた．遺伝的変異の大きさの尺度として，Nei and Li (1979)の遺伝的類似度から種内系統間の平均遺伝的距離を計算したところ，*L. peruvianum* および *L. chilense* の平均遺伝的距離は EC に属するいずれの種の平均遺伝的距離よりも大きく，その大きさは自殖性のEC種5種全体の平均遺伝的距離に匹敵した．

全ての系統間で計算した遺伝的距離に基づいて近隣結合法(neighbor-joining method)によるクラスター分析を行い，系統樹を作成したところ，トマト属は4つの主要なクラスターに分かれることが明らかになった．4つのクラスターは 1)PC，2) EC に属する自殖性種，EC に属する他殖性種である 3) *L. pennellii* および 4) *L. hirsutum* であった．PC と EC は明瞭に異なるクラスターに分けられたことから，PC と EC とは生殖的に隔離されているのみならず，両者の遺伝的背景そのものが大きく異なることが明らかになった．このことはPCがECの持たない形質を多く保有していることを意味していると考えられた．さらにPCのクラスターは *L. peruvianum* と *L. chilense* の2つのサブクラスターに分けられた．*L. peruvianum* のサブクラスターを詳細に観察したところ，ペルー中・南部に分布する系統群は共通のクラスターを形成して互いに遺伝的に近い関係を示したのに対し，ペルー北部のマラノン川流域の系統群は一つのまとまったクラスターを形成せず，その平均遺伝的距離はペルー中・南部の系統群の平均遺伝的距離よりも大きかった．*L. chilense* のサブクラスターにおいては，ペルー南部の系統群とチリ北部の系統群とはそれぞれ別のクラスターを形成し，ペルー南部の系統群の平均遺伝的距離はチリ北部の平均遺伝的距離より大きかった．以上のことから，PCの *L. peruvianum* および *L. chilense*，特にペルー北部の *L. peruvianum* およびペルー南部の *L. chilense* は遺伝的多様性が高かったことから，PCおよびこれらの地域の系統は栽培トマトの遺伝的改良に供する遺伝資源として注目に値すると考えられた．

さらに RAPD マーカーは再現性などの点でその信頼性を低く評価されることがあるが，本実験において RAPD マーカーを用いて作成した系統樹は，果実色，形態などによって分類された種ならびに自家不和合性や種間交雑不和合性によって分類されたグループと同様のクラスターを形成した．このことから，操作が簡単で短時間に多くのマーカーを扱うことのできる RAPD 分析は種や系統の遺伝的多様性や関係をある程度推定するという目的には有用な手段であると考えられた．

2．　トマト栽培種と'*peruvianum*-complex' (*L. peruvianum* および *L. chilense*)との種間雑種生産能力に関する品種間差異.

PC の有用形質を栽培種に導入する際には，種間交雑を行うと雑種胚が退化してしまう交雑不和合性が問題となるが，交雑によって得られた未熟な種子（ここでは胚珠と呼ぶことにする）を *in vitro* で培養することにより雑種を獲得することができる．しかしながら，種子親に用いる栽培品種間にその効率の差異が観察されることが報告されている．そこで，栽培種の雑種生産能力を GPF（１果実当たり得られた発芽能力を持つ雑種胚珠数），さらに GPF を GPF=OPF×GPO の式で分解した２つの指標，すなわち OPF（１果実当たり得られた胚珠数）と GPO（発芽能力を持つ胚珠数を総胚珠数で割った値，すなわち１つの胚珠が発芽する確率）で評価して実験を行なった．種子親として９品種，花粉親として *L. peruvianum*，*L. peruvianum* var. *humifusum* および *L. chilense* の各１系統，計３系統を用いて種間交雑を行ったところ，品種‘世界一’，‘ポンデローザ’および‘アーリ・ピンク’の３品種は年次によらず安定して高い GPF を示したのに対し，品種‘ベスト・オブ・オール’はいずれの年も全く雑種個体を生産しなかった．GPF，OPF および GPO に及ぼす両親の遺伝子型と年次間の要因に関する分散分析を行なったところ，OPF には種子親の遺伝子型が高度に有意に関与していること，GPO には年次間の要因（環境要因）が高度に有意に関与していること（低温条件が有利と推測），さらに GPF には種子親の遺伝子型と年次間の要因の両者が有意に関与していることが明らかになった．また，GPF，OPF および GPO のいずれにも花粉親の遺伝子型は有意な関与が認められなかった．さらに種子親の遺伝子型の関与が認められた OPF および GPF と種子親の花器・果実の形態形質との相関分析を行ったところ，OPF は花柱，子房および果実（つまり生殖器官）の幅と正の相関が認められたが，GPF と直接相関を示す花器・果実の形態形質は観察されなかった．また OPF と GPO 間には相関が認められなかったことから，種間交雑において GPF を高めるためには OPF と GPO の両者を高める必要があると考えられた．以上の実験結果から，種間交雑において GPF を高めるには，生殖器官の幅が大きい種子親を選んで高い OPF を確保し，同時に環境要因を考慮して高い GPO を確保することが重要であると考えられた.

3．　トマト栽培種と野生種 *L. chilense* 間の交雑後代における自家不和合性および一側性不和合性に関する遺伝分析.

自家和合性（SC）の栽培種を種子親に，自家不和合性（SI）の PC'種を花粉親にして種間交雑を行うと花粉管が正常に伸長して受精に至るが，逆に PC 種を種子親に栽培種を花粉親にしたときには花粉管が花柱の途中で停止する．このような一側性不和合性（UI）が存在するために 育種的に PC 種の細胞質を利用することは困難な状況にある．タバコ属においては SI を支配する遺伝子（S 遺伝子）が，同時に UI も支配している例のあることが明らかにされている

が，トマトにおいては UI とS遺伝子との関係は未だに解明されていない．また，トマト属の種間雑種後代の自家不和合性の遺伝的メカニズムについてもまだよく分かっていない．そこで，トマト栽培種‘世界一’(SK)， SK × *L. chilense* PI128652 の F_1 および F_1 を栽培種に戻し交雑して得られた $BC_1$68 個体を供試して UI および SI の遺伝的メカニズムを明らかにすることを試みた．アニリンブルー蛍光観察によって F_1 および BC_1 の花柱内における UI と SI による花粉管伸長阻害の様子を比較したところ，花柱内で UI を示す花粉管が停止する位置は花柱の長さに対して 19-25%の狭い範囲内であったのに対し，SI を示す花粉管の停止する位置は 20-90%の広い範囲であった．また，UI を示したほとんどの花粉管の先端は破れて開口していたのに対し，SI を示す花粉管の先端は破れて開口したものと膨張したものが観察された．BC_1 世代における UI と SI の分離を観察したところ，UI と SI にはそれぞれ１および２つの遺伝子が関与していること，および UI を支配する遺伝子は SI を支配している２つの遺伝子のうちの１つと同じものである可能性が示唆された．さらに SDS-PAGE および RNase 活性染色による BC_1 個体の花柱タンパク質の分析を行ったところ，30kD 付近に検出された RNase のうちの一つが UI の発現の有無と完全に連鎖していた．このことと UI の分離に１遺伝子が関与していたこととを考え併せると，トマト栽培種と *L. chilense* 間で観察される UI は RNase に支配されている可能性が考えられた．

４． トマト野生種 *L. peruvianum* のスクロース蓄積能に関する遺伝分析．

L. peruvianum は栽培種が持たないスクロース蓄積能を持つことが知られているが，この形質が育種に利用された例は未だになく，新たな食味を呈する育種素材として注目に値する．しかしながら，*L. peruvianum* のスクロース蓄積能に関する遺伝様式は明らかにされていない．そこで，*L. peruvianum* var. *humifusum* LA2153 のショ糖蓄積能を栽培種に導入する際の遺伝様式を明らかにする目的で，トマト栽培種と LA2153 の交雑後代において，Harada *et al.* (1995)が開発したインベルターゼ遺伝子の一部を増幅しインベルターゼ活性の有無を判定することができる PCR プライマー，AIT-1 および AIT-2 を用いてそのバンドパターンの分離分析を行なった．交雑組合わせや世代の違いに関わらず，PCR バンドパターンは１遺伝子の分離モデルに適合した．そこで観察された３種類のバンドパターン，栽培種ホモ型，ヘテロ型および野生種ホモ型の個体が持つスクロース蓄積能を調査したところ，栽培種ホモ型とヘテロ型個体のスクロース蓄積は全糖含量の６％以下と極めて低い値にを示したのに対し，野生種ホモ型は全糖含量の 32%以上のスクロースを蓄積した．さらにこの PCR マーカーは RFLP マーカー TG102 と完全に連鎖していることが示された．以上のことから，*L. peruvianum* のスクロース蓄積能は第３染色体に座乗する単一劣性のインベルターゼ遺伝子であることが明らかになった．

5．　トマト野生種における灰色かび病抵抗性系統のスクリーニング．

トマト灰色かび病（*Botrytis cinerea* Pers.）は世界的に大きな問題になっている病害であるが，未だに抵抗性育種素材が見つかっておらず，抵抗性品種の育成が困難な状況にある．そこでPCを含むトマト野生種 43 系統およびナス属近縁野生種 *Solanum lycopersicoides* 1 系統の中から有望な抵抗性系統を探索することを試みた．各系統において葉と茎における抵抗性を調査したところ，それらの抵抗性には相関がなかった（$r = -0.127^{ns}$）が，葉と茎の両者において強い抵抗性を示す系統として *L. peruvianum* LA2745，*L. hirsutum* LA2314 および *L. pimpinellifolium* LA1246 をスクリーニングすることができた．特に LA2745 の葉は他の野生種系統とは異なり，接種後2週間以上経っても全く病斑が広がらない極めて強い抵抗性を示した．栽培種と LA2745 との F_1 もまた LA2745 と同様の高い抵抗性を示したことから，この抵抗性は優性の遺伝子に支配されている可能性が示唆された．以上の結果から，*L. peruvianum* LA2745 は灰色かび病抵抗性品種を育成する上で有望な育種素材であると考えられた．

本研究はトマト新品種を育成するための遺伝資源として有望なPCに属する種，*L. peruvianum* および *L. chilense* を利用するための総合的な方法を提案したものである．第Ⅱ章および第Ⅲ章においてPCを用いながら有望な育種素材をスクリーニングする上で着目すべき点およびPC種の有用形質を栽培種に導入するために行う種間交雑の効率をいかに向上させるかについて検討した．第Ⅴ章および第Ⅵ章においては，実際に *L. peruvianum* 由来のスクロース蓄積能を栽培種に導入したり，灰色かび病抵抗性の育種素材をスクリーニングした例を示した．さらに第Ⅳ章においては種間交雑および交雑後代で問題になる一側性不和合性と自家不和合性の問題を取り上げた．

今後，トマト新品種に求められる広範な需要に応えるためには，このような野生種からの有用な形質を効率よく栽培種に導入するための研究がますます重要になると思われる．加えて，かけがえのない貴重な野生種や在来種をできる限り多く次の世代の人々のために保存し伝えていくことの意義は一層大きくなっていくであろう．

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