Chapter Ⅲ

Detection of New Molecular Markers Associated to the High Shoot Regeneration Capacity of the Wild Tomato Species, *Lycopersicon chilense* Using BC_1F_1 and BC_2F_1 Generations

Key Words : *Lycopersicon esculentum*, *L. chilense*, PCR-based marker, RAPD, Acid invertase gene (*inv*), shoot regeneration capacity, BC_1F_1, BC_2F_1.

1. Introduction

One of the purposes of plant tissue and cell culture is to induce shoots on explants and to regenerate plants through *in vitro* tissue culture technique. Almost all useful plants have presumably been experimentally tested for shoot regeneration. The theory that shoot regeneration from tissues in a number of plants could be controlled by the combination and the concentrations of phytohormones *in vitro* culture was proposed by Skoog and Miller (1957). However, significant variations have been observed by

several researchers among varieties, species, and genera in regards to plant regeneration capacity. In addition, the problem of enhancing the regeneration capacity of recalcitrant plants by the genetic control is as yet unsolved. Genetic studies in regards to plant regeneration have been performed for several crops (e.g., rice ; Quimio and Zapata 1990, Taguchi-Shiobara *et al.* 1997, wheat ; Lazar *et al.* 1984, Ben Amer *et al.* 1997, maize ; Willman *et al.* 1989, Armstrong *et al.* 1992, barley ; Komastuda *et al.* 1989, Komastuda *et al.* 1993, Mano *et al.* 1996, tomatoes ; Koornneef *et al.* 1987, Koornneef *et al.* 1993, alfalfa ; Yu and Pauls 1993). Koornneef *et al.* (1993) introduced the superior regeneration capacity of *Lycopersicon peruvianum* into the cultivated tomato *L. esculentum* by backcrossing. They reported that this capacity was controlled by a dominant *L. peruvianum* alleles at a locus (*Rg*-1) near the middle of chromosome 3. Torelli *et al.* (1996) obtained a cDNA (G36) from a cultivated tomato by the mRNA differential display method, which exhibited the typical features of a morphogenetic marker.

In this Chapter, we focused on the high shoot regeneration capacity of one of the wild tomato species, *L. chilense*, using the

backcross generations and analyzed the new molecular markers that may have potential roles in regulating the above mentioned trait.

2. Materials and methods

2.1 Plant materials

The wild species *L. chilense* PI128644 (which was provided by the Institute of Radiation Breeding, NIAR MAFF) was used as the pollen parent. *L. esculentum* cv. 'Kyoryoku Ogata Toko' (KOT) was used as a seed parent. Interspecific hybrids F_1-44 (KOT × PI128644), and their backcross generations, BC_1F_1-44 (KOT × F_1-44), and BC_2F_1-44-15 (KOT × BC_1F_1-44) were used for the segregation analysis of PCR-based markers and shoot regeneration capacity. The development of the F_1 and the BC_1F_1 was described in Chapter Ⅱ. BC_2F_1-44-15 plants were obtained from a backcross of KOT with a self-compatible plant (BC_1F_1-44-15) with the high shoot regeneration capacity being selected out of the BC_1F_1-44 plants. The seeds of the KOT×BC_1F_1-44-15 in the reddish fruits appeared normal, but the embryos in the seeds were

almost immature or were aborted. The normal embryos were dissected and cultured on an MS medium (Murashige and Skoog 1962) without any growth regulators.

2.2 Shoot regeneration capacity of root explant

Ten KOT plants, and 10 of the PI128644, 6 of the F_1-44, 22 of the BC_1F_1-44, and 113 of the BC_2F_1-44-15 plants were used to evaluate shoot regeneration capacity. KOT and PI128644 were germinated aseptically. All plants were maintained *in vitro* cutting on an MS medium without growth regulators in a 200 or 300 ml Erlenmeyer flask. Root segments (approximately 1 cm in length) were excised from the middle part of the adventitious roots of 1 week old shoot. The root explants were cultured on an MS medium which was supplemented with 1 mg/l of zeatin riboside (ZR), 2 % sucrose and 0.8 % agar in a 100 ml Erlenmeyer flask. The flasks were put in a culture room at approximately 25 ℃ under 16-hr photoperiods with fluorescent illumination at 2000 lux. The shoot regeneration (SR) rate (%) was determined 4 weeks after being cultured. The SR rate (%) refers to the number of root segments with adventitious shoots / the number of root segments cultured.

The experiment was performed in a randomized block design with three blocks. Each experimental plot had 5 to 6 root segments from one plant.

2.3 DNA isolation

Genomic DNA for PCR was isolated from leaf tissue from *in vitro* plants according to simple modified method of Böhm *et al.* (1993) with the following modifications. Approximately 0.1 g of fresh leaf tissue was ground in liquid nitrogen with 3 μl mercaptoethanol in Eppendorf tube 1.5 ml using handy homogenizer S-203 (Ikeda rika co.) and homogenized in 700 μl of extraction buffer (Appendix 5). The homogenate was centrifuged at 18000 g for 10 min. After decanted the supernatant, the pellet carefully resuspended 88 μl of extraction buffer with 88 μl of lysis buffer (Appendix 5) and 30 μl of 5 % (w / v) sodium N-dodecanoylsarcosinate. The suspension was incubated for 20 min at 65 ℃. The solution was extracted with chloroform-isoamyl alcohol (20 : 1 (v / v)) and centrifuged for 10 min and 18000 g at 4 ℃. The supernatant was transferred into a new tube and nucleic acids were precipitated from the supernatant by adding 1 volume

of isopropanol. After centrifuged for 10 min and 18000 g at 4 ℃, the pellet was rinsed by 70 % ethanol. The pellet was dissolved in TE (10 : 1) buffer.

2.4 RAPD analysis

The BC_1F_1-44 and BC_2F_1-44-15 plants were evaluated in regards to the segregation of the RAPD markers. The RAPD markers were detected from a preliminary experiment in which both the parents and the F_1 hybrids or a BC_1F_1-44-15 plant were used. Kit A (Operon 10-mer Kit) was used to analyze BC_1F_1-44 plants, while primer Kits A and K were used to analyze the BC_2F_1-44-15 plants. Ten μl of reaction mixture was composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.25 U TaKaRa Taq DNA polymerase, 0.4 μM primer, and 10 ng template DNA. The amplification conditions were programmed on a Gene Amp PCR System 2400 (Perkin Elmer) as follows : 1 cycle at 93 ℃ for 3 min and 45 cycles of 1 min at 93 ℃, 1.5 min at the 40 ℃ and 2 min at 72 ℃, and 5 min at a post extension of 72 ℃ . The PCR products were separated by electrophoresis in a 2 % agarose gel and were stained with

ethidium bromide.

2.5 PCR for acid invertase gene

AIT-1 (5'- CGGTGAAAAACATTCAATGAG -3') and AIT-2 (5'-TCCACAATTGAGTGATCCAC -3') (Harada *et al.* 1995) was used to amplify the acid invertase gene (*inv*). DNA amplifications were done by a Taitec Gene Thermo Unit GTU-1605. Fifteen μl reaction mixture was composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 0.4 mM each of dNTPs, 0.5 U TaKaRa Taq DNA polymerase, 1.8 μM each of primers, and 40 ng template DNA using the following method : 5 min of preheating 94 ℃, 45 cycles of 1 min 50 sec at 94 ℃, 2 min at 58 ℃, and 2 min at 72 ℃ following 10 min at a post extension of 72 ℃. The PCR products were separated by electrophoresis in a 4 % NuSieve 3 : 1 agarose gel and were stained with ethidium bromide. The size of the amplified fragments were calculated by a Gel Reader (ver. 2.05, University of Illinois).

3. Results

3.1 Segregation of shoot regeneration trait

Small green calli appeared on the root tip side of the KOT root explants approximately 2 weeks after being cultured. However, the calli soon became brown and no shoot regeneration was found (Fig. 3-1). On the other hand, green calli developed on both ends of the explants of PI128644 and F_1 within one week after being cultured. After one more week, several shoots were formed first on the calli of the stem-side of the segment, and then on the opposite side (Fig. 3-1). The SR rates of PI128644 and the F_1-44 plants were more than 80 % (Fig. 3-2A). The SR rates of the BC_1F_1-44 plants showed a two-peak distribution of frequency. BC_1F_1-44 plants are divided into 3 groups in regard to the SR rate : the first has an SR rate of 0% (similar to a tomato cultivar), the second has a rate less than 30%, and the third has a rate that is more than 80%, similar to the wild parent and F_1 plant (Fig. 3-2A). In BC_2F_1-44-15, about 70% (86 plants) of all plants did not form any shoots, and the others (27 plants) that showed shoot regeneration indicated a flat distribution of frequency more than 0% to 100% (Fig. 3-2B).

3.2 Segregation of PCR-based molecular markers

Segregation of the RAPD markers in the 20 BC_1F_1-44 plants was determined for 20 random OPA primers. The heterogeneity of the segregation rates of the plants in regards to the 22 RAPD markers was not significant (χ^2=21.72, 0.05<p, d.f.=21). The total ratio of 'presence to absence' for the RAPD markers was 261 to 179, and was skewed significantly from an expected ratio of 1:1 with an increased deviation towards the presence of the bands (χ^2=15.28, p<0.005, d.f.=1) (Table 3-1).

On the other hand, we detected 8 RAPD markers in the BC_2F_1-44-15 population using OPA and OPK primers. The segregation of 5 of the 8 RAPDs showed an enormous significant distortion toward the reduction of the marker. It is contrasted with the segregation in 3 of the 8 RAPD markers that fitted the expected ratio of 1:1 in the BC_2F_1-44-15 (Table 3-1).

The PCR products of the wild parent, *L. chilense* PI128644, which were amplified by AIT primer, were about 10~14bp longer in size than those of tomato cultivar KOT which was 188bp in size (Fig. 3-3). The BC_1F_1-44-15 plant characterized to have a high SR rate was heterozygote in the *inv* locus. Twenty of the BC_1F_1-44

plants segregated 13 plants (heterozygote) and 7 (homozygote of *inv*^{esc}) ones, which showed a deviation which increased with that had *inv*^{chi}. In the BC_2F_1 generation, the segregation of *inv*^{chi} showed a very significant distortion similar to 5 of the 8 RAPDs (Table 3-1). In addition the PCR products of the heterozygous plants that were amplified by AIT primer contained a third band which were longer than the other two parents bands in bp size.

3.3 Relationship between shoot regeneration capacity and molecular markers

In the BC_1F_1-44 and BC_2F_1-44-15 plants, the RAPD markers which were designated OPA02-1, OPA20-3 and the *inv*^{chi} were found in plants with higher SR rates, but were hardly found in the plants that were unable to regenarate shoots (Table 3-2, Fig. 3-3, 3-4A and 3-4B). BC_1F_1-44 and BC_2F_1-44-15 populations were divided into two groups, respectively : one having the markers and the other having none; and evaluated by the Mann-Whitney test (Yonezawa 1988) in regard to the relationship between the three markers and the SR rate (Table 3-3). The results indicated that the three markers in BC_2F_1 were significant at 1% level; in addition,

the OPA02-1 and OPA20-3 in BC_1F_1 were significant. This suggests that OPA02-1, OPA20-3, and *inv*^{chi} are linked with one of the genes that controll the high shoot regeneration capacity.

4. Discussion

Several researchers have pointed out on the dominance of the shoot regeneration capacity of *L. peruvianum* (Frankenberger *et al.* 1981, Adams and Quiros 1985, Thomes and Smith 1985, Koornneef *et al.* 1987, Chen and Imanishi 1991, Koornneef *et al.* 1993). In the present experiment, the shoot regeneration capacity of *L. chilense*, which is a species of the "*peruvianum*-complex", was dominant against *L. esculentum*, similar to *L. peruvianum*. BC_1F_1-44 plants could be divided into 3 groups in regards to the SR rate. On the other hand, the BC_2F_1-44-15 plants could not be divided on the SR rate. These results suggest that at least 2 loci or more may be have contributed to this capacity of *L. chilense* PI128644. Koornneef *et al.* (1993) described the *Rg*-1 dominant gene near the middle of chromosome 3. *Rg*-1 controls the shoot regeneration capacity in combination with the dominant alleles at one or two other loci which are either *L. peruvianum* or *L.*

esculentum in origin. Our results unfortunately were not adequate to construct a genetic model.

We detected three new molecular markers, OPA02-1, OPA20-3 and *inv*^{chi}, which may be closely linked with the high shoot regeneration capacity genes which were derived from the wild species, *L. chilense* PI128644. Two of these markers, OPA20-3 and *inv*^{chi}, showed a skewed segregations towards the reduction of the marker, corresponding to the reduction in the number of plants with a high shoot regeneration capacity in the BC_2F_1-44-15. As a result, the linkage relationships among the markers and the relationship between the markers and the loci that are related to shoot regeneration capacity gene could not be completely evaluated. However, the PCR-based markers that we detected may facilitate the selection of plants with high shoot regeneration capacity in efforts to introduce this trait into the tomato cultivars.

In addition, *Rg*-1 is closely linked with the RFLP markers of TG391 and TG222, and *r* (yellow fruit) gene on chromosome 3 (Koornneef *et al.* 1993). Likewise, the *r* gene is closely linked with the *sucr* gene of acid invertase (*inv*) (Chatelat *et al.* 1995). In general, the *Lycopersicon* species shares the same loci on the same

location in each chromosome (Bonnema *et al.* 1997). Therefore, *inv*^{chi} may have a linkage with one of the dominant genes that controls high shoot regeneration capacity on *Rg*-1 locus or near the *Rg*-1 in *L. chilense* PI128644. Therefore, one of the dominant genes that controls high shoot regeneration capacity is closely related to the new molecular markers that are involved in *L. chilense* PI128644 and may be on *Rg*-1 locus or near the *Rg*-1.

Table 3-1
Segregation of the wild species-specific RAPD and *inv*^{chi} bands in BC_1F_1 and BC_2F_1 generations

Progeny	Name of bands	Size(kb)	Wild species-specific band		χ^2	Probability
			Presence	Absence		
BC_1F_1-44[1]	RAPD [3]		261 (plants)	179 (plants)	15.28	$p<0.005$(d.f.=1)
					21.72[5]	$0.05<p$(d.f.=21)
	inv^{chi} [4]	0.18	13	7	1.80	$0.05<p$
BC_2F_1-44-15[2]	OPA02-1	0.90	49	60	1.11	$0.05<p$
	OPA02-2	0.46	11	98	69.44	$p<0.005$
	OPA18	0.93	20	85	40.24	$p<0.005$
	OPA19	0.59	10	99	72.67	$p<0.005$
	OPA20-1	0.82	27	82	27.75	$p<0.005$
	OPA20-3	0.62	21	88	41.18	$p<0.005$
	OPK17	1.02	45	64	3.31	$0.05<p$
	OPK19	0.96	55	55	0.00	$0.05<p$
	inv^{chi}	0.18	16	87	48.94	$p<0.005$

[1] cv. 'Kyoryoku Ogata Toko' (KOT)×F_1 (KOT×*L. chilense* PI128644)

[2] KOT×BC_1F_1-44-15 (BC_1F_1-44-15 : a self-compatible and high shoot regeneration rate plant)

[3] 22 of RAPD markers detected by PCR analysis with OPA kit

[4] Acid invertase gene of *L. chilense* PI128644

[5] Heterogeneity χ^2

Table 3-2
Frequency of plants with the new molecular markers in the BC_1F_1 and BC_2F_1 depending on the shoot regeneration rate

Generation	BC_1F_1-44[1]						BC_2F_1-44-15[2]					
Marker name	OPA02-1[3]		OPA20-3[4]		*inv*[chi 5]		OPA02-1		OPA20-3		*inv*[chi]	
Range of shoot regeneration rate	marker presence	marker absence	marker presence	marker absence	marker presence	marker absence	marker presence	marker absence	marker presence	marker absence	marker presence	marker absence
90<a≦100%	6	0	6	0	3	3	4	0	4	0	4	0
80<a≦90%	2	0	2	0	2	0	2	0	2	0	2	0
70<a≦80%	0	0	0	0	0	0	0	0	0	0	0	0
60<a≦70%	0	0	0	0	0	0	3	1	3	1	3	1
50<a≦60%	0	0	0	0	0	0	1	0	1	0	1	0
40<a≦50%	0	0	0	0	0	0	3	1	2	1	3	1
30<a≦40%	0	0	0	0	0	0	1	0	1	0	1	0
20<a≦30%	3	1	4	0	2	2	2	0	1	1	1	1
10<a≦20%	0	1	0	1	0	1	2	1	2	1	2	0
0<a≦10%	1	0	1	0	1	0	1	1	0	2	0	2
0%=	2	4	0	6	5	1	25	54	3	75	0	81

[1] *L. esculentum* cv. 'Kyoryoku Ogata Toko' (KOT) × F_1 (KOT × *L. chilense* PI128644)

[2] KOT × BC_1F_1-44-15 (BC_1F_1-44-15 : a self-compatible and high shoot regeneration rate plant)

[3] 0.9 kb band amplified by OPA02.

[4] 0.62 kb band amplified by OPA20

[5] Acid invertase gene of *L. chilense* PI128644

Table 3-3
Mann-Whitney test on the relationship between shoot regeneration capacity and molecular markers

Generation	Marker name	n_1[1)]	n_2	R1[2)]	R2	U	Z
BC_1F_1	OPA02-1	14	6	177	33	72*	
	OPA20-3	13	7	181	29	90**	
	inv^{chi}	13	7	129	81	53ns	
BC_2F_1	OPA02-1	44	58	2747	2506	795	4.513**
	OPA20-3	19	81	1583	3467	146	7.552**
	inv^{chi}	17	86	1597.5	3758	17.5	8.689**

[1)] n_1 indicates the no. of plants with the marker and n_2 indicates the no. of plants without the marker.

[2)] R1 and R2 indicate the total of the signed rank of n_1 and n_2 plants, respectively.

*, ** Significant at the 5 % and 1 % levels respectively, ns: not significant.

Mann-Whitney test was conducted by Yonezawa *et al.* (1988).

Z was calculated as follows

$$|Z| = |U - n_1n_2/2| \Big/ \sqrt{\left[\{n_1n_2/(n_1+n_2)(n_1-n_2-1)\} \cdot \{(n_1+n_2)^3 - (n_1+n_2) - C\}/12\right]}$$

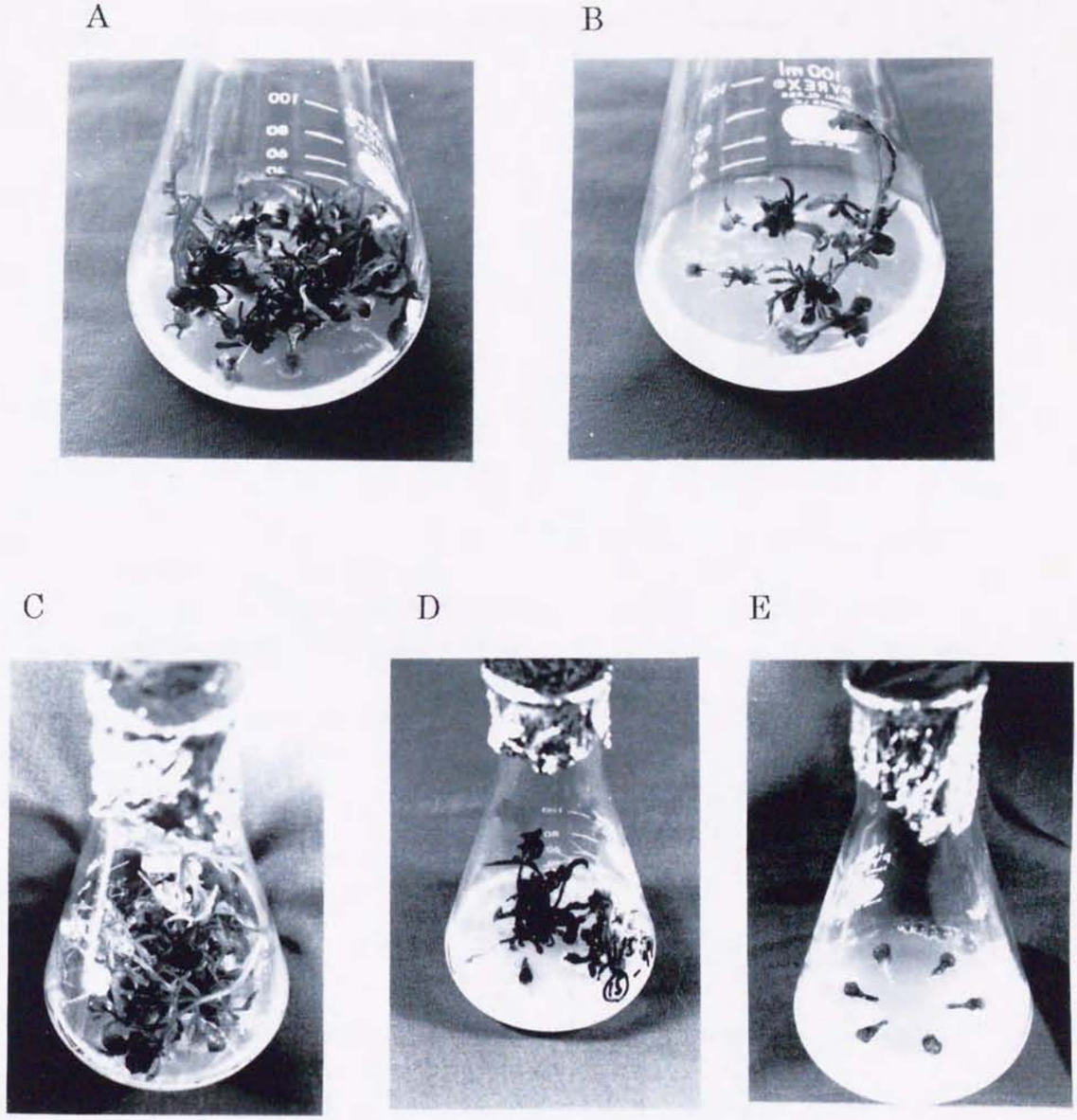


Fig. 3-1 Shoot regeneration from root explants in the F_1 plants and BC_2F_1-44-15 plants.

The percentage in the parenthesis indicates the shoot regeneration rate. A; F_1-44 after 4 weeks culture (100 %), B; F_1-44 after 3 weeks culture (100 %), C; BC_2F_1-44-15-12 (100 %), D; BC_2F_1-44-15-99 (50 %), E; BC_2F_1-44-15-18 (0 %)

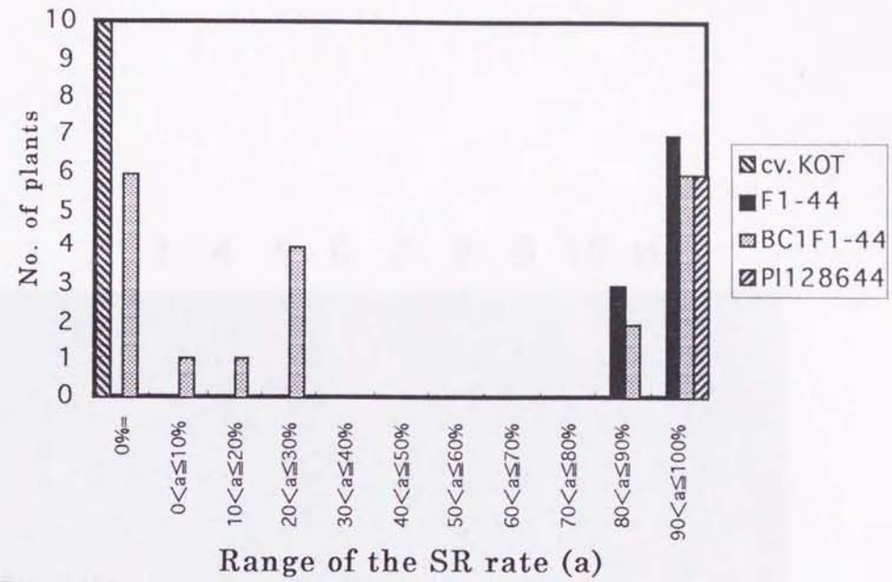


Fig. 3-2A

Frequency distribution of parent, F1 and BC1F1 plants on the shoot regeneration rate

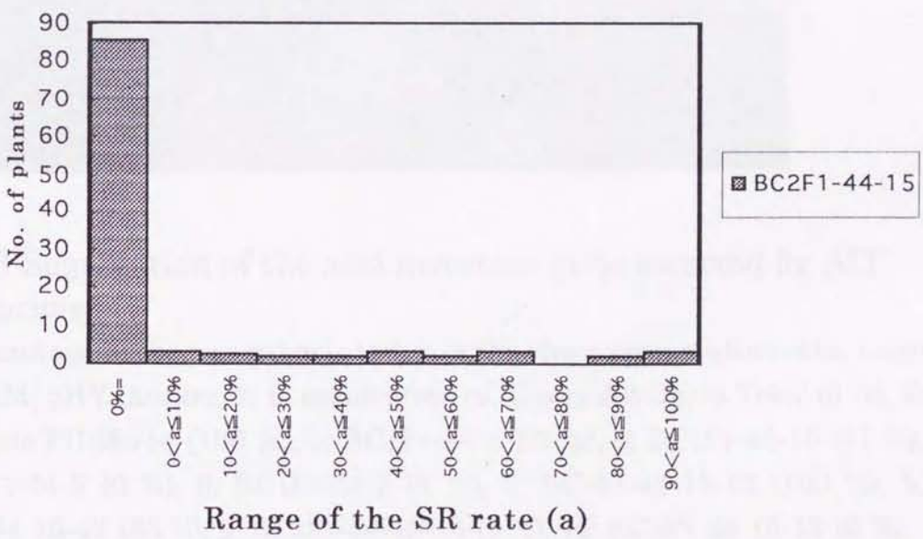


Fig. 3-2B

Frequency distribution of BC2F1-44-15 plants on the shoot regeneration rate

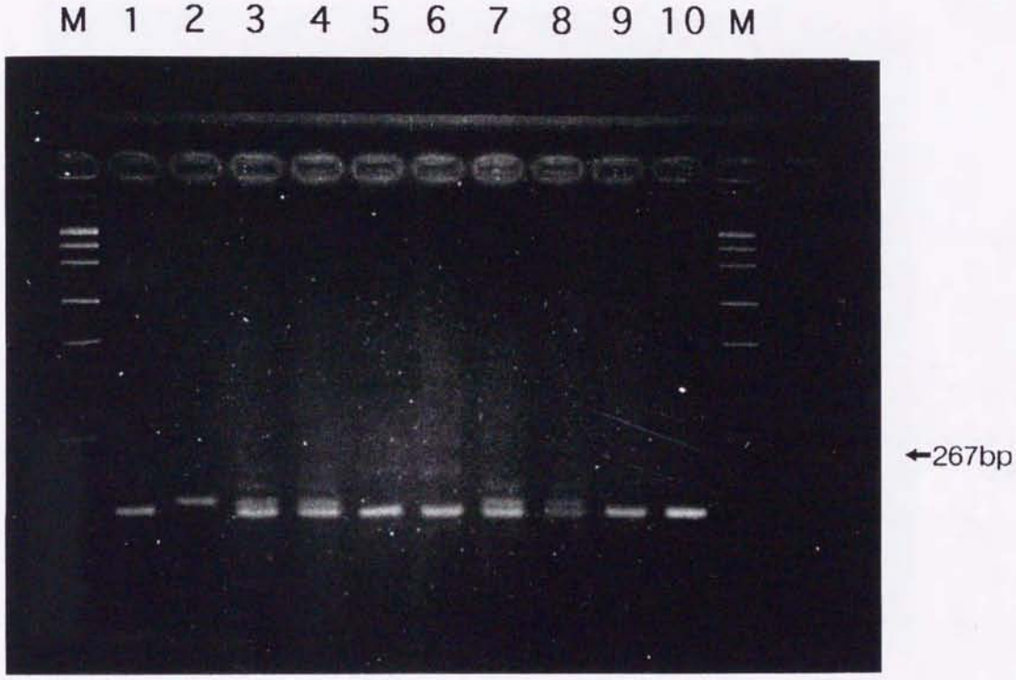


Fig. 3-3 Segregation of the acid invertase gene detected by AIT primer

The percentage in the parenthesis indicate the shoot regeneration rate. Lane number M; pHY marker, 1; *L. esculentum* cv. 'Kyoryoku Ogata Toko' (0 %), 2; *L. chilense* PI128644 (100 %), 3; BC_1F_1-44-1 (95 %), 4; BC_1F_1-44-15 (87 %), 5; BC_1F_1-44-5 (0 %), 6; BC_1F_1-44-7 (0 %), 7; BC_2F_1-44-15-12 (100 %), 8; BC_2F_1-44-15-43 (83 %), 9; BC_2F_1-44-15-18 (0 %), 10; BC_2F_1-44-15-19 (0 %)

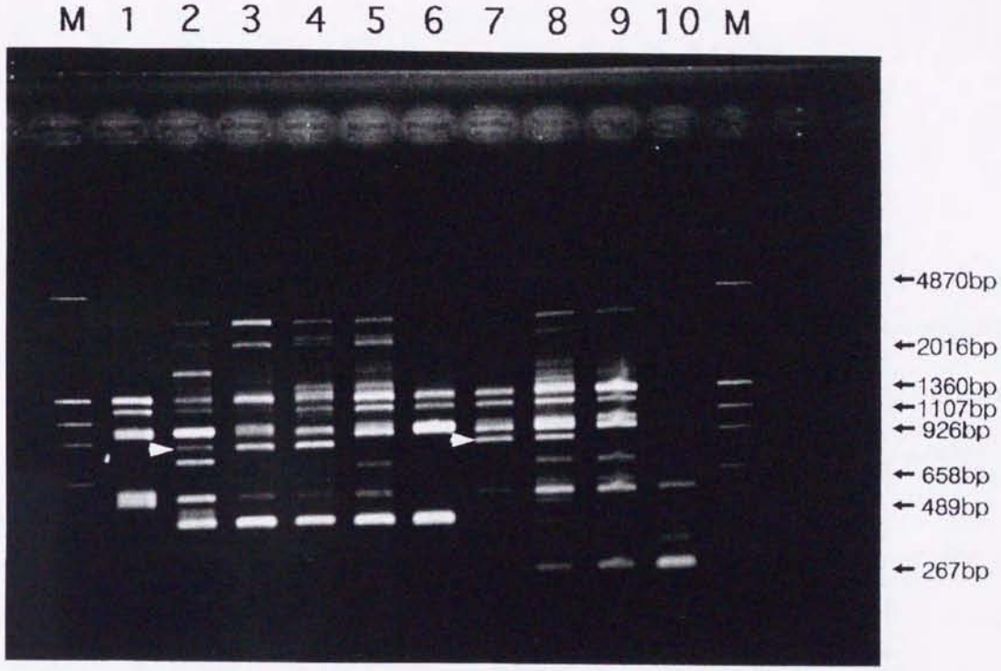


Fig. 3-4A Segregation of the RAPD marker OPA02-1.
The arrow heads indicate the RAPD marker OPA02-1 (0.9 kb). The percentage in the parenthesis indicate the shoot regeneration rate. Lane number M; pHY marker, 1; *L. esculentum* cv. 'Kyoryoku Ogata Toko' (0 %), 2; *L. chilense* PI128644 (100 %), 3; BC_1F_1-44-1 (95 %), 4; BC_1F_1-44-15 (87 %), 5; BC_1F_1-44-5 (0 %), 6; BC_1F_1-44-7 (0 %), 7; BC_2F_1-44-15-12 (100 %), 8; BC_2F_1-44-15-43 (83 %), 9; BC_2F_1-44-15-18 (0 %), 10; BC_2F_1-44-15-19 (0 %)

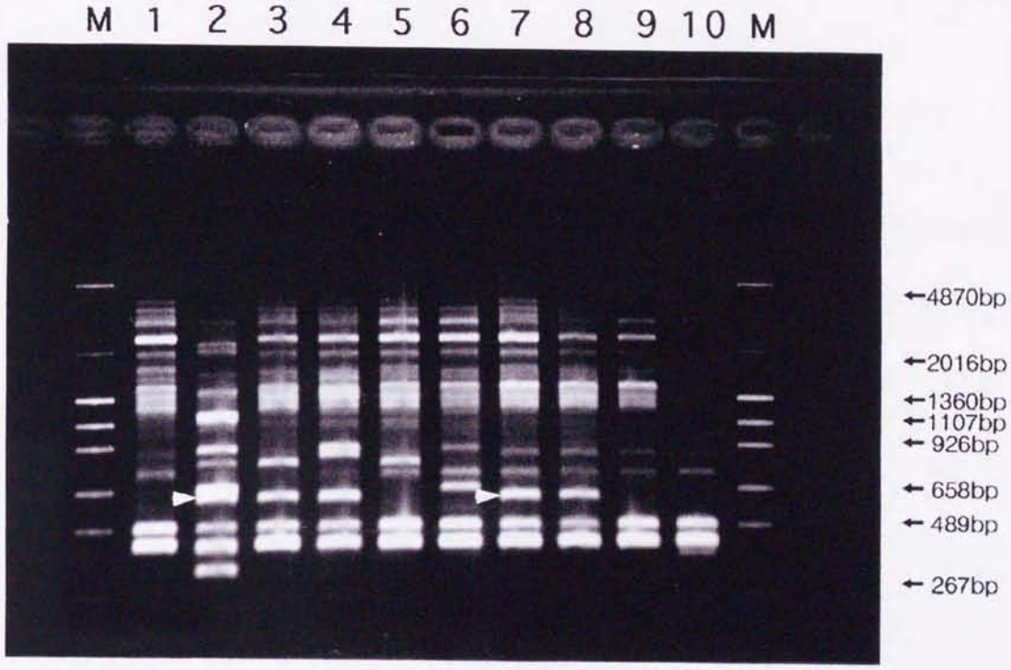


Fig. 3-4B Segregation of the RAPD marker OPA20-3.

The arrow heads indicate the RAPD marker OPA20-3 (0.62 kb). Lane number M; pHY marker, 1; *L. esculentum* cv. 'Kyoryoku Ogata Toko', 2; *L. chilense* PI128644, 3; BC_1F_1-44-1, 4; BC_1F_1-44-15, 5; BC_1F_1-44-5, 6; BC_1F_1-44-7, 7; BC_2F_1-44-15-12, 8; BC_2F_1-44-15-43, 9; BC_2F_1-44-15-18, 10; BC_2F_1-44-15-19

Chapter IV

Identification of *Rg* Locus Controlling the Superior Shoot Regeneration Capacity Derived from *L. chilense* in the BC_1F_2 Generation

Key Words : *L. chilense*, shoot regeneration capacity, PCR-based marker, RAPD, Acid invertase gene (*inv*), BC_1F_1, BC_1F_2, RFLP, linkage map.

1. Introduction

The totipotency of various plants have been studied, but the genetic and physiological mechanism that induce shoot and/or root regeneration from plant tissues have been yet unclear. A genotypic component in the response of plants to *in vitro* culture is observed in virtually all tissue culture manipulations. In most studies, the nature of the genotypic effect is only vaguely defined, because the relative, not direct, responses of different genotypes to a particular tissue culture technique has been evaluated. In recent years, the rapid progress in molecular biology has gradually changed the

direction of the area of studies into genetic analysis and gene cloning in regard to plant regeneration.

The introgression of the superior regeneration capacity of the wild species *Lycopersicon chilense* PI128644 to cultivated tomato has been progressed by backcrossing (Chapter Ⅲ). BC_1F_1 and BC_2F_1 generations were developed. The segregation analysis of PCR-based markers, two RAPD markers (OPA02-900 and OPA20-620) and acid invertase gene (*inv* ^{chi}), linked to the superior shoot regeneration capacity of *L. chilense* PI128644 were described in Chapter Ⅲ.

However, the skewness of segregation were often observed in the progenies between *L. esculentum* and wild species (Vallejos and Tanksley 1983, Zamir and Tadmor 1986, Paterson *et al.* 1988, Paterson *et al.* 1991, Paran *et al.* 1995, Foolad 1996, Grandillo and Tanksley 1996). The skewness also was observed in the BC_2F_1 generation (described in Chapter Ⅲ). Therefore, the genetic model of the shoot regeneration capacity and the relationship on linkage between the markers and the trait were not sufficiently evaluated.

In this Chapter, it was pursued to detect another new RAPD markers which linked with the shoot regeneration capacity, and to

construct a linkage map on the trait and markers using the BC_1F_2 generation.

2. Materials and Methods

2.1 Plant materials

The wild species *L. chilense* PI128644, tomato cultivar *L. esculentum* 'Kyoryoku Ogata Toko', F_1-44 plants and the BC_1F_1-44-15 plant, and their progeny BC_2F_1-44-15 population were used (described in Chapter Ⅲ). In addition, BC_1F_2-44-15 population was used. This population was obtained from a selfing of the BC_1F_1-44-15 plant which have high shoot regeneration capacity like the wild species.

2.2 DNA isolation

Genomic DNA for PCR analysis was extracted from 0.1 g of fresh leaf tissues from *in vitro* plants by CTAB method that was simply modified (Böhm *et al.* 1993) (described in Chapter Ⅲ). Genomic DNA for southern hybridization was isolated as follows : 0.1~0.2 g of fresh leaves were pulverized well in liquid nitrogen with 2~4 μl of mercaptoethanol and a small amount of quartz sand

in a mortar and were homogenized in 700 μl of the extraction buffer (Appendix3-1). The homogenate was incubated for 1~2 hours at 37 ℃. After adding 250 μl of phenol and 250 μl of chloroform, the sample was vortexed for 30 sec and centrifuged at 13000 g for 5 min. Then after adding 500 μl of CIA (chloroform : isoamylalcohol =24 : 1) into the supernatant, the sample was vortexed for 30 sec again and centrifuged at 13000 g for 5 min. Nucleic acids were precipitated from the supernatant by adding 0.1 volume 3 M sodium acetate and 2 volume 100 % ethanol. The pellet was rinsed by 80 % ethanol after centrifuged at 13000 g for 5 min at 4 ℃. The pellet was dissolved in 300 μl of TE buffer and incubated for 10 min at 65 ℃. After adding 10 μl of RNase A, the sample was incubated for 30 min at 37 ℃, then 300 μl of CTAB buffer was added, the sample was incubated for 15 min at 65 ℃ again. Thereafter, the solution was extracted with 500 μl of CIA and centrifuged for 5 min and 13000 g at a room temperature. Nucleic acids were precipitated from the supernatant by adding 600 μl of isopropanol. The pellet was rinsed with 80 % ethanol and was dissolved in the TE buffer.

2.3 PCR analysis

Random primer OPB and OPC kits were used to detect the new RAPD markers that linked with high shoot regeneration capacity. A preliminary experiment was done to detect the wild species-specific RAPD markers using KOT, PI128644 and the BC_1F_1-44-15 plant. The relationship between the RAPD markers detected and shoot regeneration capacity was evaluated by comparing 2 groups of BC_2F_1 plants : one was characterized with high SR rate and the other with 0 % of SR rate (described in Chapter Ⅲ). Ninety plants of the BC_1F_2-44-15 generation were evaluated in regards to the segregation of the RAPD markers linked with high shoot regeneration capacity. PCR-RAPD condition was as follows : 15 μl of a reaction mixture was composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.375 U TaKaRa Taq DNA polymerase, 0.4 μM primer, and 15 ng template DNA. The amplification conditions were programmed on a TaKaRa PCR Thermal Cycler TP2000 as follows : 1 cycle at 93 ℃ for 3 min and 45 cycles of 1 min at 93 ℃, 1.5 min at the 40 ℃ and 2 min at 72 ℃, and 5 min at a post extension of

72 ℃.

The *inv* gene was amplified according to Chapter Ⅲ with the following modifications. Twenty μl reaction mixture was composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.5 U TaKaRa Taq DNA polymerase, 0.3 μM each of primers, and 20 ng template DNA. The DNA products were analyzed as shown in Chapter Ⅲ.

2.4 Evaluation of shoot regeneration capacity

The middle part of the adventitious root (approximately 1 cm) of BC_1F_2-44-15 plants were used to evaluate the shoot regeneration capacity according to the method in Chapter Ⅲ. Shoot regeneration (SR) rate (%) was determined 4 weeks after being cultured on MS medium supplemented with 1 mg / l zeatin riboside. The SR rate (%) refers to the number of root segments with adventitious shoots / the number of root segments cultured. The F_1 hybrids and KOT were used as control. Each experimental plot had 6 root segments derived from one plant and the experiment was repeated two times.

2.5 Southern hybridization

For Southern hybridization, 10~15 μg of DNA isolated from leaves were digested by restriction enzymes ; EcoRI, EcoRV, Hind Ⅲ, Hae Ⅲ, BamHI and Pst Ⅰ, according to the manufacturer's (TaKaRa) instruction. The enzymes were applied in rather excess (2.4~3.6 U / μg DNA) to ensure complete digestion. Restricted DNA fragments were separated in 0.7 % agarose gels (Agarose LO3 TaKaRa). Following denaturation and neutralization, DNA was transferred onto Positive Charge membrane filter (Boehringer mannheim) using Bacu gene (Phamacia) according to the manufacturer's (Boehringer Mannheim) instruction. The membranes were baked to fix at 120℃ for 30 min. Tomato genomic clone TG102 and TG288 were used as probes. These probes were labeled with digoxigenin (DIG) (Boehringer Mannheim) by the random priming method.

Hybridization were carried out at 42 ℃ in Hybridization solution (2 % w/v Blocking reagent, 0.1 % w/v N-laurylsarcosine Na salt, 2×SSC, 0.02 % w/v SDS) for 6 to 12h. Washing of the membrane filters was performed by two conditions: one is 2 times of

10 min, 2×SSC, 0.1 % SDS at a room temperature and the other is 2 times of 15 min, 0.1×SSC, 0.1 % SDS at 42 ℃. The signal was detected by chemiluminescence following the manufacturer's instructions (Boehringer Mannheim) (Appendix 7).

2.6 Linkage analysis

MAPL (Ukai *et al.* 1995) and Mapmaker (Lander *et al.* 1987) were used to construct a linkage map.

3. Results

3.1 Segregation of shoot regeneration capacity

Forty-two of the BC_1F_2-44-15 plants that were chosen at random were evaluated in regards to shoot regeneration capacity. The SR rates of KOT and F_1 hybrid was 0 % and 92 %, respectively. The SR rates of the BC_1F_2-44-15 plants indicated the distinct two-peak distribution which were divided into two groups : one has more than 40 % of SR rate and the other has less than 20 % (Fig. 4-1). The segregation ratio was 31 for the former : 11 for the latter and fitted the expected ratio 3 : 1 ($\chi^2 = 0.03$, $p > 0.9$), suggesting that the superior shoot regeneration capacity derived from *L.*

chilense PI128644 would be controlled by a dominant major gene that could designate *Rg*. The plants which were characterized as those with higher shoot regeneration capacity formed calli on the both ends of root segments and developed adventitious shoots on the calli of the stem side of root segment ends like the wild species PI128644 and the F_1-44 plants. On the other hand, the plants as those with non-shoot regeneration capacity formed no or few shoot but only callus. (Fig. 4-2).

3.2 Segregation of PCR-based marker linked with shoot regeneration capacity

Using OPB and OPC kits, new 11 RAPD markers which were specific for the wild species were detected in a BC_1F_1-44-15 plant (Table 4-1). Three of them, OPA12-480, OPC02-450 and OPC19-680, were found only in the group of BC_2F_1 plants characterized with high shoot regeneration capacity (Table 4-1). The three RAPD markers would be linked with the dominant major gene related with high shoot regeneration capacity. The segregation of BC_1F_2-44-15 generation was determined for 6 markers including OPA02-900, OPA20-620 and *inv* ^{chi} (described in Chapter Ⅲ).

Table 4-2 shows the segregation of the RAPD markers and *inv* ^{chi} in the BC_1F_2 generation (refer to Appendix 8). A co-dominant marker, *inv* ^{chi} , and dominant RAPD markers indicate a good fit to the expected ratio 1 : 2 : 1 or 3 : 1, respectively.

3.3 Co-segregation of RFLP markers with shoot regeneration capacity and invertase gene

In the TG102, using the genomic DNA digested by EcoRV, a polymorphism between *L. esculentum* and *L. chilense* PI128644 was detected (Appendix 9). BC_1F_1-44-15 plant was heterozygous in TG102 locus that is located on the chromosome 3 (Tanksley *et al.* 1992). But any polymorphism have not been detected for TG288 locus that is also located on the chromosome 3 after using the 6 enzymes. Co-segregation of TG102 and shoot regeneration capacity indicates that there is a close relationship ($\chi^2 = 13.46$, d.f. = 2, $p < 0.005$) between TG102 marker and the high shoot regeneration capacity (Table 4-3). And it also indicates that there is a close relationship ($\chi^2 = 47.6$, d.f. = 4, $p < 0.005$) between TG102 marker and invertase gene (*inv*)(Table 4-4).

3.4 Linkage analysis

The linkage analysis in the BC_1F_2 generation revealed that all 6 PCR-based markers based on about 90 plants, shoot regeneration capacity based on 42 plants and TG102 of RFLP marker were located on the same linkage group. The genetic length of the linkage group were about 51 cM (Fig. 4-3). *Rg* was mapped in the middle between OPA02-900 and OPB12-480 with a distance of about 5 cM (Fig. 4-3).

4. Discussion

BC_2F_1-44-15 generation indicated huge skewed segregation in some markers (described in Chapter Ⅲ). The genetic model of the shoot regeneration capacity could not be evaluated in the BC_2F_1-44-15 generation, because the frequency of distribution in regard to SR rate had a flat and continuous distribution and did not fit any genetic model. But it was suggested that at least 2 loci would contribute to the shoot regeneration capacity. On the other hand, BC_1F_2-44-15 generation did not indicate any skewness of segregation in the markers which linked to the superior shoot regeneration capacity. The segregation of BC_1F_2-44-15 plants in

regard to SR rate indicated that it could be clearly divided into two groups : one is characterized with high SR rate and the other is with non or low SR rate (Fig. 4-1). And the segregation ratios fitted the expected ratio 3 : 1 which is the segregation ratio of a dominant gene in F_2. These results are consistent with that the shoot regeneration capacity of *L. peruvianum* is controlled by a major dominant gene, *Rg*-1 (Koornneef *et al.* 1993).

Sugiyama (1997) reported the model of redifferentiation in the hypocotyl culture of *Arabidopsis thaliana*. He divided the material condition into 3 types that are incompetent (IC) for differentiation, competent for root redifferentiation (CR) and competent for shoot and root redifferentiation (CSR). The hypocotyl that is in IC condition has to change to the CSR condition via CR condition to redifferentiate shoots. But the root that is already in CR condition has to change to the CSR condition to redifferentiate shoots by *SRD* 3. It seems that shoot formation from root explants of tomato is similar to that of *Arabidopsis thaliana* that is controlled by a major dominant gene.

In this Chapter, new 3 RAPD markers were detected using OPB and OPC kits that consisted of 40 random primers . Using 4

kits (OPA, OPB, OPC and OPK), 19 RAPDs that are specific for the *L. chilense* PI128644 were detected in the BC_1F_1-44-15 plant. As a result of segregation analysis in the BC_2F_1-44-15 and BC_1F_2-44-15 generations, total 5 RAPD markers that linked with *Rg* locus were detected. Therefore, approximately one new marker will be detected about per one kit. All the detected markers, *inv* ^{chi} and 5 RAPDs, that are linked with the shoot regeneration capacity segregated with the ratio 3 :1 on the same linkage group. This suggests that the superior shoot regeneration capacity, cultured on MS medium with 1 mg / l zeatin riboside, may be controlled by a major gene and/or additional plural genes which are located within this linkage group.

The *Rg*-1 was located with a distance of 9 cM from TG222 on the chromosome 3 using the backcross progeny derived from *L. peruvianum* (Koornneef *et al.* 1993) (Fig 4-4). TG222 was located on between TG288 and TG102 (TG288-TG222 is 0.8 cM, TG222-TG102 is 5.6 cM) in the F_2 population of *L. esculentum* × *L. pennellii* (Tanksley *et al.* 1992)(Fig 4-4). However, Chatelat *et al.* (1995) reported TG288 was located on between TG102 and TG222 (TG102-TG288 is 1.9 cM, TG288-TG222 is 5.5 cM) and TG102 was

tightly linked to the *sucr* gene that is a recessive sucrose accumulator gene in the backcross generation between *L. esculentum* and *L. chmielewskii*. Acid invertase gene of the green fruited wild species is also a recessive sucrose accumulator gene and *sucr* would be an invertase gene (Chatelat *et al.* 1995). The genetic distances between RFLP markers were different. The order of RFLP marker, TG102, is different between backcross progeny of *L. esculentum* × *L. chmielewskii* and others. However, it would be considered that the order may be *Rg*-1, *r*, and *sucr* with TG102. And there is possibility that *Rg*-1 of *L. peruvianum* (Koornneef *et al.* 1993)and *Rg* of *L. chilense* in the present study are the same locus.

In the present study, though Chatelat *et al.* (1993) could not resolve *sucr* and TG102, the distance between *inv* and TG102 is increased about 20 cM. Chatelat *et al.* (1993) pointed out the restraint of recombination in this area. Many researchers reported that the recombination frequency is different among the progenies between *L. esculentum* and the other wild species (Paterson *et al.* 1988, 1991 ; Van Ooijen *et al.* 1994 ; Bonnema *et al.* 1997). In the BC_1F_2 generation of *L. esculentum* ×*L. chilense* PI128644, the

recombination could occur more easily in this area than in the backcross progeny of *L. esculentum*× *L. chmielewskii*. In the future, the relationship among TG66, TG542, TG605, *r*, OPA01-900, OPB12-480 and *Rg* must be clear to decide the order of linkage map.

These PCR-based markers that were detected in the present study are very useful to introduce the superior shoot regeneration into tomato cultivar. However, the regeneration gene of *L. chilense*, *Rg*, was still on about 5 cM from the nearest RAPD markers at both sides. It is desired to detect more tightly linked markers and to construct the higher density molecular map for marker-assist selection and map-based cloning.

Table 4-1
Percentage of marker present in high SR rate group and 0 % of SR rate group in the BC_2F_1 generation

Marker	High SR rate gruop[1)]	0 % of SR rate gruop[2)]
OPB12-480	92 (%)	0 (%)
OPC02-450	92	0
OPC19-680	100	0
inv^{chi}*	92	0
OPA02-900*	92	0
OPA20-620*	91	0
OPB03	69	63
OPC20	62	13
OPB11	42	38
OPB18	39	25
OPC10	39	50
OPB10	23	13
OPB07	8	13
OPC05	8	38

[1)] consist of 13 plants which showed more than 60 % of SR rate

[2)] consist of 8 plants

* detected in Chapter III

Table 4-2
Segeregation of PCR-based markers related to the shoot regeneration capacity of *L. chilense* PI128644 in the BC_1F_2 generation

Marker	no. of plants			χ^2	p value
	wild type	heterozygote	tomato type		
inv[1)]	21	42	26	0.84	$p > 0.3$
OPA02-900	72	-	17	1.65	$p > 0.2$
OPA20-620	67	-	23	0.01	$p > 0.9$
OPB12-480	67	-	23	0.01	$p > 0.9$
OPC02-450	67	-	23	0.01	$p > 0.9$
OPC19-680	66	-	23	0.03	$p > 0.9$

[1)] co-dominant marker of acid invertase gene

Table 4-3
Co-segregation of RFLP marker TG102 and *Rg* gene and χ^2 test for contingency

	TG102			
	++	+−	−−	
Rg+	4	6	0	10
Rg−	0	1	6	7
	4	7	6	17

$\chi^2 = 13.46$ (d.f. = 2) : $p < 0.005$

Table 4-4
Co-segregation of RFLP marker TG102 and *inv* gene and χ^2 test for contingency

		TG102			
		++	+−	−−	
	++	4	0	0	4
inv	+−	0	13	0	13
	−−	0	3	9	12
		4	16	9	29

$\chi^2 = 47.6$ (d.f. = 4) : $p < 0.005$

TG102; ++: wild type, +−: heterozygote, −−: tomato type

Rg+: dominant gene, *Rg*−: recessive gene

inv: ++: wild type, +−: heterozygote, −−: tomato type

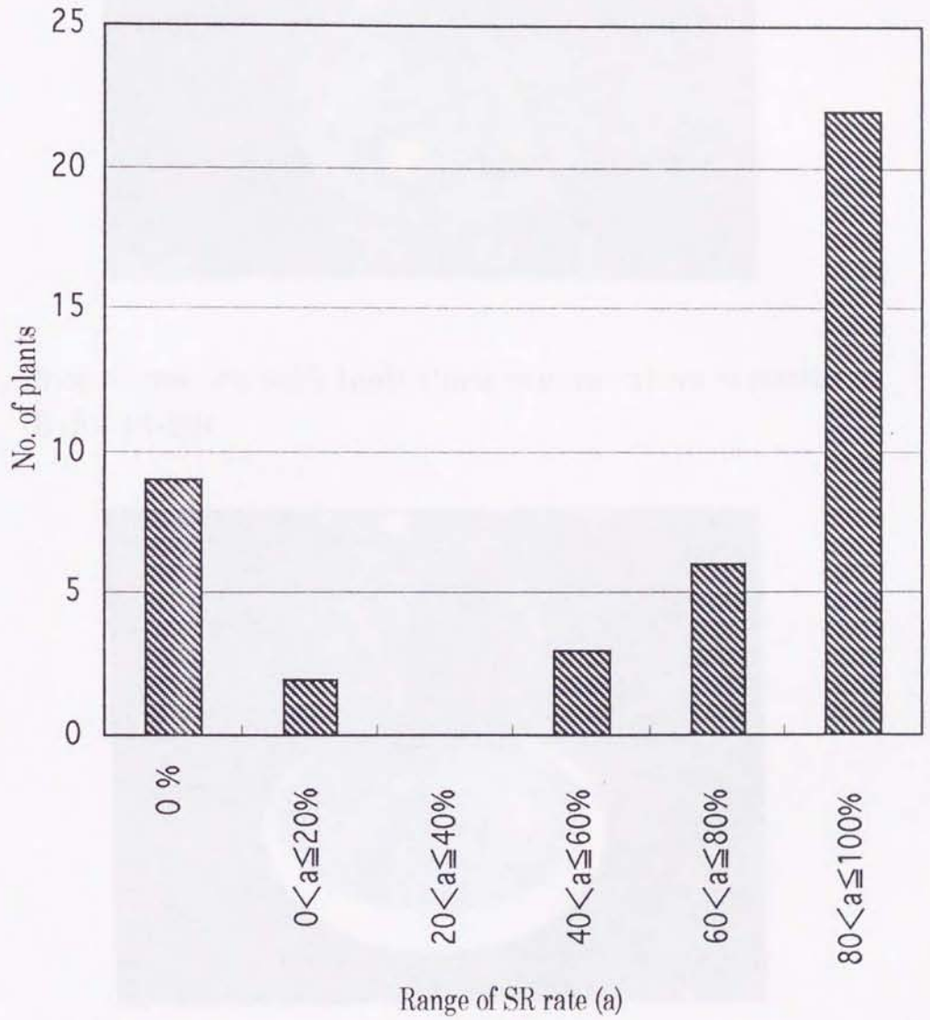
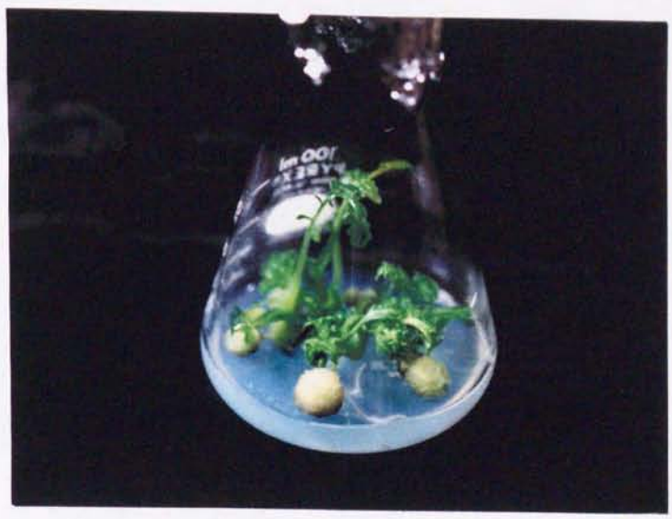


Fig. 4-1
Frequency of distribution of SR rates in BC1F2 generation.



Root segments with high shoot regeneration capacity
(B_1F_2-44-20)

Root segments with non shoot regeneration capacity
(B_1F_2-44-55)

Fig. 4-2

Shoot regeneration from root segments after 4 weeks of culture

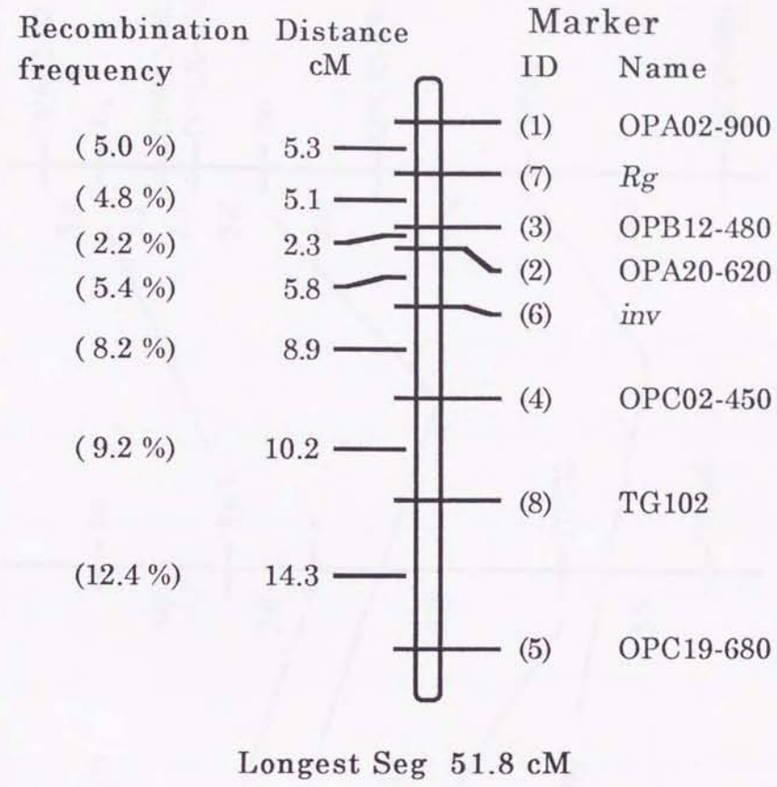


Fig.4-3
Linkage map of *Rg*, *inv* and 6 molecular markers in chromosome 3

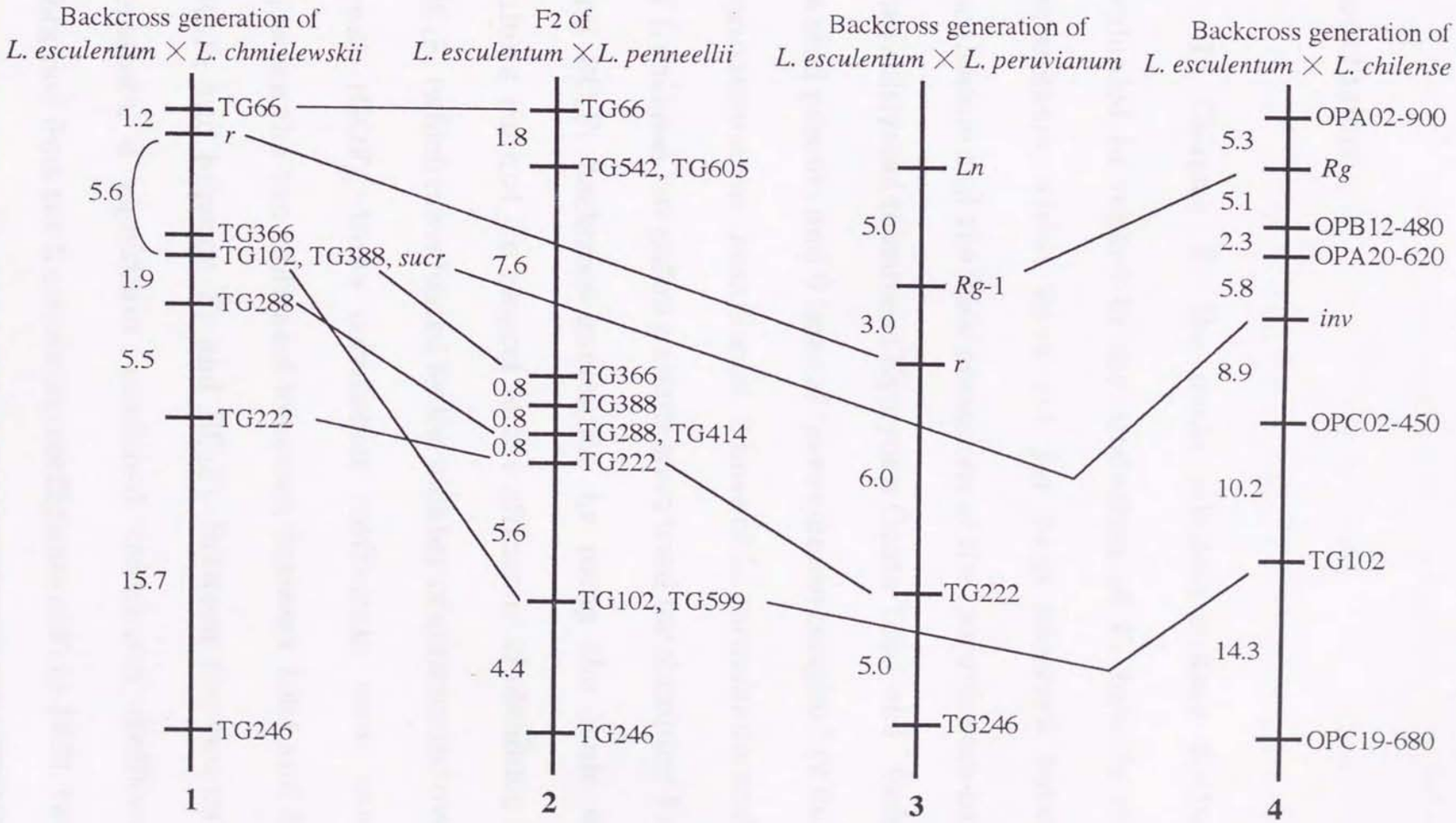


Fig. 4-4. Comparison of the linkage map of the chromosome 3 in tomato

1: the RFLP map by Chetelat *et al.* (1995), 2: by Tanksley *et al.* (1992), 3: by Koornneef *et al.* (1993), 4: by the present study