New Molecular Markers Linked with the High Shoot Regeneration Capacity of the Wild Tomato Species *Lycopersicon chilense*

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Summary

The goal of this research was to identify molecular markers that could assist in the selection of high shoot regeneration capacity from the progeny of a cross between a tomato cultivar and a 'peruvianum-complex'. The BC₁F₁ and BC₂F₁ generations between a tomato cultivar, 'Kyoryoku Ogata Toko' (KOT) and *L. chilense* PI128644 (together with both the parents and the F₁ hybrids) were used as materials. BC₂F₁ generation was developed by a cross between KOT and a self-compatible BC₁F₁ plant that had high shoot regeneration capacity. Root explants of KOT cultured on an MS medium supplemented with 1 mg/l zeatin riboside did not form any shoots, while those of the PI128644 and the F₁ hybrids showed a high shoot regeneration (SR) rate of more than 80 %. The BC₁F₁ generation could be divided into 3 groups (0%, less than 30% and more than 80%) for SR rate. The BC₂F₁ generation could be divided only into 2 groups; about 70% of the plants had 0% SR rate and the other 30% had a continuous and non-peak frequency distribution up to 100%. The segregation of the RAPD markers and the acid invertase gene marker (*inv*^{chl}) which were specific to the PI128644, were evaluated in BC₁F₁ and BC₂F₁ to determine a possible relationship between these markers and the ability to regenerate shoots. The Mann-Whitney test showed that the RAPD markers, OPA02-1, OPA20-3 and *inv*^{chl} were mostly present in the plants with the high shoot regeneration capacity. It was suggested that these three molecular markers are closely linked to the high shoot regeneration gene of *L. chilense* PI128644.

Key Words : *Lycopersicon esculentum*, *L. chilense*, PCR-based marker, shoot regeneration capacity.

Introduction

The theory that shoot regeneration from tissues in a number of plants could be controlled by the combination and the concentrations of phytohormones in *in vitro* culture was proposed by Skoog and Miller (1957). However, significant variations in plant regeneration capacity have been observed by several researchers among varieties, species, and genera. In addition, the problem of enhancing the regeneration capacity of recalcitrant plants by the genetic control is as yet

unsolved. Genetic studies on the regeneration of plants have been reported in 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; Komatsuda *et al.* 1989, Komatsuda *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. The trait was apparently controlled by a dominant *L. peruvianum* allele at a locus (*Rg-1*) in chromosome 3. Torelli *et al.* (1996) obtained a cDNA (G36) from cultivated tomato by mRNA differential display method, which exhibited the typical features of a morphogenetic marker.

In this paper, 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.

Materials and methods

Plant materials

The wild species *L. chilense* PI128644 was used as the pollen parent. *L. esculentum* cv. 'Kyoryoku Ogata Toko' (KOT) was used as the seed parent. Interspecific hybrids F₁-44 (KOT × PI128644), and their backcross generations, BC₁F₁ (KOT × F₁-44), and BC₂F₁ (KOT × BC₁F₁-44-15) were used for the segregation analysis of PCR-based markers and shoot regeneration capacity. The development of F₁ and BC₁F₁ was described by Takashina *et al.* (1997). BC₂F₁ plants were obtained from a backcross of KOT with a self-compatible plant (BC₁F₁-44-15) with the high shoot regeneration capacity selected from the BC₁F₁ plants. The seeds of KOT × BC₁F₁-44-15 from reddish fruits appeared normal, but some of the embryos in the seeds were immature or aborted. The normal embryos were dissected and cultured on an MS medium (Murashige and Skoog 1962) without any growth regulators.

Shoot regeneration capacity of root explants

Ten KOT, 10 PI128644, 6 F₁, 22 BC₁F₁, and 113 BC₂F₁ plants were used for evaluation of shoot regeneration capacity. KOT and PI128644 were germinated aseptically. All plants were maintained as cutting *in vitro* on an MS medium without growth regulators in 200 or 300

Received June 9, 1997. Accepted March 9, 1998.

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 zeatin riboside, 2 % sucrose, and 0.8 % agar in 100 ml Erlenmeyer flask. The cultured explants were incubated at approximately 25 °C under 16-hr photoperiod with fluorescent illumination at 2000 lux. The shoot regeneration (SR) rate (%) was determined 4 weeks after culture. 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 derived from one plant.

DNA isolation

Total DNA was extracted from approximately 0.1 g of fresh leaf tissue from *in vitro* plants by a simple modified CTAB method (Böhm *et al.* 1993).

RAPD analysis

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. Primer Kit-A (Operon 10-mer Kit) was used for analyzing the BC_1F_1 plants, while Primer Kits-A and K were used to analyze the BC_2F_1 plants. The reaction mixture (10 μ l) contained 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 μ M 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 °C for 3 min and 45 cycles of 1 min at 93 °C, 1.5 min at 40 °C, 2 min at 72 °C, and 5 min at a post extension of 72 °C. The PCR products were separated by electrophoresis in a 2 % agarose gel and were stained with ethidium bromide.

PCR for acid invertase gene

AIT-1 (5'-CGGTGAAAAACATTCAATGAG-3') and AIT-2 (5'-TCCACAATTGAGTGATCCAC-3') (Harada *et al.* 1995) were used to amplify the acid invertase gene (*inv*). DNA amplifications were done by a Taitec Gene Thermo Unit GTU-1605. The reaction mixture (15 μ l) contained 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 μ M of each primers, and 40 ng of template DNA. The amplification conditions were as follows : 5 min of preheating at 94 °C, 45 cycles of 1 min 50 sec at 94 °C, 2 min at 58 °C, and 2 min at 72 °C following 10 min at a post extension of 72 °C. The PCR products were separated by electrophoresis in a 4 % NuSieve 3 : 1 agarose gel and were stained with ethidium bromide. The sizes of the amplified fragments were calculated by a Gel Reader (ver. 2.05, University of Illinois).

Results

Small green calli developed on the root tip side of KOT root explants approximately 2 weeks after culture. However, the calli soon became brown and no shoot regeneration was found. On the other hand, green calli developed on both ends of the explants of PI128644 and F_1 within one week after culture. After one more week, several shoots were formed on the calli of the stem-side of the segment, and then on the opposite side. The SR rates of PI128644 and the F_1 plants were more than 80 % (Fig. 1). The BC_1F_1 plants were divided into 3 groups : the first has an SR rate of 0% (similar to tomato cultivar), the second has a rate less than 30%, and the third has a rate more than 80% (similar to the wild species and F_1 plants) (Fig. 1). In BC_2F_1 , about 70% (86 plants) of the plants did not form any shoots, and the other 27 plants showed a continuous and a non-peak

Fig. 1. Frequency distributions of parents, F_1 , BC_1F_1 and BC_2F_1 plants on shoot regeneration rate.

Fig. 2. Segregation of OPA02-1 (0.9 kb) in BC₁F₁ and BC₂F₁.

M; pHY marker, 1; *L. esculentum* cv. 'Kyoryoku Ogata Toko', 2; *L. chilense* PI128644, 3; BC₁F₁-44-1, 4; BC₁F₁-44-15, 5; BC₁F₁-44-5, 6; BC₁F₁-44-7, 7; BC₂F₁-12, 8; BC₂F₁-43, 9; BC₂F₁-18, 10; BC₂F₁-19.

The plants of lanes 3, 4, 7 and 8 indicated more than 80 % and the plants of lanes 5, 6, 9 and 10 indicated 0 % in SR rate, respectively.

frequency distribution in the SR rate up to 100% (Fig. 1).

In the preliminary RAPD analysis, we detected 22 RAPDs which were specific to the wild parent in the BC₁F₁ generation using OPA kit. The BC₁F₁-44-15 plant had 6 of the 22 RAPDs. On the other hand, 2 RAPDs were detected using OPK kit in the BC₁F₁-44-15 plant. A total of 8 RAPDs were used for segregation analysis in the BC₂F₁ generation. The segregation of 5 of the 8 RAPDs showed a significant distortion from the expected segregation rate (1:1). The PCR products of *L.*

chilense PI128644 (*inv*^{chi}) amplified by AIT primers were about 10~14bp longer than those of KOT which was 188bp in size. The BC₁F₁-44-15 plant characterized to have a high SR rate was a heterozygote in the *inv* locus. In the BC₂F₁ generation, the segregation of *inv*^{chi} showed a very significant distortion similar to 5 of the 8 RAPDs.

In the BC₁F₁ and BC₂F₁ 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 regenerate shoot (Fig. 2). BC₁F₁ and BC₂F₁ 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 1). The results indicated that the correlation between the presence of the three markers and the high SR rate in BC₂F₁ was significant at 1% level; in addition, the presence of OPA02-1 and OPA20-3 in BC₁F₁ was significantly related to high SR rate. This suggests that OPA02-1, OPA20-3, and *inv*^{chi} are linked with one of the genes that control the high shoot regeneration capacity.

Discussion

Several researchers have pointed out the dominance of the high shoot regeneration capacity of *L. peruvianum* (Frankenberger *et al.* 1981, Adams and Quiros 1985, Tomes 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 that of *L. peruvianum*. BC₁F₁ plants could be divided into 3 groups in regards to the SR rate. On the other

Table 1. Mann-Whitney test on the relationship between shoot regeneration capacity and molecular markers

Generation	Marker name	N1 ¹⁾	N2	R1 ²⁾	R2	U	Z
BC ₁ F ₁	OPA02-1	14	6	177	33	72*	
	OPA20-3	13	7	181	29	90**	
	<i>inv</i> ^{chi}	13	7	129	81	53ns	
BC ₂ F ₁	OPA02-1	44	58	2747	2506	795	4.513**
	OPA20-3	19	81	1583	3467	146	7.552**
	<i>inv</i> ^{chi}	17	86	1597.5	3758	17.5	8.689**

¹⁾N1 indicates the no. of plants with the marker and N2 indicates the no. of plants without the marker.

²⁾R1 and R2 indicate the total of the signed rank of N1 and N2 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;

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

hand, the BC₂F₁ plants which showed shoot regeneration could not be divided based on the SR rate. These results suggest that at least 2 loci or more may contribute to this capacity of *L. chilense* PI128644. Koornneef *et al.* (1993) described the *Rg*-1 dominant gene to be located in chromosome 3 of tomato. *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.

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. Since two of these markers showed a severely skewed segregation, linkage among the markers and loci controlling shoot regeneration could not be fully 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. And this is the first report that these RAPD markers which may be closely linked with the high shoot regeneration capacity genes were detected in tomato.

In addition, *Rg*-1 is closely linked to *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*) (Chetelat *et al.* 1995). In general, the *Lycopersicon* species share the same loci on the same location in each chromosome (Bonnema *et al.* 1997). Therefore, *inv*^{chi} may have a linkage with the gene of the high shoot regeneration capacity in *L. chilense* PI128644 which may be the *Rg*-1 locus.

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