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Construction of a Linkage Map in *Capsicum annuum* L. Using RAPD Markers and Identification of Two QTLs.

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Abstract

A linkage map of *Capsicum annuum* L. was constructed by random amplified polymorphic DNA (RAPD) markers followed in a backcross population of an intraspecific cross between cultivars HDA210 and Yatsufusa. A total of 420 random primers were tested and 311 polymorphic bands were generated by 158 random primers. Among them, 86 Yatsufusa specific bands generated by 52 primers were examined for mapping. Most bands except three segregated in Mendelian fashion fitting the expected 1:1 ratio. The total length of the map was 533 cM distributed in 15 linkage groups. The map distance between adjacent markers ranged 0 to 32.8 cM, with an average distance of 9.1 cM (63 markers). Some markers were clustered and this may be due to the amplification of a repetitive sequence by the RAPDs. Primer pairs for a sequence characterized amplified region (SCAR) were developed and the segregation scores by the SCAR primers were in accordance with the RAPD data. Two QTL markers for number of axillary shoots and for early flowering were developed. One QTL for early flowering located in the linkage group 3 and explained 61 % of the phenotypic variation. The other QTL for the number of axillary shoots located in the linkage group 4 explained 55 % of the phenotypic variation.

Introduction

Development of RAPD markers is technically simple. It can be performed quickly, and requires only small amounts of DNA. Linkage maps based on RAPD markers were established in azuki bean (Kaga et al., 1996), sweet potato (Thompson et al., 1997), blue berry (Rowland and Levi, 1993), pine (Devey et al., 1996), apple (Hemmat et al., 1994), and peach (Chapparo et al., 1994). The optimal conditions for developing reproducible RAPD markers in pepper has been reported (Yang and Park, 1998a).

Two previous linkage maps of pepper have been created. An interspecifc cross (*C. annuum* x *C. chinense*) was used for the first map (Prince et al., 1993; Tanksley et al., 1988). The second was constructed using doubled haploid (DH) progenies derived from an intraspecific cross of *C. annuum* (Lefebvre et al., 1995). A number of genes including *L* (resistance to TMV), *up* (erect habit of the fruit) (Lefebvre et al., 1995), *C* (fruit pungency), QTL for multiple flower per node (Prince et al., 1993), and *fc* (anther filament color) (Tanksley et al., 1988) were assigned into the linkage groups on the two molecular maps. Intraspecific crosses have advantages over interspecific crosses for mapping because most of the breeding programs use the intraspecific variability and recombination is suppressed in interspecific crosses (Lefebvre et al., 1995). The use of DH progenies allows the continued addition of markers to the map and simultaneous mapping of numerous monogenic and polygenic traits in the same population. However, there are statistical limits to linkage detection because some chromosomal region are

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selected during the androgenetic process resulting in clustered and skewed markers at some chromosomal regions (Lefebvre et al., 1995).

To assure a good representation of the whole genome, multiple maps based on several crosses should be developed, and these maps could be integrated into a single map. In this report, a map using a backcross population of an intraspecific cross was constructed and two quantiative trait loci (QTL) controlling the number of axillary shoots and early flowering, were positioned in the linkage groups.

Materials and methods

Plant materials

Twenty two pepper lines were planted and evaluated for their morphological and genetic differences. Among them, two pepper lines, HDA210 (*C. annuum* L.) and Yatsufusa (*C. annuum* L. var. *fasciculatum* Ilish) were chosen as parental lines and their progenies, F_1 and $BC_1(P_1)$ populations (HDA210/Yatsufusa//HDA210) were used for mapping. Their F_6 lines were also used for confirmation of the of developed markers.

RAPD analysis

All the methods were followed as described previously (Yang and Park, 1998a). A total of 427 primers (300 random decamers: UBC no. 1~100, 301~400, 701~800, 90 SSR primers: UBC 801~890, and 37 mtD primers: 16~28mer) from Univ. of British Columbia, Vancouver and 20 random decamers (OPG 1~20) from Operon Co. were used.

Genetic similarity

RAPD bands were scored as 1 for presence and 0 for absence. A total of 18 primers were used: 17 decamer (UBC no. 127, 147, 303, 308, 312, 319, 329, 336, 338, 341, 345, 348, 349, 350, 351, 356, and 359) and one SSR primer UBC no. 872, $(GATA)_4$ and reproducible bands were counted. The genetic similarity coefficients among accessions assayed were quantified based on Nei's (1987) formula using NTSYS-pc software (Rohlf, 1992). The cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA).

DATA analysis

The presence or absence of specific RAPD markers in the backcross population distinguished each plant. Some markers were distinguishable by band intensity in homozygous and heterozygous plants in the backcross population but were not included for mapping. Only Yatsufusa specific bands were used for mapping because only the crossing over frequencies in the paternal parent could be counted in $BC_1(P_1)$ (HDA210//HDA210/Yatsufusa) population. The linkage relationships among the molecular and phenotypic markers were tested using MAPMAKER version 3.0 (Lincoln et al., 1993). A minimum LOD score of 3.02 and a maximum recombination rate of 0.5 were chosen and linkage distance was calculated using Kosambi map function (Kosambi, 1944).

QTL analysis

Number of axillary shoot and days to first flowering from the BC_1 plants were measured. The association between markers and QTL related with these two traits was tested using the interval mapping method (Lander and Botstein, 1989) with MAPMAKER-QTL (Lincoln et al., 1992). A LOD score of 3.0 was chosen as a minimum to declare the presence of a QTL in a given region. The LOD peak was used to estimate the most likely QTL position on the RAPD linkage map.

Cloning and SCAR PCR

Mapped markers were recovered from the agarose gels by freeze and spin method (Marshall and Lew, 1994), and cloned into pGEM-T vector (Promega Co.) and transformed into *E.coli*, JM109. The cloned fragments were sequence characterized and a pair of SCAR primers of 24-mer, were constructed according to their sequences at both ends. 56℃ of annealing temperature were used for SCAR PCR.

Results and discussion

Genetic diversity and selection of parental lines for mapping

Twenty two *C. annuum* accessions were classified by their morphological characteristics and RAPD patterns. Even though more polymorphic bands were observed between different species, fair amounts of intra-specific polymorphic bands were produced (Figure 1). Therefore it was thought that an intraspecifc cross of *C. annuum* could be used as mapping population. Reproducible and polymorphic bands such as shown in Figure 2 were counted for cluster analysis. One marker, $u872_{1500}$, $(GATA)_4$, was unique in the six Asian lines among 22 accessions (Figure 2) and this band was quite common in Korean commercial cultivars, i.e. in 9 among 14 comm-

ercial F_1 hybrids (this marker was located on the linkage group 3 in Figure 5). On the other hand, the others dispersed variously among accessions. Thirty five reproducible and polymorphic bands among 121 bands were generated by 18 primers and were analyzed to generate a matrix of similarity (Figure 3). Based on the results, two lines, HDA 210 and Yatsufusa, were selected as parental lines for the mapping cross because they showed large differences morphologically and at the DNA level.

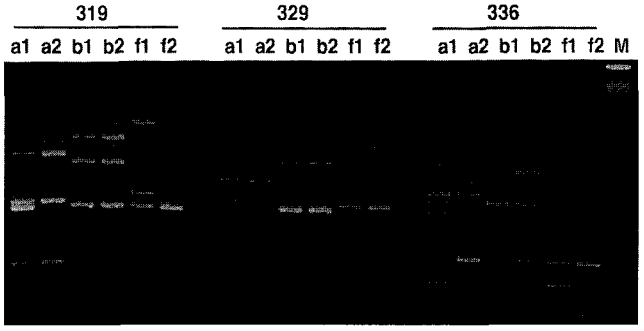

Figure 1. Comparison of RAPD profiles three species of *Capsicum* spp. Numericals, 319, 329, and 336, indicate UBC primer no. and a,b, and f indicate *C. annuum*, *C. baccatum*, and *C. frutescens*, respectively. M: DNA size marker, λDNA digested with *Eco*RI and *Hin*dIII.

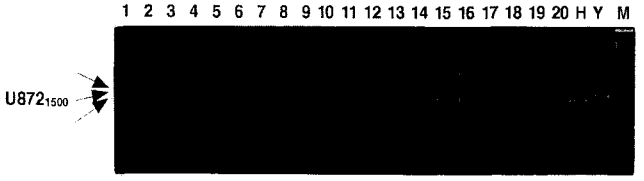

Figure 2. RAPD profile by UBC primer no. 872, $(GATA)_4$. Numericals indicate the accession names in Figure 3. Three bands pointed by arrows were counted for cluster analysis. M: DNA size marker, λDNA digested with *Eco*RI and *Hin*dIII.

Detection of RAPD markers

A total of 311 polymorphic bands were generated by 158 primers, one to six bands by each primer. These were also detected in the F_1 plant, indicating their transmission to the next generation. Among them, 86 P_2 (Yatsufusa) -specific, polymorphic bands generated by 52 primers (Table 1) were examined for their segregation in BC1F1 populations. Three bands among them deviated significantly from the expected ratio of 1:1 ($P<0.05$).

Sixty six appeared to be P_2-specific dominant markers such as those shown in Figure 4A, while the remaining twenty seemed to be codominant markers (Figure 4B) considering that band intensity was affected by other P_1-specific band. It was pointed out that codominant bands could arise from heteroduplex formation (Novy and Vorsa, 1995). However it was assumed that the codominant bands were formed due to difference of DNA fragment length. Some P_1-specific bands were scored reproducibly by band intensity in $BC_1(P_1)$ population and by band presence or absence in $BC_1(P_2)$ population. Demeke and Adams (1994) also reported that homozygous and heterozygous genotypes with regard to the specific bands could be distinguished by band intensity. Umt32 primer generated three polymorphic bands, one Yatsufusa specific and two HDA210 specific bands. These two HDA210 specific bands segregated as presence or absence in F_6 lines. These results show that the polymorphic bands between parental lines are consistent and reproducible for mapping although all are HDA210 specific bands. The P_1-specific bands were not included for mapping since HDA210 was used as the recurrent parent for the backcross population development.

Table 1. Total, polymorphic and scored bands for mapping according to kinds of primers

Primers	No. of primers		No. of bands			
	Tested	Polymorphic	Total	Ploymorphic	Scored[a]	Mapped[b]
Decamer	320	97	2,976	159	70 (35)	51 (31)
mtD	37	13	231	28	22 (12)	17 (11)
SSR (2base)	70	47	559	107	12 (6)	12 (6)
SSR (3-5base)	21	11	105	17	6 (4)	6 (4)
Total	448	158	3,871	311	110 (57)	86 (52)

[a]Number of bands segregated in mapping population and numbers in ( ) are the no. of primer
[b]Number of polymorphic bands scored for mapping and numbers in ( ) are the no. of primer

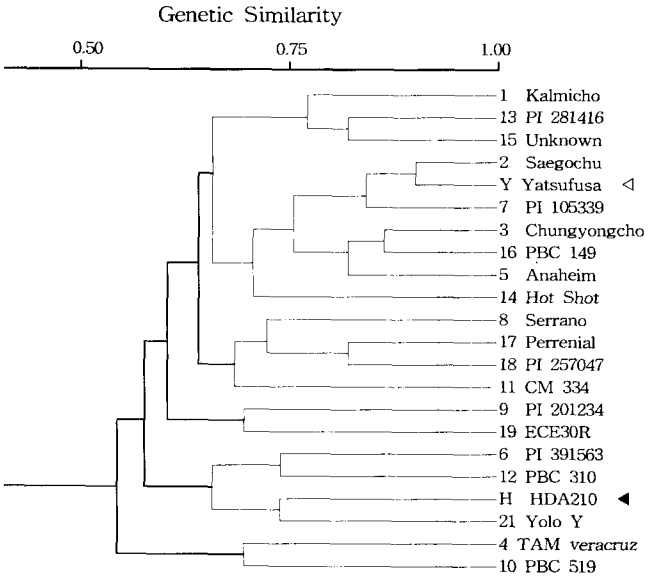

Figure 3. Genetic similarity of the 22 *C. annuum* accessions based on UPGMA analysis using 35 polymorphic bands. The parental lines used for mapping were HDA210(◀) and Yatsufusa(◁) and those were differentiated at the level of similarity index 0.6.

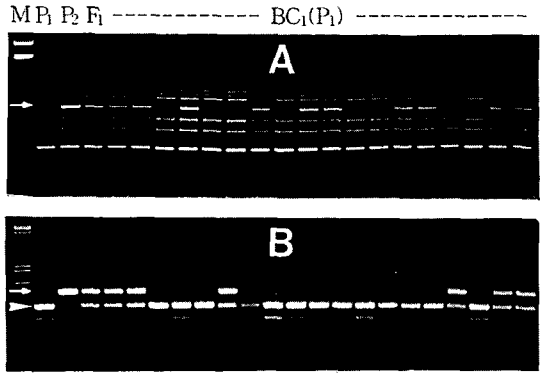

Figure 4. Kinds of markers: P_2-specific (A: $mtD10_{1020}$) and co-dominant (B: $u755_{1375/1100}$) markers. The band pointed by arrow could be scored by presence or absence and the arrowhead could be distinguished by band intensity in each plant. Lane M: DNA size marker, λDNA digested with *EcoRI* and *HindIII*, lanes P_1, P_2: HDA210 and Yatsufusa, respectively.

Linkage mapping

A genetic linkage map was constructed based on the segregation of 86 RAPD markers using MAPMAKER (Lincoln et al., 1993). Nine linkage groups containing more than three markers and five linkage groups containing two markers were established. It covered a total map distance of 532.5 cM (Figure 5). The distance between adjacent markers ranged from 0 to 32.8 cM with an average distance of 9.1 cM (613 markers). LG 15, was composed of two markers whose fragments are the same size even though they were generated by two SSR primers, UBC811, $(GA)_8C$, and UBC840, $(GA)_8YT$ (Y is mixed base with C and T). They appeared to be the same bands which were generated by the recognition of the same region, $(GA)_8CT$. LG 146 was established by inspection of segregation of two morphological characters in F_2 and BC_1 population. But the traits did not show linkage to any other RAPD markers. More abundant markers could be positioned and the markers linked to the characters could be developed.

The pepper molecular maps constructed by previous researchers covered 720 cM with 19 linkage groups (Prince et al., 1993) and 820 cM with 14 linkage groups (Lefebvre et al., 1995). These maps were estimated to cover 36~59 % of the total pepper genome because the genome size was estimated to be 1,390 cM or 1,498~2,268 cM (Lefebvre et al., 1995).

The inheritance of many agriculturally important genes such as five genes controlling monogenic traits such as growth habit, fasciculate flowering, purple anther color, immature fruit color, and shape of fruit apex were inspected in the segregating population. Also two quantitative QTLs controlling plant height and main stem length were inspected for the genetic segregation mode (Yang and Park, 1998b) but none of these were included on the linkage map. The map including those genes will be saturated by using other molecular markers such as RFLP and AFLP and could be integrated with the previous pepper maps in the future (Prince et al., 1993; Lefebvre et al., 1995).

QTL for days to first flowering

Yatsufusa grows fast in the early stage and has determinate growth with fruit clusters of 8 to 10 flowers at a node. First flowering of Yatsufusa is 30 days earlier than HDA210. This early flowering trait was shown to be governed by a single incomplete dominant gene or a few major genes in the backcross populations (Yang and Park, 1998b). Two QTLs for early flowering trait in *Brassica oleracea* (Camargo and Osborn, 1996) and three QTLs for early harvest in tomato have been reported (Lindhout, 1994).

One QTL was found in the LG3 with the LOD score, 9.0 when days to first flowering was subjected to QTL mapping (Figure 3, 6). This QTL explained 61 % of phenotypic variance and a marker locus, u38750-2 turned out to be its most likely location. The first flowering date of HDA210- homozygous class was 10 days later than the heterozygous class (Table 2).

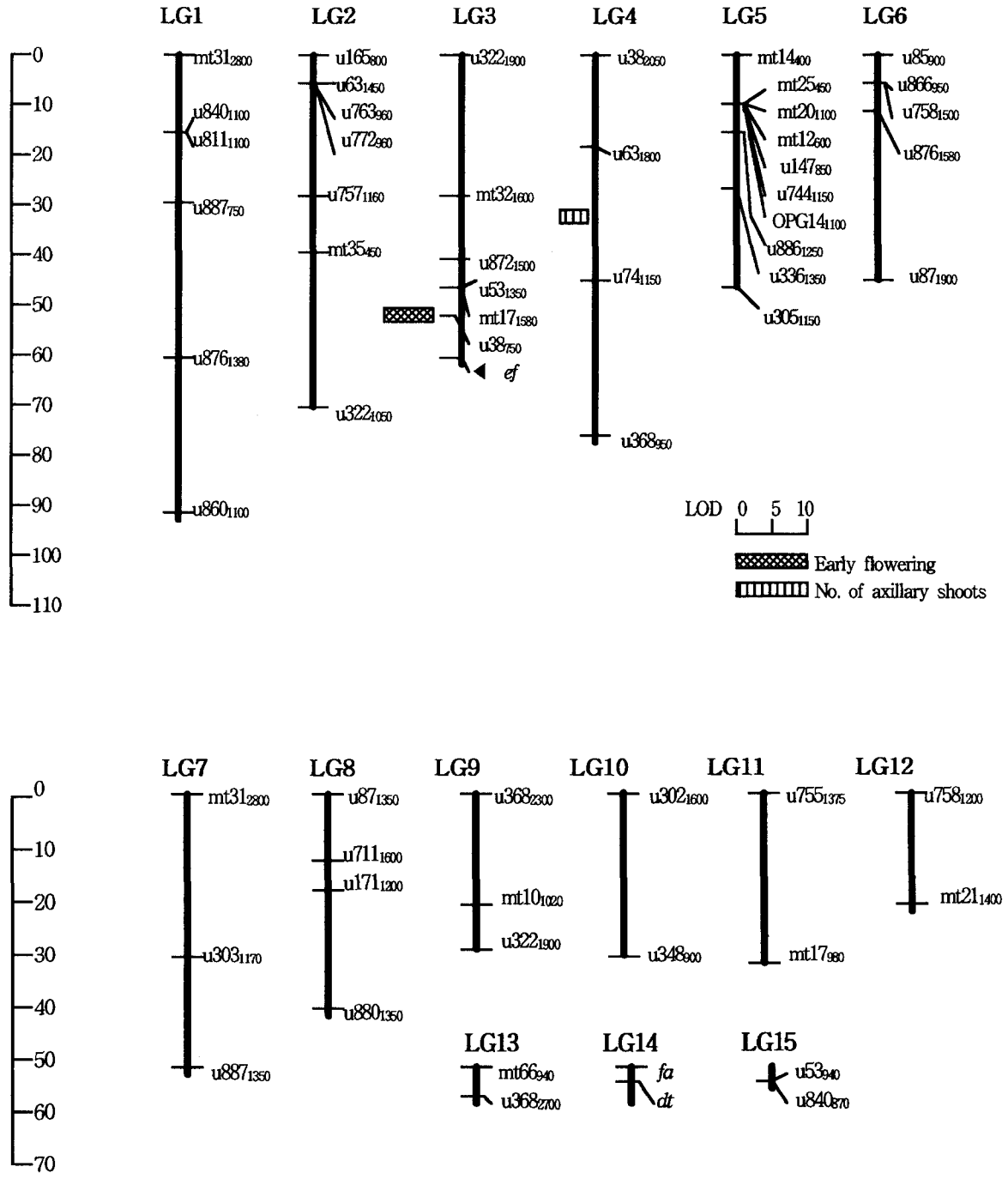

Figure 5. The genetic linkage map of *Capsicum annuum* based on 86 RAPD markers using BC_1F_1 population of an intraspecific cross, HDA210 x Yatsufusa. Sixteen linkage groups represented by solid lines were formed at the level of LOD score 3.0 and their numbering is indicated at the top. Two linkage groups, LG8 and LG13 might be coalesced into one linkage group (dotted line). Two putative QTLs of compact axillary shoot and early flowering were designated. Twenty nineseven markers did not belong to any linkage groups: OPG14$_{1570}$, u17$_{1600}$, u53$_{1600}$, u53$_{1100}$, u56$_{1150}$, u56$_{870}$, u63$_{1050}$, u66$_{1350}$, u66$_{560}$, u147$_{500}$, u354$_{1200}$, u 360$_{1400}$, u368$_{1300}$, u730$_{880}$, u755$_{1375}$, u758$_{650}$, u771$_{450}$, u811$_{700}$, u840$_{400}$, u860$_{950}$, u866$_{950}$, u891$_{950}$, mtD25$_{1400}$, mtD25$_{950}$, mtD25$_{2100}$, mtD30$_{1200}$, and mtD35$_{940}$. Map distances are presented at the left side in centimorgans (Kosambi function).

Table 2. Means for days to first flowering of different genotypic classes in BC_1F_1 population. Numbers in ( ) = no. of individuals examined.

Markers	Genotype		Difference in means	LOD score
	P_1/ P_1	P_1/ P_2		
$u38_{750}$	25.68	15.00	10.68	8.1
	(19)	(20)		
$mt17_{1580}$	26.22	15.64	10.58	6.5
	(22)	(17)		

Table 3. Means for number of axillary shoots of different genotypic classes in BC_1F_1 population. Numbers in ( ) = no. of individuals examined.

Markers	Genotype		Difference in means	LOD score
	P_1/ P_1	P_1/ P_2		
$u63_{1800}$	1.56	3.07	1.51	1.1
	(25)	(15)		
$u74_{1150}$	1.52	3.39	1.87	1.8
	(13)	(27)		

QTL for multi axillary shoots

Yatsufusa produces extensive axillary shoots at the main stem. The number of axillary shoots was controlled by few major genes with environmental effect (Yang and Park, 1998b). The QTL peak was located between loci $u74_{1150}$-1 and $u63_{1800}$-1 with LOD score 3.0 in the linkage group 4 (Figure 5, 7). Means for numbers of axillary shoots of different genotypic classes in regards to each marker were significantly different and 55 % of the phenotypic variance were explained by the QTL (Table 3).

Clusters of the RAPD markers

A RAPD marker, $u147_{850}$, showed complete linkage relationships with other five markers, $OPG14_{1100}$, $u744_{1150}$, $mtD12_{660}$, $mt20_{1100}$, and $mt25_{450}$. These six markers did not show any cross-over events between them as shown in Figure 8. Two markers, $u811_{1100}$ and $u840_{1100}$, located at a same position in LG1 were same size even though they were generated by two SSR primers, UBC811, $(GA)_8C$, and UBC840, $(GA)_8YT$ (Y is mixed base with C and T). They appeared to be the same bands which were generated by the recognition of the same region, $(GA)_8CT$. These type of clustered markers were also found at other fourive linkage groups (Figure 3) and this may be due to the amplification of repetitive region by RAPD. These phenomenons were also detected in the peach RAPD map (Chapparo et al., 1993). For the confirmation of the clustered markers, $u147_{850}$, one of the cosegregating markers, was cloned, sequence- characterized, and SCAR primer were designed (Jang, 1997). A single band was generated and showed polymorphism by band presence or absence. The segregation score were same with the scores of these six markers, $u147_{850}$, $OPG14_{1100}$, $u744_{1150}$, $mtD12_{660}$, $mt20_{1100}$, and $mt25_{450}$ (Figure 8).

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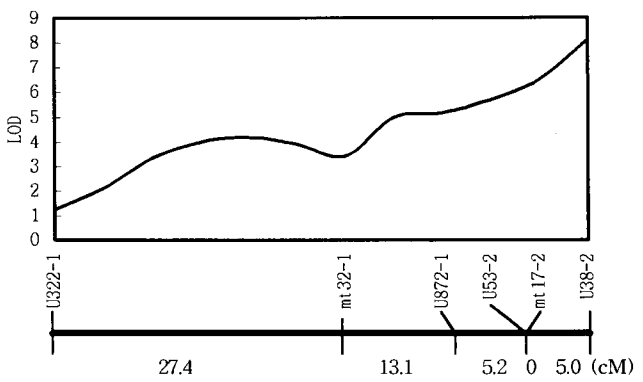

Figure 6. QTL likelihood plots for 'days to first flowering' (linkage group 3). Vertical axis indicates the LOD scores based on MAPMAKER/QTL and the numbers between adjacent markers indicate the map distance in Kosambi map function.

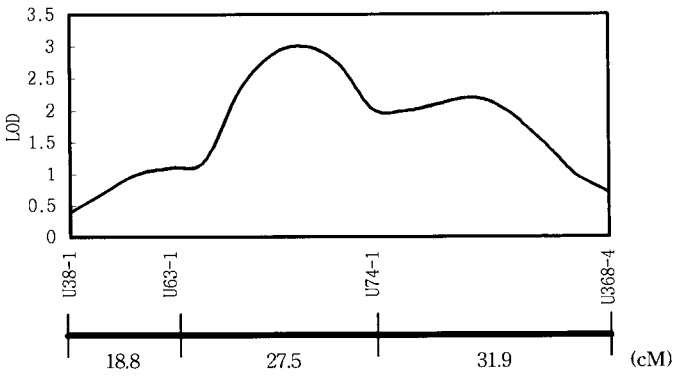

Figure 7. QTL likelihood plots for 'number of axillary shoots' (linkage group 4). Vertical axis indicates the LOD scores based on MAPMAKER/QTL. The numbers between adjacent markers indicate the map distance in Kosambi map function.

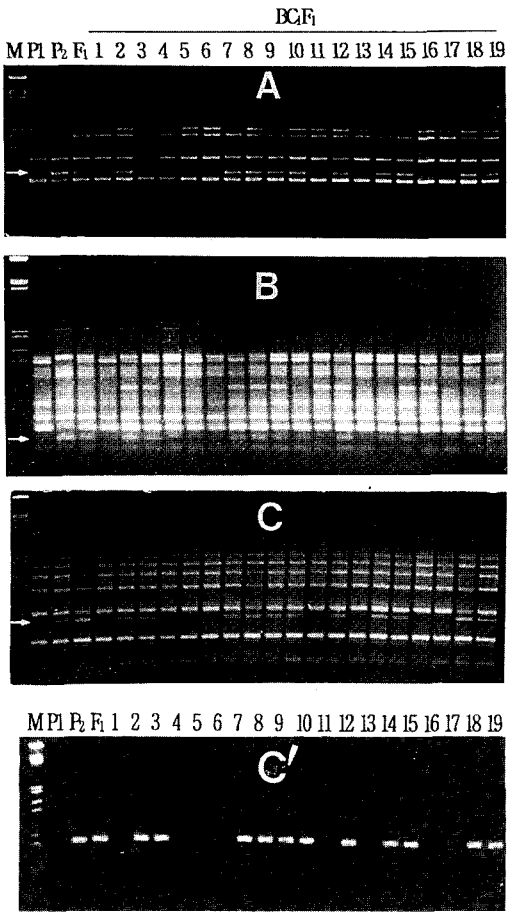

Figure 8. Co-segregation of RAPD markers (arrows): $mt20_{1100}$ (A), $mt25_{450}$ (B), $u147_{850}$ (C), and $u147_{850}$ SCAR marker (C') (only 19 BC1F1 plants are shown). C' profile is generated by SCAR PCR using a pair of SCAR primers, u147850-F: 5'-GTGCGTCCTCATAAAAACAATTGC-3', $u147_{850}$-R: 5'-GTGCGTCCTCTTAGTAATTTTTCT-3'. Lane M: DNA size marker, λDNA digested with *Eco*RI and *Hin*dIII. Lanes P_1 and P_2 are HDA210 and Yatsufusa, respectively.

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