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Construction of a linkage map and QTL analysis of horticultural traits for watermelon [*Citrullus lanatus* (THUNB.) MATSUM & NAKAI] using RAPD, RFLP and ISSR markers

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Abstract We have been constructing linkage maps for watermelon (*Citrullus lanatus*) on the basis of random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), inter-simple sequence repeats (ISSRs) and isozymes using an F_2 population derived from a crossing between a cultivated inbred line (H-7; *C. lanatus*) and an African wild form (SA-1; *C. lanatus*). A total of 120 F_2 plants was used for construction of a linkage map using 477 RAPDs, 53 RFLPs, 23 ISSRs and one isozyme markers. Linkage analysis revealed that 554 loci could be mapped to 11 linkage groups that extended for 2,384 centimorgans (cM). While a BC_1 population [(H-7 × SA-1) × H-7] consisting of 60 individuals was grown and scored for quantitative traits. Another linkage map with a total length of 1,729 cM was constructed in the BC_1 using genetic markers found to segregate in the F_2 population. A QTL analysis was applied by means of interval mapping for locating such agronomic traits as hardness of rind, Brix of flesh juice, flesh color (red and yellow) and rind color. The relative order of markers in the BC_1 map was essentially the same as that on the linkage map in the F_2. A total of five QTLs for four agronomic traits was detected. The QTL for hardness of rind was mapped on group 4. The linkage group 8 contained the QTL for sugar content of the flesh as expressed in Brix of the juice. The QTL for red flesh color was detected on groups 2 and 8. The QTL for rind color mapped on the group 3. The present map and QTL analysis may provide a useful tool for breeders by introducing valuable wild watermelon genes to cultivars.

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Introduction

Watermelon (*Citrullus lanatus*) originated in the arid areas of southern Africa, and is now cultivated throughout the world as a valuable fruit vegetable (Mohr 1986). In addition edible seed cultivars are bred and grown in China (Zhang 1996). Various breeding efforts have focused on fruit qualities, such as the fruit sugar content, size, maturity and rind color. Disease resistance is also a target of breeding (Crall et al. 1979). Fusarium wilt caused by *Fusarium oxysporum* f. sp. *niveum* is one of the main diseases (Smith 1899). However, genetic diversity in cultivated watermelons is not so high. While, a wide variety of wild watermelons are found in the native arid areas of southern Africa (Shimotsuma 1963; Ming and Xing-ping 1988), some wild African watermelons possess horticultural advantages, such as resistance to Fusarium wilt (Henderson et al. 1970; Martyn 1991), resistance to the zucchini mosaic virus (Provvidenti 1991), tolerance to a cracking of the fruit (El Mekki 1992), variation in fruit-size and a high percentage of female flowering. Use of these wild watermelons as a gene source may enlarge the breeding capacity.

Two linkage maps have been constructed to-date for watermelon (*C. lanatus*). One linkage map constructed with RAPDs, isozymes and RFLPs spanning 524 cM revealed the loci for rind color and flesh color (Hashizume et al. 1996). Another one, covering 354 cM and based on isozymes and seed protein, revealed the loci for flesh color (Navot and Zamir 1986; Navot et al. 1990). However, these linkage maps covered only a small part of the genome. The parentage between the wild form and the cultivated watermelon indicated plenty of DNA polymorphism for mapping (Hashizume et al. 1996). More-densely populated linkage maps covering a wide range of the genome should be constructed for QTL analysis of watermelon.

In the present study, we have used F_2 and BC_1 populations derived from a cross between a cultivated watermelon and an African wild form. The former population was used for construction of a linkage map using RAPD, RFLP, ISSR and isozyme markers, and the latter for scoring quantitative traits. A QTL analysis based on the data of the BC_1 population allows detection of additive, dominant and overdominant loci.

Materials and methods

Plant materials

The F_2 population was generated from H-7, a cultivated inbred used for the production of commercial F_1 cultivars, and SA-1, a white-fleshed and non-sweet wild race from South Africa. These were used to make a linkage map (Hashizume et al. 1996). Forty percent of the F_2 population did not bear fruit by self-pollination because of the genetic influence from SA-1. Consequently, the F_2 population was not suitable for a QTL analysis of the fruit. H-7 was backcrossed to F_1 for mapping the QTLs of agronomic traits of fruit. All H-7, SA-1 and F_1 (H-7 × SA-1) plants, and 120 F_2 plants, and 60 BC_1 plants [(H-7 × SA-1) × H-7], were grown in a greenhouse. The plants were individually sampled and assayed for segregating RAPDs, RFLPs, ISSRs, isozymes and morphological traits, such as hardness of rind, Brix of flesh juice, flesh color and rind color.

Measurement of agronomical characteristics

Hardness of rind was measured with a hand-operated fruit hardness meter (Mokuya Co., Ltd., Tokyo, Japan) equipped with a blunt-end rod plunger (6 mm in diameter), and expressed as puncture force. Brix of flesh juice was measured with a hand refractometer (Atago Co., Ltd., Tokyo, Japan). The color values, Hunter L*, a*, b*, of the flesh and rind were measured with a color-difference meter (model CR-100; Minolta Camera Co., Ltd., Osaka, Japan). The a* and b* values in the flesh were measured as red and yellow values respectively. The L* value was measured as the rind color indicating the brightness.

RAPD assays

DNA was extracted from young true leaves (approximately 500 mg) of 20 day old plants by the method described (Murray and Thompson (1980) with some modification (Hashizume et al. 1996). A total of 460 primers (10-mers and 12-mers) was surveyed for segregation between the cultivated inbred H-7 and the wild line, SA-1. As a result, a total of 280 primers showing polymorphism was used for construction of the linkage map. The DNA-amplification procedure was a modification of that reported earlier (Hashizume et al. 1993, 1996). The reaction mixture (10 μl) for PCR was composed of 10 mM Tris–HCl (pH 8.9), 80 mM KCl, 1.5 mM $MgCl_2$, 0.1% sodium cholate, 0.1% Triton X-100, 0.2 mM of each dNTP, 0.4 mM primer, 10 ng of template DNA and 0.2 units of the Tth DNA polymerase (Toyobo, Osaka, Japan). Amplification was carried out in a Program Temperature Control system (PC-700; Astec, Shime, Japan) by preheating for 15 s at 94 °C, and then heating employing 45 cycles for 25 s at 94 °C, for 2 min at 40 °C, for 3 min at 72 °C and post-heating for 7 min at 72 °C. After all the cycles had been completed, 9 μl of the product were analyzed on a 1.5% agarose gels.

ISSR analysis

A total of 15 primers selected from UBC set#9 (University of British Columbia) showing polymorphism between H-7 and SA-1, was used for construction of the linkage map. The sequence of used primers were as follows: ISSR1; $(AG)_8T$, ISSR2; $(AG)_8G$, ISSR3; $(GA)_8T$, ISSR4; $(GA)_8C$, ISSR5; $(GA)_8A$, ISSR6; $(CT)_8A$, ISSR7; $(CT)_8G$, ISSR8; $(CA)_8T$, ISSR9; $(CA)_8A$, ISSR10; $(CA)_8G$, ISSR11; $(GT)_8A$, ISSR12; $(GT)_8C$, ISSR13; $(GT)_8T$, ISSR14; $(TC)_8A$ and ISSR15; $(TC)_8C$. The reaction mixture was same as the above, and the PCR was performed by preheating for 15 s at 94 °C, and then heating employing 35 cycles for 25 s at 94 °C, for 1 min at 50 °C, for 2 min at 72 °C and post-heating for 5 min at 72 °C. The PCR products were separated on 2.2% agarose gels, and stained with ethidium bromide.

RFLP and isozyme analysis

Genomic DNA (2 μg) from each sample was digested with *Bam*HI, *Hin*dIII, or *Eco*RV fractionated in 1.0 % agarose, and blotted onto a nylon membrane in 0.4 N NaOH, and used for hybridization. cDNA clones from true leaf of the H-7, or the SA-1 true leaf, were prepared with the Time Saver cDNA Synthesis Kit (Pharmacia, Uppsala, Sweden) and used as probes. Plasmid pRR217, containing a rDNA fragment (PRR) from rice (Takaiwa et al. 1984) was also used as a probe. Labeling was carried out with positively charged complexes of peroxidase and glutaraldehyde (Whitehead et al. 1983; Renz and Kruz 1984). Hybridization and detection were performed according to the instructions supplied with the ECL direct nucleic-acid labeling and detection systems (Amersham Life Science, Amersham, England).

The isozyme patterns of glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1) were determined according to the procedures of Vallejos (1983) and Hirai (1986).

Linkage analysis and QTL analysis

Segregation of a 3:1 or 1:2:1 (F_2) and a 1:1 (BC_1) ratio of RAPDs, RFLPs and isozymes was tested by chi-square analysis. Segregation ratios that differed from the expected values (significant at $p = 0.05$ or less) were classified as distorted. The linkage map was constructed with the MAPMAKER ver. 3.0 program (Lander et al. 1987; Massachlisetts, USA) with linkage criteria of LOD > 3.0 or a recombination frequency of < 0.3 and the Kosambi (1944) mapping function. Interval QTL mapping was carried out using the software MAPMAKER/QTL version 1.1 (Paterson et al. 1988; Lincoln et al. 1993). A LOD score of 2.4 would be needed as the significance threshold for QTLs (Lander and Bostein 1989).

Results and discussion

Construction of a linkage map

A total of 477 RAPD markers amplified with 280 primers selected for showing polymorphism between the parental lines H-7 and SA-1 were used for construction of the linkage map in the F_2 population (Fig. 1). A total of 53 RFLP markers including pRR and cDNA, 23 ISSR markers and one isozyme marker were located in the linkage map. Most of the molecular markers gave the expected Mendelian ratio of 3:1 or 1:2:1 ($P > 0.05$). However, 59 markers (11%) exhibited significant skewing ($P < 0.05$) from the expected ratios.

A total of 554 polymorphic loci for which genotypes were determined in the segregating F_2 population were

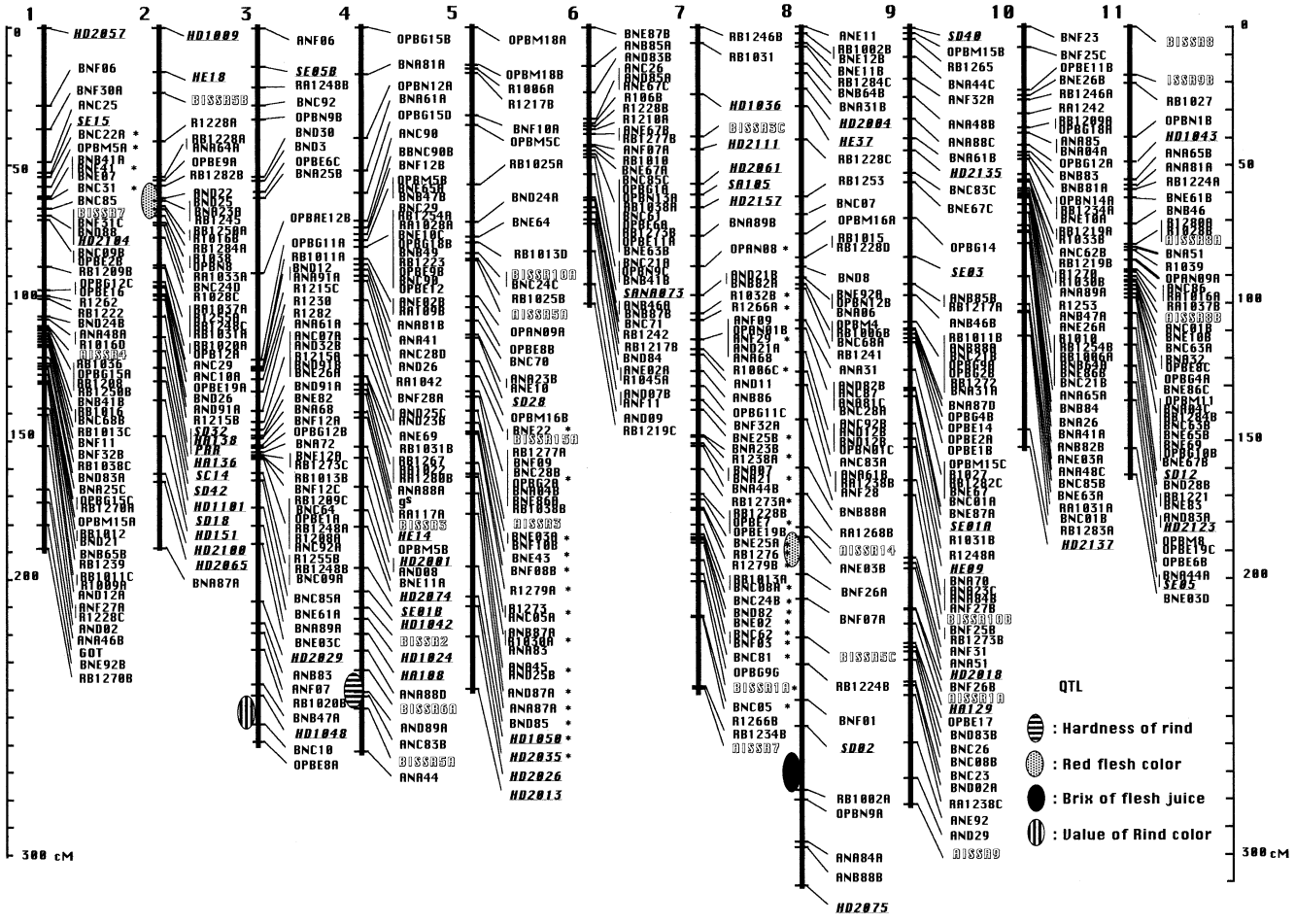

Fig. 1 A linkage map and QTLs in watermelon using RAPD, RFLP and ISSR markers. *Asterisks* indicate skewed loci. RFLP markers are *underlined italic*, and ISSR markers are *shadowed*

used to calculate linkages. The loci, including the genetic marker reported (Hashizume et al. 1996), were arranged into 11 linkage groups and the order of the loci in each linkage group was determined by the method of maximum likelihood. The resulting order of the loci and map distances between the markers in centimorgans (cM) are shown graphically as linkage maps in Fig. 1. In our previous study of the linkage map using another BC_1 [(H-7 × SA-1) × SA-1] population, the analysis of the segregation of RAPD markers allowed us to identify at least 11 linkage groups (Hashizume et al. 1996). Linkage groups 1 to 11 in the present study corresponded to linkage groups 1 to 11 in the previous study. The length of all linkage groups was extended in the present study. The morphological locus *gs* cannot be detected in the present population. The approximate location of *gs* is shown in Fig. 1. The loci R1030A, R1273 and R1279A, at the end of group 7 in the previous study, were rearranged and placed at the end of group 5 in this study. According to the map distances, a total of 2,384 cM in the F_2 was covered with an average interval length of 4.3 cM. Some parts on each group of the map were densely populated and tightly linked, showing no recombinants, but some regions were sparsely populated. More than half of the molecular markers on group 5 and group 7 exhibited significantly distorted ratios. A lot of RFLP markers (cDNA in the true leaf) tended to be mapped on the end of the each group. ISSR markers were impartially located on the groups. The RFLP and ISSR markers were useful to integrate the part of each group. As a result of the integration, the number of linkage groups could correspond to the chromosome number of the haploid genome of *C. lanatus* (n = 11). However, there are few gaps more than 30 cM between the markers on group 3 and group 10. Though some part of the genome would be uncovered, the linkage map is densely populated and extended to include more parts than reported previously (Navot et al. 1990; Hashizume et al. 1996). QTL analysis for agronomic traits in watermelon could be carried out using the map based on the molecular markers which segregated in the F_2 population.

Using the BC_1 [(H-7 × SA-1) × H-7] population, another linkage map was constructed for QTL analysis utilizing 240 molecular markers selected from each group on the linkage map in the F_2 population. The linkage map spanned 1,729 cM, which was more compact than that of

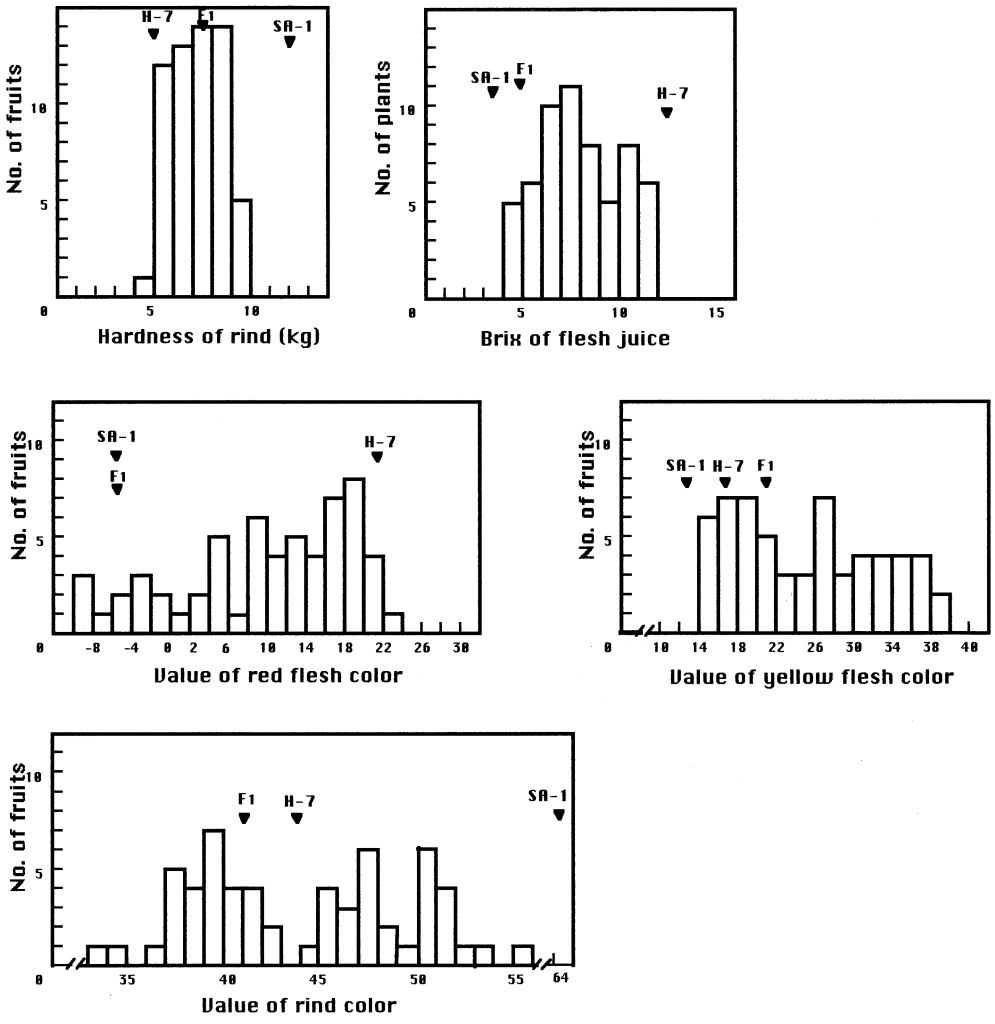

Fig. 2 Distributions for agronomic traits in the BC_1 progeny

the map based on the F_2. The average distance between markers was 7.2 cM. The relative order of markers in the BC_1 was essentially the same as that on the linkage map in the F_2 population.

Segregation and genetic analysis of agronomic characteristics

A BC_1 population [(H-7 × S-1) × H-7] of 60 individuals was grown and scored for its quantitative fruit traits for QTL mapping. Fifty nine out of the 60 BC_1 plants bore fruit, while the remaining one did not bear any. The absence of fruit would be the result of a genetic influence of the SA-1 parent because the wild form does not often bear fruit. Distribution of quantitative traits in the BC_1 is shown in Fig. 2. Four out of five agronomic traits were characterized by continuous variation, which suggests polygenic inheritance. The wild form showed high values of hardness of the rind at around 12 kg, and that of the cultivated inbred line was low. The means of the F_1s and the BC_1 fell close to that of the mid-parent. A QTL for hardness of the rind was detected on group 4 and explained 25.8% of the phenotypic variance with a LOD score = 2.8.

The cultivated inbred line, H-7, showed a high Brix value at around 12° Brix, while that of the wild line was low. That for the F_1 was near to that of the wild line, suggesting a partial dominance of the wild parental allele. The values for BC_1 fell between the parental mean values (Fig. 2). A QTL was detected on group 8 for the Brix of flesh juice. The QTL on group 8 accounted for 19% of the phenotypic variance (LOD = 2.6). The effect of the wild-form allele was in the direction expected, with a decrease in the Brix of flesh juice.

H-7 and SA-1 had red and white flesh-color respectively, while the F_1 was pale yellow. The flesh color of the backcrossed population showed a wide diversity among red, pink, yellow and pale yellow. The mean values for the yellow flesh color of the F_1 and the BC_1 were higher than those of the parents, suggesting an overdominance of the allele from the cultivated line. The main flesh pigments in cultivated watermelons are lycopene, carotene and xanthophyll. A correlation is observed for the red flesh color and lycopene contents (Sato et al. 1981). The yellow color is mainly composed of xanthophyll (Watan-

abe et al. 1980; Sato et al. 1981). However, the measurement of the value of yellow (b*) is disturbed by the red color in the flesh (Watanabe et al. 1980; Sato et al. 1981). Therefore, the overdominance observed here may derive from the disturbance of measurement. A QTL for flesh color was detected on group 2, showing the same QTL positions mapped for the yellow and red values. The QTL explained 55.2% (LOD = 10.3) and 35.8% (LOD = 5.7) of the phenotypic variance for yellow and red respectively. In this region, the wild-form alleles for flesh color were in the direction for increasing yellow and decreasing red. Therefore, the QTL on linkage group 2 is for red, and that for the yellow color observed here comes from the masking effect discussed above. To understand genes that render the yellow flesh color, the xanthophyll content in the fruit should be measured by fractionation, or else another cross between the yellow-colored flesh of the cultivated and the white flesh of the wild line would be required.

Another QTL was detected on group 8 for the red value of the flesh, accounting for 35.5% (LOD = 5.2) of the phenotypic variance. The wild allele acts in the direction expected, decreasing the value. In our previous study using another BC_1 [(H-7 × SA-1) × SA-1], we detected a locus for white flesh color (*w*) on group 6 (Hashizume et al. 1996). This locus cannot be detected in the present backcrossed population, because the present BC_1 population did not have white flesh due to the dominant gene for pale-yellow flesh color in the cultivated watermelon. The flesh color would, therefore, be controlled by more than three loci. It is noteworthy that QTLs for red flesh color and that of sugar content were found in a close range of linkage group 8. Most of the cultivated watermelon has red flesh, although various flesh colors, white, yellow and red, are known in genetic resources. The present finding may give the answer to the question why cultivated watermelon is red. Selection for sweeter watermelon for cultivation may result in selection for red flesh ones.

The rind color of the cultivated line H-7 was dark green, while that of the wild line was light green. The F_1 showed darker colors than the H-7, indicating an overdominance (Hashizume et al. 1996). The distribution of rind color of the BC_1 showed three peaks: dark green, green and light green (Fig. 2.). A QTL for the rind color value was mapped to group 3 and explained 32.4% (LOD = 4.7) of the phenotypic variance. Though the SA-1 mean value for rind color was higher than the value of the BC_1 population, the effect of the wild-form allele detected in this study was in the direction expected decreasing the value. Another locus for rind color, *gs*, was detected on group 4 in our previous study. The allele *gs* for the dark-green rind in the cultivated line is dominant over light green in the wild. The allele from the cultivated line decreased the color value, namely promoted darkness of the rind (Hashizume et al. 1996). The effect of the locus *gs* was not detected in the present population. This comes from the presence of the dominant allele of *gs* in all individuals analyzed. Therefore, the QTL for rind color in group 3 detected in this study would affect the *gs* for promoting the darkness of the rind. The rind color should be controlled by more than two loci

Linkage map of watermelon

To our knowledge this is the first report on a comprehensive linkage map for watermelon. In the present map, the number of the linkage groups corresponded to the haploid chromosome number (n = 11). The total map distance (2,384 cM) recorded here is comparable to the distances reported on melons (1,390 cM, Baudracco-Arnas et al. 1996; 1,942 cM, Wang et al. 1997). Moreover, some of the important agronomic traits, such as sugar content, rind color and flesh color, are recorded in the present map. The present linkage map may give a useful framework for mapping of agronomic traits in watermelon and for studies on the syntheny of various cucurbit crops. In spite of the importance of watermelons in horticulture, genetic studies of watermelon have been rather poor. Only 38 morphological or physiological genes are currently listed (Rhodes and Zhang 1995). One of the difficulties of genetics is having the space for planting, and the labor required for the cultivation of watermelons. The planting density of watermelons is 3,000–8,000 plants per hectare in Japan. Those of tomatoes and rice are 30,000 and 700,000 respectively. Genetic study using a large number of watermelon plants requires space and is laborious. In the present study we used 120 F_2 plants for the construction of the linkage map, and 60 BC_1 plants for the QTL analysis. The other difficulty is low DNA polymorphism among cultivated watermelon. In our previous study, only 3% of the amplified DNA bands differed from the parents of commercial hybrid cultivars (Hashizume et al. 1993), while African wild forms showed a wide difference (37%) from cultivated watermelon. Therefore, the combination of wild and cultivated watermelon is suitable for construction of the linkage map of watermelon.

Selection markers for sugar content

Fruit sugar content is one of the most valuable traits in a wide variety of fruit crops, such as melon, watermelon and tomato. Since this trait is generally thought to be polygenic and largely affected by environmental conditions, such as temperature or soil water, improvement of the sugar content requires a complex and prolonged breeding process. Mapping the genes affecting the fruit sugar content may greatly help the breeding and also provide valuable information on genetics and physiology. In tomato, introgression of a 7 M chromosomal segment from the wild species *Lycopersicon chmielewskii* to cultivated tomato was found to increase in soluble solids (Azanza et al. 1995). The present study suggests that at least a region in the linkage map affects the sugar content of watermelon, since soluble sugar content accounts for

Table 1 QTLs detected in the BC_1 population using interval mapping, Phenotypic effect = H-7/SA-1means – H-7/H-7means

Trait	Marker interval	QTL position (cM)	H-7/H-7 means	LOD score	% Var	Weight
Red flesh color	RB1245-BNA23A (group 2)	0.0	15.6	5.68	35.8	–8.7
	BNF26A-BNB88A (group 8)	2.0	15.4	5.18	35.5	–8.6
Yellow flesh color	RB1245-BNA23A (group 2)	0.0	19.3	11.03	55.2	11.0
Brix of flesh juice	RB1002A-SD02 (group 8)	8.0	8.8	2.61	18.8	–1.8
Value of rind color	OPBE8A-BNC10 (group 3)	2.0	47.0	4.74	32.4	–6.3
Hardness of rind	BISSR5A-BISSR6A (group 4)	5.6	6.4	2.76	25.8	1.3

about 90% of the Brix value in watermelon (Kurata 1971). The nearest marker to the QTL, RB1002A on the group 8, can be used for selection of sugar content. When the marker was applied for the selection in a BC_2 (BC_1 × H-7) progeny, most of the individuals selected with the marker showed values above 7 degrees (data not shown). Since cultivation of watermelon plants to fruit maturity is labor-intensive and time-consuming, and the sugar content of the fruit is largely affected by environmental factors, pre-selection using these DNA markers at the seedling stage may be helpful for breeders. Marker-assisted selection may increase the number of plants handled by breeders in backcrossing and make more-complicated breeding possible. Since QTLs found in the present study explained only a part of the phenotypic variance of sugar content, more detailed study using more advanced generations is required.

Use of African watermelon for breeding

DNA polymorphism among cultivated watermelons is rather poor (Hashizume et al. 1993), and that among African wild forms is also low. However, 37% of random amplified DNA showed polymorphism between the cultivated and African wild races (data not shown). Therefore, the molecular markers located on the linkage map can be utilized for QTL analysis in other crosses between cultivated watermelon and the African wild form. Wild watermelons collected in South Africa showed a wide diversity of shape: round, oval, conical or flat-shaped like a pumpkin. Rind color varied from dark green to light green. Both striped and non-striped fruit were recorded in this area. Flesh color also varied from pale yellow to white. Seed color was red, green or brown. Some African wild watermelons showed resistance to Fusarium wilt. However, the majority of the fruit had a low sugar content of 2 to 4 degrees Brix and also many undesirable characters, such as white flesh color and bitterness in the fruit. Therefore, an extensive backcross is required to remove these undesirable traits, if using one of these lines as a parent. The present map and markers would be useful in the backcross.

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