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Research Report

Construction of a Genetic Linkage Map Using a Frame Set of Simple Sequence Repeat and High-Resolution Melting Markers for Watermelon (*Citrullus* spp.)

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Abstract. Backcross breeding programs are widely used for rapid cultivar development by introgression of valuable traits from wild-type germplasm into elite lines. For efficient introgression from a distant cross, a genetic map is useful to select markers for maintaining the genetic background of elite lines. Construction of a genetic map in watermelon has been limited on one hand, by the relatively narrow genetic background within watermelon cultigens that results in little genetic polymorphism, and on the other hand, by high segregation distortion rates (SDR) of highly polymorphic markers found in a distant cross. In this study, we tried to develop a genetic map using expressed sequence tag (EST)-based simple sequence repeat (SSR) markers; however, only 33 EST-SSRs were anchored to the map, mainly because of multi-copies of repetitive sequences and SDR in the F2 mapping population derived from a cross of PI 189225 (*Citrullus amarus*; previously *C. lanatus* ssp. *citroides*) and 'TS' (*Citrullus lanatus* ssp. *lanatus*). To obtain more candidate markers, the whole genome of both parental lines was resequenced, and 2.5 and 0.3 million single-nucleotide polymorphisms (SNPs) were identified in PI 189225 and 'TS', respectively. By comparing these SNPs to the reference genome, we developed 200 high-resolution melting (HRM) candidate markers for genotyping the F2 progeny. Ultimately, 103 HRM markers were located on the genetic map, and 42 EST-SSR markers were consolidated in the map. The total map length was 1178.3 cM, the average length of the linkage groups was 107.1 cM, and the average inter-marker distance was 8.24 cM. Although these markers are not evenly distributed along the genome, they are considered to be successfully anchored. The EST-SSR and HRM markers in this genetic map will be useful in breeding programs as frame markers for foreground and background selection.

***Additional key words*:** MABC, MAS, NGS, resequencing, segregation distortion

Introduction

Watermelon, *Citrullus lanatus* (Thunb.) Matsum. et Nakai, is an important crop and provides phytochemicals such as lycopene, citrulline, arginine, and glutathione, as well as water and minerals essential for human health (Inthichack et al., 2014; Ren et al., 2012; Soteriou et al., 2014).

Watermelon is a specialty crop that belongs to the xerophyte genus *Citrullus* with a genome size of 425 Mb (2n = 2x = 22) (Levi et al., 2013; Xu et al., 2013). Prior to genome sequencing of watermelon, a genetic linkage map was constructed using molecular markers derived from isozymes (Navot and Zamir, 1987; Zamir et al., 1984), randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism, amplified fragment length polymorphism (AFLP), and sequence-related amplified polymorphism (Levi et al., 2006). In addition, high-frequency oligonucleotide-targeting active genes (HFO-TAGs), which are recently developed markers, have been reproducible and useful in the genetic mapping of watermelon (Levi et al., 2013). Similar to other major crops, known sequence-based markers retrieved from GenBank (http://ncbi.nlm.nih.gov), expressed sequence tag (EST)-based simple sequence repeats (SSRs), were used for genetic linkage mapping of watermelon, but only few SSRs were anchored to the map. After initiating watermelon genome sequencing at the end of 2008, as a part of the International Watermelon Genome Initiative, the draft genome sequence yielded large numbers of SSR, insertion and deletion (InDel), and structure variation (SV) markers (Ren et al., 2012). Using these markers, which are sequence-based and codominant markers, a high-density genetic map was successfully constructed, providing anchors and orientation of assembled scaffolds. The genetic map was constructed using recombinant inbred lines (RIL) generated from line

97103 (Chinese elite line) × PI296341-FR (wild-type, *Fusarium* wilt-resistant line) and consisted of 953 markers that covered 798-cM genetic distance and 330.4-Mb scaffold size. Overall, 234 scaffolds were anchored on the map, and the coverage of the 97103 genome was approximately 93.5%.

When using arbitrary markers without any information on the watermelon genome sequence, very low numbers of markers (< 400 loci) from RAPD, SSR, and AFLP were anchored on the map because of the lack of polymorphisms, generating a few linkage groups caused by narrow genetic backgrounds of cultivated watermelon (Hashizume et al., 1996; Hashizume et al., 2003; Levi et al., 2001). In contrast, experimental populations generated from the cross between the cultigen and wild-type line, which have a genetically long distance, helped in identifying many polymorphic markers, but demonstrated severe segregation distortions of the markers and resulted in low numbers of anchoring markers and linkage groups (Levi et al., 2002).

The main factors that limited the construction of the genetic linkage map have been addressed by the publication of draft genome sequences for watermelon (Xu et al., 2013). Recent development of resequencing by next-generation sequencing (NGS) has accelerated the identification of variant sequences such as single-nucleotide polymorphisms (SNPs) and InDels, which can be used as codominant markers capable of genotyping each progeny of a mapping population. To identify large data sets of single nucleotide variants (SNVs) by resequencing, alleles with high polymorphism information content (PIC) values and informative SNVs were analyzed using representative genotyping platforms, Illumina GoldenGate Array (Illumina Inc., USA) and Fluidigm Biomark (Fluidigm Inc., USA), which have been mainly used for other crops. To date, for genetic linkage mapping, QTLs, and genome-wide association studies of watermelon, genotyping by sequencing (GBS) (Elshire et al., 2011; Nimmakayala et al., 2014a; Nimmakayala et al., 2014b; Reddy et al., 2014) and DArT-Seq™ (Petroli et al., 2012; Ren et al., 2015), known as rapid SNP discovery methods by reduction of genome complexity, have been reported. These methods for high-throughput (HT) genotyping reduce time and labor required for genotyping alleles and constructing high-density genetic linkage maps. Furthermore, the extensive SNP data overcome the limits of low polymorphisms and high segregation distortion.

Recently, SNP-based high-resolution melting (HRM) was introduced for discriminating genotypes of alleles as a type of digital PCR (Li et al., 2010; Sonnante et al., 2011). The analysis platform using HRM without fluorescent dye-labeled probes can be an alternative to other HT genotyping methods, especially for selection from a large number of makers and in relatively small-scale experiments.

In this study, we resequenced parent plant genome of an F2 mapping population using Illumina HiSeq 2000 (Illumina Inc., USA) and obtained candidate markers of HRM SNPs from NGS data. A genetic linkage map for watermelon was constructed using selected reliable HRM markers and the F2 population. EST-SSR markers that escaped segregation distortion in the F2 progeny were also anchored to consolidate the genetic map.

Materials and Methods

Plant Materials and DNA Extraction

F1 and 252 F2 progeny derived from a cross between a wild watermelon, US PI 189225 (*C. amarus*; previously *C. lanatus* ssp. *citroides*), and a cultivated inbred, 'TS' (*C. lanatus* ssp. *lanatus*), were developed by Partner Seed Company (Ansung, Korea). The plants were grown at 20-30°C with 12-14 h of natural light in a greenhouse. Leaves of the plants were individually sampled and stored at -80°C in a deep freezer, until DNA extraction and assay for segregating EST-SSR and HRM markers. Genomic DNA of each plant sample was extracted using a modified CTAB method (Stewart and Via, 1993).

Detection of EST-SSR Loci

Four hundred primer sets of EST-SSRs, ranging in size from 150 to 400 bp, and computationally generated by the "International Cucurbit Genomics Initiative" (ICuGI; http://www.icugi.org), were kindly donated by Dr. Y. Park (Pusan University, Korea). Primers that produced DNA fragments were used for the polymorphism test of the parents and F1 and 252 F2 progeny. A total of 125 polymorphic SSR primers were obtained and used for genetic mapping. PCR was performed as follows: denaturation at 95°C for 2 min, followed by 35 cycles of 45 s at 94°C, 45 s at 50-59°C (the annealing temperature in each PCR experiment ranged from 50°C to 59°C on the basis of the T_m for each primer pair, as recommended by the ICuGI), and of 60 s at 72°C, with a final extension at 72°C for 5 min. PCR products were separated using 3% agarose gel (Agarose SFR™, Amresco, USA) in 1× TBE buffer.

SNP Identification and HRM Marker Development

We resequenced the whole genome of inbred line 'TS' and PI 189225 using Illumina HiSeq 2000 (Illumina, Inc., USA) at Microgen Inc. (Korea). Each sequencing depth had approximately 30× coverage, and all high-quality short pair-end reads were aligned to the reference genome of watermelon (http://www.iwgi.org) using the BWA tool (Li and Durbin, 2009) and BAM (http://bio-bwa.sourceforge.net) files also generated by the SAM tools (http://samtools.sourceforge.net). GATK (http://www.broadinstitue.org/gatk/index.php) identified SNPs and short InDels and generated single- and multi-variant

Table 1. Summary of whole-genome resequencing results of mapping parents, PI 189225 (*Citrullus amarus*) and cultigen 'TS' (*Citrullus lanatus* ssp. *lanatus*) of watermelon

Parental lines	Total reads (bp)	Mapped reads (bp)	Properly paired reads (bp)	Properly paired rate (%)	Average genome coverage[z]
PI 189225	211,377,532	171,115,481	140,256,789	66.35	31.5×
TS	162,922,891	145,061,895	122,434,489	75.15	30.6×

[z]Calculated using the total number of bases generated/size of the sequenced genome; the reference genome size is 353 Mb (line 97130) (Ren et al., 2012)

call format (VCF) files (http://vcftools.sourceforge.net). In total, 38,826,320 SNPs were detected across the genome sequences of the parental lines. Resequencing data for the parental lines are shown in Table 1.

Using multiVCF files, representative 106 SNPs located on the genome at relatively regular intervals in each of the 11 chromosomes were selected for HRM marker generation. The PCR primer set for HRM was designed by Primer3web (http://primer3.ut.ee/), PCR products ranged in size from 80 to 120 bp, and annealing temperature was set at 60°C. Two hundred primer pairs that harbored SNPs, designated as H1-H11 (HRM marker for chromosome 1) according to the physical location in the reference genome, were used for PCR-coupled HRM assay with LightCycler® 96 (Roche Life Science, Inc., USA). The HRM assays were performed using 10-μL reactions containing 1× LightCycler® 480 High Resolution Melting Master (Roche Life Science), 3 mM $MgCl_2$ (Roche Life Science), 0.5 μM of each primer, and 1 ng of dsDNA template. The PCR conditions were as follows: preincubation at 95°C for 10 min, followed by 55 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. A preconditioning step for DNA melting of 60 s at 95°C, 60 s at 40°C, and 60 s at 65°C followed the PCR stage, and then HRM was performed at 65-97°C, with 0.07°C per 1 s of temperature increment; dsDNA melting was detected by 15 readings per 1°C. The HRM data were analyzed using the LightCycler® 96 SW 1.1 program (Roche Life Science, Inc., USA), and the sizes of all amplified products were confirmed using 1.5% agarose gel electrophoresis.

Construction of the Genetic Linkage Map

The genetic map was constructed using the genotyping results for F2 (PI 189225 × TS) mapping population with SSR and HRM markers, which were genotyped to the 252 F2 and 90 F2 progeny, respectively. Segregation distortion at each marker locus was tested against the expected ratio for F2 (1:2:1) using a chi-square test, and significantly biased markers were excluded ($P \leq 0.05$) for the construction of the genetic map. The genetic linkage map was generated with JoinMap 4.1 (van Ooijen, 2006). Markers were grouped with LOD > 6, except for chromosomes 5 and 10. Distances in centimorgan (cM) were calculated from the recombination frequencies using the Kosambi mapping function (Kosambi, 1944).

Results and Discussion

The primary frame markers developed in this study have direct relevance to the modern breeding programs, including linked marker development for marker-assisted selection (MAS) (Kim et al., 2013) and marker-assisted backcross breeding (MABC) (Hasan et al., 2015). PI 189225, known as a valuable wild germplasm with resistance to anthracnose (Boyhan et al., 1994), powdery mildew (Tetteh et al., 2013), and gummy stem blight (Gusmini, 2005), has shown high polymorphism for EST-SSR and SNP marker development. Once the frame markers are established, foreground selection for MAS and background selection for MABC would be helpful for establishing efficient breeding programs.

Genotyping with EST-SSRs and Linkage Map Construction

In this study, 420 primer pairs of EST-SSRs were tested to apply codominant markers for discernable genotypes of PI 189225 (P1), 'TS' (P2), and F1 plants. Primer sequences and detailed information on the EST-SSRs were obtained from the "International Cucurbit Genomics Initiative" (ICuGI; http://www.icugi.org). A total of 212 (50.5%) primer pairs showed reproducible polymorphic genotypes in the F1 progeny. When screening polymorphic EST-SSRs in the 252 F2 progeny, 121(28.8%) primer pairs produced clear and scorable bands and determined putative codominant loci (Fig. 1). The number of markers for codominant alleles is similar to that reported for *Cucumis* EST-SSRs (Fernandez-Silva et al., 2008); however, it is probably low, considering that the tested F1 progeny is expected to have high polymorphism by a wide distant cross by PI 189225 (*C. amarus*) in this study. Subsequently, the selected primer pairs were used for linkage analysis, but only 33 (7.9%) EST-SSR markers were mapped to 12 linkage groups, with 17 remaining ungrouped loci after excluding markers that showed segregation distortion against the Mendelian segregation law (1:2:1) in the F2 progeny (Fig. 2). This quite low mapping result due to severe segregation distortion may also be caused by the distant cross to obtain high genetic polymorphisms (Ren et al., 2015). The total

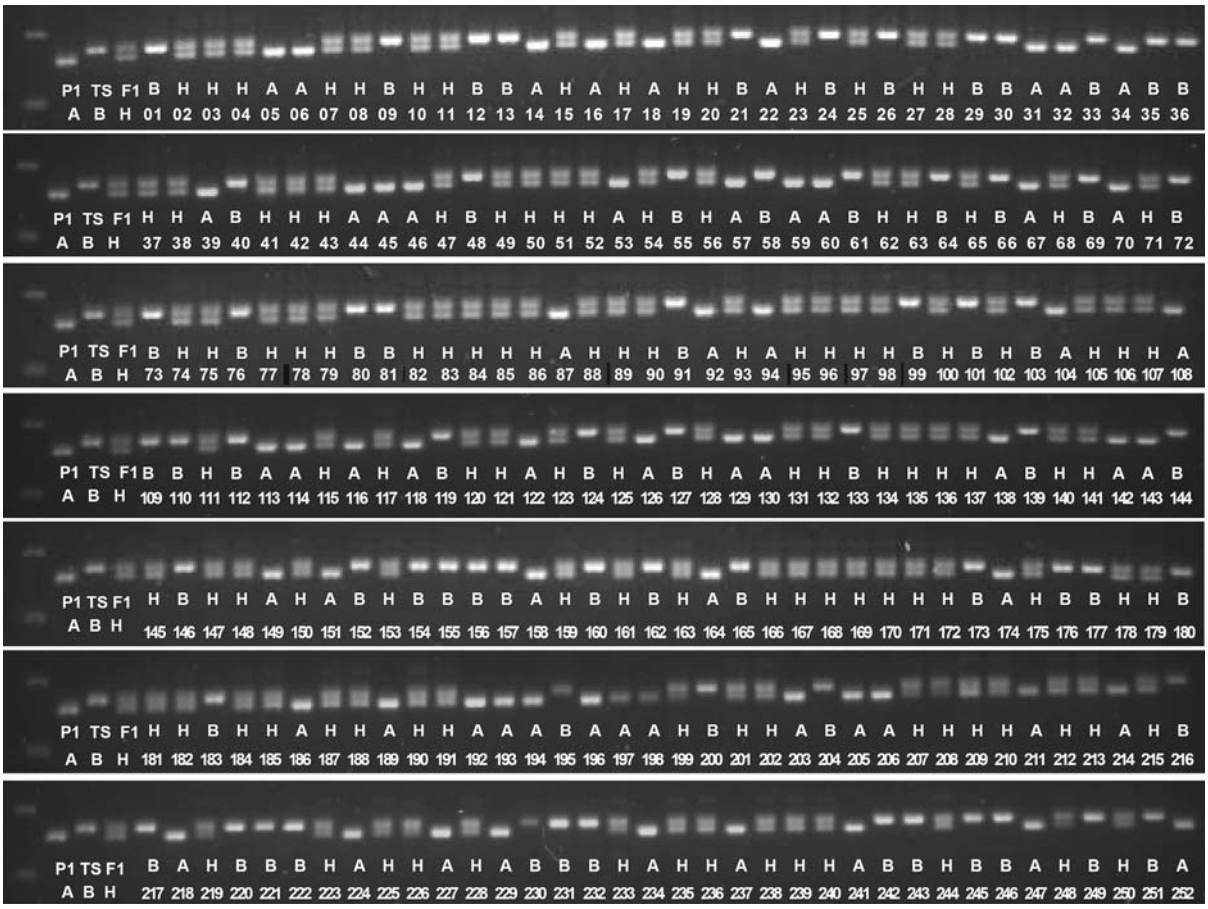

Fig. 1. Agarose gel electrophoresis results for EST-SSRs. S_14204 primer pairs (5′-TTTTCCCCAAAATTTTCAACC-3′ and 5′-TTGGAAGAAA TCGGCAAAAC-3′) were used for genotyping 252 F2 progeny. Genotypes A, B, and H represent PI 189225, 'TS', and F1 progeny, respectively.

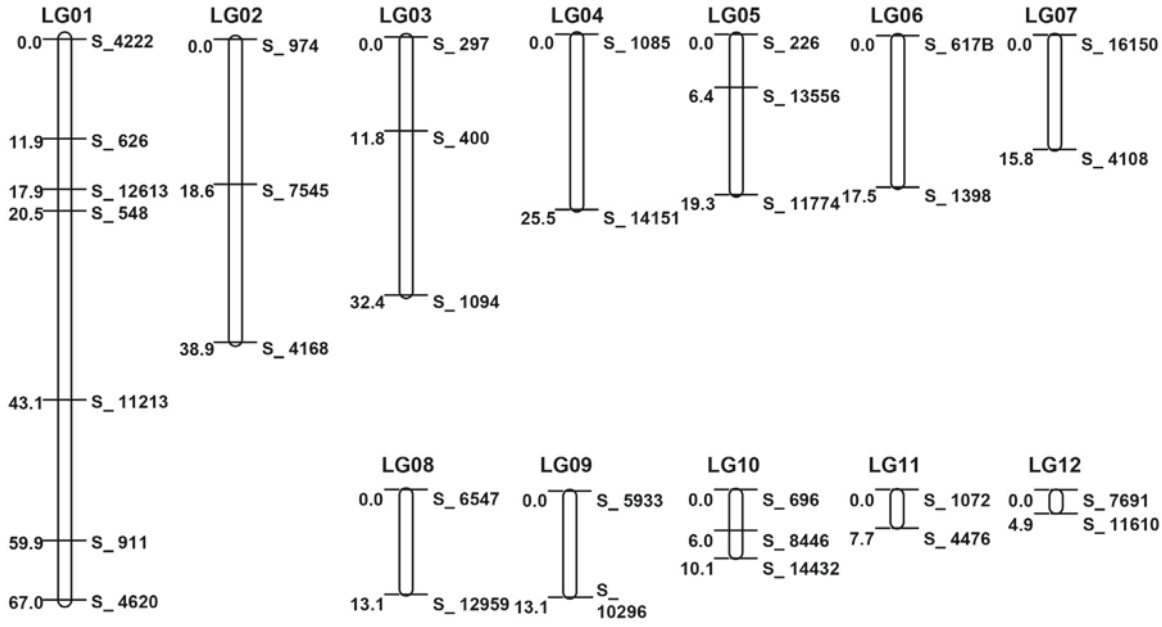

Fig. 2. Construction of a genetic linkage map using EST-SSR markers. Information on each marker is provided in the ICuGI website(http://www.icugi.org).

map length was 265.2 cM (Fig. 2), and the average length of a linkage group was 40.8 cM. This map length and marker coverage would be insufficient to use a downstream work including background selection for MABC. We need more representative reliable markers with regular marker intervals in each chromosome. Thus, we resequenced the parental lines using an NGS platform, identified many SNPs, and validated representative HRM markers for each chromosome.

SNP Identification and HRM Marker Development

The whole genome of each parental line was resequenced, and the filtered reads were aligned and sequence variation was compared to the international reference genome of the 97103 line (Ren et al., 2012). The resultant mapping data from more than 30× of genome coverage showed 66.35% of PI 189225 and 75.15% of 'TS' at a properly paired rate (Table 1). Distribution of SNVs, excluding InDels, was analyzed in coding, splicing, promoter, and intergenic regions (Table 2). Wild-type germplasm of PI 189225 accumulates more than 10-fold higher SNPs in all the genomic regions than the cultigen of 'TS', and, naturally, more SNPs were found in the intergenic region than in the coding region. The number of SNPs was sufficient for developing markers for constructing the genetic map.

On the basis of the indexed SNPs of VCF files, candidate regions for HRM analysis were selected by approximately 10 points, with possibly regular intervals along each chromosome. For developing a genotyping platform, the degree of throughput for large numbers of samples and data points should be studied. However, while the higher throughput could be better for genotyping, the initial development cost for most of the popular HT genotyping platforms is higher than that of conventional genotyping platforms such as gel-based markers, including InDels, sequence characterized amplified region, and cleaved amplified polymorphic sequences markers. Although gel-based genotyping could be a lower throughput platform, it offers easy access for researchers who perform small-scale research (Verma and Arya, 2008). The HRM marker system is considered to be positioned between HT digital PCR and gel-based analyzing systems,

Table 2. Distribution of single-nucleotide variant numbers in whole-genome resequencing of the parental lines

Chr.	Coding region		Splicing region		Promoter region		Intergenic region		Total	
	PI189225	TS	PI189225	TS	PI189225	TS	PI189225	TS	PI189225	TS
1	7378	371	16	1	21687	888	298659	29683	327740	30943
2	5830	527	21	2	17562	1360	258752	28194	282165	30083
3	4124	363	13	1	11563	638	217933	20477	233633	21479
4	2610	179	7	1	7913	480	197053	25000	207583	25660
5	7497	438	13	2	22201	1211	303248	29137	332959	30788
6	4146	360	12	3	12224	817	203227	27859	219609	29039
7	3998	339	12	1	11828	729	194002	33285	209840	34354
8	3943	312	18	4	11643	875	135086	21463	150690	22654
9	5255	454	14	3	15801	1221	298033	39535	319103	41213
10	4942	352	20	3	15204	794	129771	21556	149937	22705
11	4442	291	9	2	13344	776	120721	21211	138516	22280

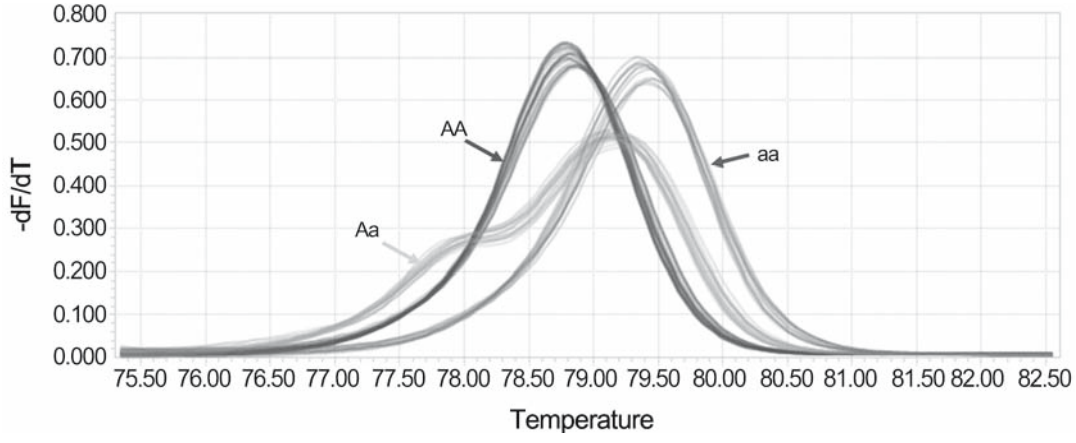

Fig. 3. Patterns of the HRM marker H1_31983755 developed in this study. AA, PI 189225; aa, 'TS'; Aa, heterozygous

and it is relatively cheaper and easier than other HT genotyping platforms. In addition, information on HRM markers is easily transferable to the SNP-based microfluidic PCR system of Fluidigm's BioMark, a recently highlighted genotyping platform. Thus, the developed HRM markers in this study would be valuable for small-scale research and breeding programs that screen SNP positions for discriminating germplasm, commercial lines, and F1 hybrids before upgrading to the HT genotyping system.

Linkage Map Construction using EST-SSR and HRM Markers

Two hundred candidate HRM markers were screened with P1, P2, and F1 plants for clear genotyping, and 172 (86%) markers were successfully selected for genetic linkage mapping. Like for EST-SSR markers, segregation distortion was found in the F2 progeny, and the number of markers was reduced to 103 (51.5%), with 25 remaining ungrouped loci. Determined loci of HRM markers were consolidated with previously developed EST-SSR markers, which were mapped to more loci on the basis of the increased number of HRM markers in the mapping procedure. The total length of the newly constructed map was 1178.3 cM, the average length of the linkage groups was 107.1 cM, and the average inter-marker distance was 8.24 cM (Fig. 4). The total map length is somewhat longer than that reported in a previous study (798 cM) (Ren et al., 2012), but this may be attributable to different populations derived from a wide distant cross (Ren et al., 2015). Bioinformatic locations from original data for EST-SSRs were well fitted to the physical chromosomal position as well as HRM markers from VCF files. In case of chromosomes 5 and 10, separated linkage groups were difficult to unite

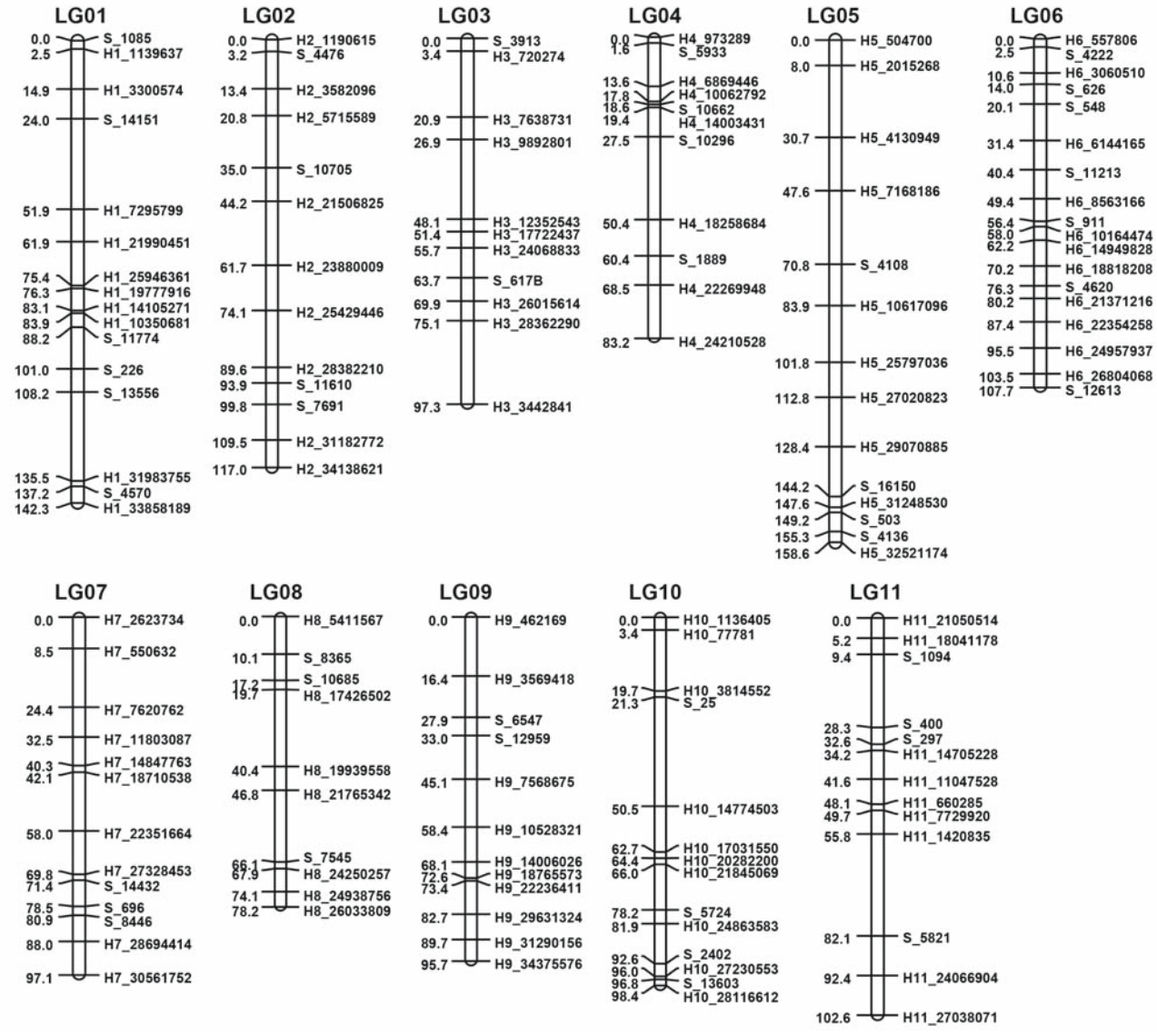

Fig. 4. Construction of a genetic linkage map using HRM markers, which are consolidated by EST-SSR markers.

into one group for each chromosome with a low LOD score under 4, since the recombination suppression region around the centromere would hinder in determining the marker location. Information on the primer sets for HRM is summarized in the supplementary data (Suppl. Table1). The EST-SSR and HRM markers of this map are considered to be solid due to escaping segregation distortion at least in this F2 population.

In this study, we successfully constructed a genetic map for frame loci of EST-SSR and HRM markers. Since new varieties that possess disease resistance are required for seed markets of watermelon, the developed markers would be very useful for finding novel disease loci by MAS and introducing elite lines by MABC.

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