Integration of Cytogenetic and Genetic Linkage Maps Unveils the Physical Architecture of Tomato Chromosome 2

Dal-Hoe Koo,^{*,†,1} Sung-Hwan Jo,^{*,‡,1} Jae-Wook Bang,[§] Hye-Mi Park,^{**}
Sanghyeob Lee^{*,††} and Doil Choi^{*,**,*2}

^{*}Plant Genome Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, 305-600, Korea, [§]School of Bioscience and Biotechnology, Chungnam National University, Daejeon 305-764, Korea, ^{**}Department of Plant Sciences, College of Agricultural and Life Sciences, Seoul National University, Seoul, 151-742, Korea, [†]Department of Functional Genomics, University of Science and Technology, Daejeon, 305-333, Korea, ^{††}Department of Horticulture, University of Wisconsin, Madison, Wisconsin 53706 and ^{††}Dongbu Advanced Research Institute, Dongbu Hitek, Daejeon, 305-708, Korea

Manuscript received March 25, 2008
Accepted for publication May 10, 2008

ABSTRACT

We report the integration of the linkage map of tomato chromosome 2 with a high-density bacterial artificial chromosome fluorescence *in situ* hybridization (BAC-FISH)-based cytogenetic map. The euchromatic block of chromosome 2 resides between 13 and 142 cM and has a physical length of 48.12 μ m, with 1 μ m equivalent to 540 kb. BAC-FISH resolved a pair of loci that were 3.7–3.9 Mb apart and were not resolved on the linkage map. Most of the regions had crossover densities close to the mean of $\sim$ 200 kb/cM. Relatively hot and cold spots of recombination were unevenly distributed along the chromosome. The distribution of centimorgan/micrometer values was similar to the previously reported recombination nodule distribution along the pachytene chromosome. FISH-based physical maps will play an important role in advanced genomics research for tomato, including map-based cloning of agronomically important traits and whole-genome sequencing.

ECONOMICALLY, the Solanaceae compose the third most important plant taxon, and consist of >3000 species. Distinctive aspects of development and the variety of phenotypes and habitats make the Solanaceae good models for investigation of the genetic bases of diversification and adaptation. To this end, the “International Solanaceae Genome Project (SOL)” was launched (MUELLER *et al.* 2005). Tomato is well suited to represent the Solanaceae because it has a relatively small genome and a strong genetics, genomics, and cytogenetics foundation.

PETERSON *et al.* (1999) provided an overview of the DNA content and physical length of all 24 chromosome arms. Tomato has pericentromeric heterochromatin, as do other Solanaceae. The synaptonemal complex karyotype data indicate that 77% of the tomato genome is located in heterochromatin and 23% in euchromatin (PETERSON *et al.* 1996). The genome size (1C) is $\sim$ 95 pg of DNA (MICHAELSON *et al.* 1991), implying 212 Mb of euchromatin (BENNETT and SMITH 1976; <http://www.sgn.cornell.edu>; tomato sequencing scope and completion criteria).

Excellent morphological and molecular genetic maps of the tomato genome are available (RICK and YODER 1988; TANKSLEY *et al.* 1992). For example, >1000 restriction fragment length polymorphisms (RFLPs), mutants, and isozymes have been located on a map that totals >1276 cM (TANKSLEY *et al.* 1992). In addition, 67 RFLP and 1175 amplified fragment length polymorphism (AFLP) markers were used to construct a RFLP-AFLP map that totals 1482 cM (HAANSTRA *et al.* 1999). To date, 2037 markers have been used to create a map that totals 1460 cM; this map is available from the Solanaceae Genome Network (SGN) database (<http://www.sgn.cornell.edu>; EXPEN 2000 map) and is used for the “SOL” project. This linkage map, which represents all the chromosomes, does not provide sufficient detail to support genome sequencing. Because linkage map distances are not simply related to physical distances, physical mapping is needed to determine the locations of markers on chromosomes. For this purpose, bacterial artificial chromosome (BAC) fingerprinting and overgo hybridization have been applied. Currently, 3439 contigs have been anchored on the EXPEN 2000 map.

As participants in the international SOL consortium, we are responsible for sequencing the euchromatic region of chromosome 2, the third largest chromosome of tomato (SHERMAN and STACK 1992). Critical steps in this process are identification of the boundaries of the euchromatin and determination of the physical loca-

¹These authors contributed equally to this study.

²Corresponding author: Department of Plant Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul, 151-742, Korea. E-mail: doil@snu.ac.kr

tions of markers. Pachytene chromosome analysis indicates that the physical size of the euchromatin is 22–26 Mb (PETERSON *et al.* 1996; CHANG 2004). The characteristic morphology of chromosome 2, with its nucleolar organizing region (NOR) and acrocentric structure, makes it easily distinguishable from the other chromosomes. Furthermore, the entire euchromatic block is located on the distal region of the long arm of the chromosome and is clearly separated from the pericentromeric heterochromatin. The linkage map of chromosome 2 has been well defined using 308 molecular markers, and its size is estimated as 143 cM (EXPEN 2000 map). A physical map has also been constructed for chromosome 2 using 75 marker-anchored BAC clones (EXPEN 2000 map). However, neither map provides sufficient detail of the physical locations of markers to initiate genome sequencing. “Molecular cytogenetics” can contribute significantly to the genome map by resolving the order of closely linked markers and confirming the physical positions of markers on the linkage groups (ANDERSON *et al.* 2004; VAN DER KNAAP *et al.* 2004; CHANG *et al.* 2007).

Fluorescence *in situ* hybridization (FISH) is the most versatile and accurate method for determining the euchromatic–heterochromatic boundaries, the locations of chromosome-specific BAC clones, and the locations of repetitive and single-copy DNA sequences (FRANSZ *et al.* 2000; CHENG *et al.* 2001a,b; WANG *et al.* 2006). Here, we report the cytological and physical structure of tomato chromosome 2 in relation to the linkage map, using BAC–FISH mapping.

MATERIALS AND METHODS

Plant material: Tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants were grown in a controlled-environment room at $26^{\circ} \pm 1^{\circ}$ under 16 hr light/8 hr dark.

BAC probe preparations: All BAC clones used for BAC–FISH were kindly provided by S. Tanksley and J. Giovannoni at Cornell University, Ithaca, New York. Tomato BAC probes were labeled with digoxigenin-11-dUTP or biotin-16-dUTP by nick translation according to the protocols provided by the manufacturer of the labeling kits (Roche, Basel, Switzerland). The Arabidopsis pAtT4 clone (RICHARDS and AUSUBEL 1988) and the wheat pTa71 clone containing a 9.1-kb fragment of 45S rDNA (GERLACH and BEDROCK 1979) were used to detect telomeric and rDNA regions, respectively.

Chromosome preparation: Pollen mother cells (PMCs) were separated using the method of FRANSZ *et al.* (2000) with some modification. Immature flower buds were fixed in ethanol:acetic acid (3:1) for 2 hr and stored at 4° . These were rinsed in distilled water and incubated in an enzyme mix (0.3% pectolyase, 0.3% cytohelicase, and 0.3% cellulase) in citrate buffer (10 mM sodium citrate, pH 4.5) for 2 hr. Each bud was softened in 60% acetic acid on an uncoated, ethanol-cleaned microscopic slide kept at 45° on a hot plate. The contents were smeared on the slide, fixed with ice-cold ethanol:acetic acid (3:1), and dried.

FISH: The FISH procedure was previously reported by Koo *et al.* (2004). In brief, chromosomal DNA on the slides was

denatured with 70% formamide at 70° for 2.5 min, followed by dehydration in a 70, 85, 95, and 100% ethanol series at -20° for 3 min each. The probe mixture containing 50% formamide (v/v), 10% dextran sulfate (w/v), 5 ng/ μ l salmon sperm DNA, and 50 ng/ μ l labeled probe DNA was heated at 90° for 10 min and then kept on ice for 5 min. A 20- μ l aliquot of this mixture was applied to the denatured chromosomal DNA and covered with a glass coverslip. The slides were then placed in a humid chamber at 37° for 18 hr. Probes were detected with avidin–FITC and anti-digoxigenin Cy3 (Roche). Chromosomes were counterstained with 1 μ g/ μ l 4',6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis). The signals were detected using a cooled CCD camera (CoolSNAP; Photometrics, Tokyo). The images were obtained with a Leica epifluorescence microscope equipped with FITC–DAPI two-way or FITC–rhodamine–DAPI three-way filter sets (Leica, Tokyo) and were processed with Meta Imaging Series TM 4.6 software. The final printed images were prepared using Photoshop 7.0 (Adobe, San Jose, CA).

Fiber-FISH: Leaf nuclei were prepared as described by JACKSON *et al.* (1998). A suspension of nuclei was deposited at one end of a poly-L-lysine-coated slide (Sigma) and air dried for 10 min. STE lysis buffer (8 μ l) was added, and the slide was incubated at room temperature for 4 min. A clean coverslip was used to slowly drag the contents along the slide. The preparation was air dried, fixed in ethanol:glacial acetic acid (3:1) for 2 min, and baked at 60° for 30 min. The DNA fiber preparation was incubated with a probe mixture, covered with a 22×40 -mm coverslip, and sealed with rubber cement. The slide was placed in direct contact with a heated surface in an oven at 80° for 3 min, transferred to a wet chamber that had been prewarmed at 80° for 2 min, and then transferred to 37° for overnight incubation. The posthybridization washing stringency was the same as in FISH of chromosome spreads. Signal detection was performed according to Koo *et al.* (2004).

Chromosome identification and measurement: The images of 20 DAPI-stained pachytene bivalents at approximately the same stage were captured from different PMCs to study the distributions of heterochromatin, positions of FISH signals, and lengths of pachytene chromosomes. The images were measured directly on the screen using the FISH Image System (Meta Imaging Series TM 4.6).

RESULTS

Cytological architecture of chromosome 2: The pachytene chromosome 2 of tomato is easily distinguished from the other chromosomes because it is acrocentric and bears a large secondary structure, the NOR, on the short arm (PETERSON *et al.* 1996). The DAPI staining of the pachytene chromosome demonstrated striking differences between the euchromatin and the heterochromatin. Brightly fluorescing heterochromatic regions were detected next to the centromere of the long arm and over the entire short arm (Figure 1A). Weakly fluorescing euchromatin was observed on the long arm (Figure 1A). Chromosome 2 at meiotic prophase I was a fully paired bivalent with a mean length of 70.22 μ m, based on 20 independent measurements. The lengths of the euchromatic and heterochromatic regions (including the NOR) were 48.12 ± 3.17 and 22.1 ± 1.23 μ m, respectively. Previous studies estimated the size of the euchromatic region of

FIGURE 1.—Cytological architecture of tomato chromosome 2. (A) DAPI-stained pachytene chromosome 2. (B) FISH pattern on pachytene chromosome 2 using probes for both digoxigenin-labeled BAC clone LE_HBa0303I24 and biotin-labeled BAC clone LE_HBa0177F12. DAPI-stained chromosomes and FISH signals were converted to a black-and-white image to enhance the visualization of distribution of euchromatin and heterochromatin on the pachytene chromosome. NOR, nucleolar organizing region; CEN, centromere; PH, pericentromeric heterochromatin; TEL, telomere; EU, euchromatin; HETERO, heterochromatin. Bar, 10 μ m.

chromosome 2 as 22–26 Mb (SHERMAN and STACK 1992; PETERSON *et al.* 1996; CHANG 2004). We used the largest size estimation (26 Mb), following guidelines of the International Tomato Genome Sequencing Project. Thus, we considered the euchromatin of the pachytene chromosome to have an average of 540 kb/ μ m.

Determination of the euchromatin borders: Several BAC probes anchored at each end of linkage group 2 were hybridized to pachytene bivalents, and the physical locations of the BAC–FISH signals were examined. The marker cLER-1-H17 was mapped onto 0.0 cM, which is the north end of linkage group 2. The FISH signal for the BAC clone LE_HBa0007F24 anchored by cLER-1-H17 was detected on the distal end of the short arm of chromosome 2 where it covered the NOR (Figure 2A), and minor signals were also detected on the pericentromeric regions of some pachytene chromosomes (data not shown). The other BAC clones anchored to molecular markers located between 0 and 12 cM gave multiple FISH signals in the pericentromeric heterochromatic regions of all chromosomes (data not

shown). The BAC–FISH signal of the T1238 (13 cM)-anchored LE_HBa0303I24 BAC was seen only near the boundary of the euchromatin and pericentromeric heterochromatin. This was located at $3.5 \pm 1.3 \mu$ m from the pericentromeric heterochromatic region of the long arm (Figure 1B). The south end of the euchromatin was verified by the T1554 (142 cM)-anchored BAC clone LE_HBa0177F12. The FISH signal for LE_HBa0177F12 was detected at the distal end of the long arm of the pachytene chromosome 2 (Figure 1B). Sequencing revealed that SL_MboI0006E22, containing telomere-specific repeated sequences, is located 100 kb from LE_HBa0177F12 (Figure 2, B–D). The biotin-labeled SL_MboI0006E02 (green) was detected at the distal ends of several pachytene chromosomes, including chromosome 2 (Figure 2B). The digoxigenin-labeled Arabidopsis telomere-specific probe (pAtT4, red) was colocalized with green signals generated from SL_MboI0006E02 (Figure 2C). These data taken together identified the euchromatin between 13 and 142 cM as suitable for our study.

FIGURE 2.—Physical coverage of the genetic linkage map of tomato chromosome 2. (A) The FISH signal from the BAC clone located at the 0-cM position was detected on the nucleolar organizing region (NOR). Digoxigenin-labeled LE_HBa0007F24 (red) anchored by cLEC-7-P21 (0 cM) was observed at the distal ends of the short arm, which is marked by a 45S rDNA locus. (B) Pachytene chromosomes of tomato were hybridized with biotin-labeled SL_MboI0006E22 (green). Arrowheads indicate pachytene chromosome 2. (C) The pachytene chromosomes in B were hybridized with digoxigenin-labeled Arabidopsis pAtT4 containing the telomere-specific sequence. (D) Sequencing results indicate that the telomere-specific repeat sequence containing SL_MboI0006E22 is 100 kb away from LE_HBa0032J10. Bar, 10 μ m.

TABLE 1
List of BAC clones used to integrate the genetic and cytogenetic maps

Position in linkage group 2	BAC
13	LE_HBa0303I24
18	LE_HBa0025N15
28	LE_HBa0025A22
37	LE_HBa0155C04
38	LE_HBa0160F05
46	LE_HBa0072A04
66	LE_HBa0059M17
70	SL_MboI0019I01
72	LE_HBa0167J21
74.5	LE_HBa0320D04
77.5	LE_HBa0291P19
82	LE_HBa0013N18
83.5	LE_HBa0009K06
88	LE_HBa0164H08
88	LE_HBa0213A01
89.3	LE_HBa0134G09
92	LE_HBa0016A12
94	LE_HBa0011A02
96	SL_MboI0014P22
106	LE_HBa0172G12
108	LE_HBa0046M08
110.9	LE_HBa0150M11
112	LE_HBa0111M10
120	LE_HBa0073P13
130	LE_HBa0155D20
140	LE_HBa0064B17
141	LE_HBa0194L19
142	LE_HBa0177F12

Integration of the cytogenetic and linkage maps: To construct an integrated high-density cytogenetic map of chromosome 2, we selected 28 BAC clones anchored to molecular markers that were dispersed along the entire linkage group 2 (Table 1). These BAC-derived probes yielded strong FISH signals in pachytene chromosome 2 and clearly demonstrated the corresponding position and order of the selected BAC clones (Figure 3, A1–A4). The order of the selected marker-anchored BAC clones was the same as in the linkage map, except for an inversion of the markers located at 66 and 70 cM (Red dotted circles in Figure 3, A2). In addition to the identification of this inversion, the BAC–FISH map sometimes resolved loci that were not resolved on the linkage map. For example, T1555 and T1535 resided at the same position in the linkage map, but, in the BAC–FISH map, the signals anchored to the two markers were visibly separated (pink dotted circles in Figure 3, A3).

Physical gaps in linkage map 2: The initial selection of marker-anchored BAC clones suggested four gaps (*i.e.*, the absence of molecular marker-anchored BAC regions) that were ≥ 10 cM long: 18–28 cM, 46–66 cM, 96–106 cM, and 112–130 cM. However, the BAC–FISH analysis demonstrated that the gap that occurred

between 46 and 70 cM (note the inversion of loci at 66 and 70 cM) was the only real physical gap (blue dotted circle in Figure 3, A2). The distance between the two FISH signals observed from LE_HBa0072A04 (46 cM) and LE_HBa0329G05 (70 cM) was 7.28 μm (data not shown), implying a physical distance of ~ 3.93 Mb (*i.e.*, $7.28 \mu\text{m} \times 540 \text{ kb}/\mu\text{m}$). On the basis that 1 cM = 185 kb, the 20-cM interval is estimated to be 3.70 Mb. Thus, the two calculations gave similar physical distances for the large gap on chromosome 2.

Estimation of the base pair/centimorgan relationship: Molecular marker-anchored BAC–FISH mapping was used to determine the relationship between base pairs and centimorgans (CHENG *et al.* 2002; CHANG *et al.* 2007). We used five BAC-derived probes for the global determination of the base pair/centimorgan relationship. The five BAC–FISH signals were detected and easily separated into four physical blocks, G1, G2, G3, and G4 (Figure 3, A), according to genetic distances of 33, 27, 33, and 36 cM, respectively. The physical portions of these four blocks composed 24% (G1, 5.8 Mb), 20.9% (G2, 5.1 Mb), 35.3% (G3, 8.5 Mb), and 19.6% (G4, 4.7 Mb) of the entire euchromatic portion of chromosome 2. The base pair/centimorgan relationships calculated from these observations were 176 kb/cM (G1), 189 kb/cM (G2), 258 kb/cM (G3), and 131 kb/cM (G4).

In addition to these rather global measurements of the base pair/centimorgan relationship, we measured more local base pair/centimorgan relationships using 28 BAC clones covering the entire chromosome 2 (Table 2). For most of chromosome 2, the base pair/centimorgan relationship was < 200 kb/cM (Figure 4). Some hot spots were detected between 120 and 140 cM, and some cold spots between 72 and 73 cM (Figure 4). Both ends of the euchromatin block have less recombination than the rest of chromosome 2 (Figure 4).

Recombination nodules (RNs) represent real cross-overs in the genome and are available for tomato (SHERMAN and STACK 1995). Therefore, we compared the centimorgan/micrometer relationship with the average number of RNs along chromosome 2. The calculated centimorgan:micrometer ratio trends are similar to the RN distribution redrawn from SHERMAN and STACK (1995) (Figure 5B).

BAC–FISH-identified chromosome 2-specific BAC clones: Because plant genomes contain many repetitive and redundant sequences, the first task in sequencing the entire euchromatic region of chromosome 2 is the selection of chromosome 2-specific anchored BAC clones. Thus, we first selected all BAC clones anchored to molecular markers located between 13 and 142 cM in linkage group 2. In total, 69 BAC clones were selected from the SGN database (<http://www.sgn.cornell.edu>). BAC–FISH analyses of these BAC clones demonstrated several different types of hybridization patterns (Table 3): a single FISH signal was located on pachytene chromosome 2 for 37 BACs; multiple FISH signals were

FIGURE 3.—Integration of cytogenetic and genetic maps. (A) Pachytene chromosome hybridized using five biotin- or digoxigenin-labeled BAC clones (LE_HBa0303I24, 13 cM; LE_HBa0072A04, 46 cM; LE_HBa0204D01, 74.5 cM; LE_HBa0172G12, 106 cM; LE_HBa0177F12, 142 cM). G1–G4 represent designated genetic distance blocks of 33, 27, 33, and 36 cM, respectively. (A1–A4) Twenty-eight BAC clones hybridized to pachytene chromosome 2. The green and red dots on each line indicate BAC–FISH results from the left and the right, respectively; the distance is not proportional to the actual distance. The blue dotted circle indicates the physical gap. The red dotted circle indicates the reversed order of loci between the genetic and the cytogenetic maps. The pink dotted circle indicates two separate loci that were colocalized in the genetic map.

located on the pericentromeric heterochromatin regions of the pachytene chromosomes, including chromosome 2, for three BACs; no FISH signal was located on pachytene chromosome 2, but was located on other chromosomes for one BAC; and no FISH signal was observed on any chromosome for 28 BACs. BAC–FISH

identified 37 BAC clones that could be used as “seed” BACs for sequencing. This analysis also indicated that one BAC clone (LE_HBa0258N07) that was previously assigned to chromosome 2 by overgo hybridization actually occurs on other chromosomes, but not on chromosome 2 (data not shown).

TABLE 2

Comparison of cytogenetic and genetic distances between loci

BAC clones (position in genetic map)	Mean distance $\pm$ SD (μ m)	Linkage distance (cM)	kb/cM	cM/ μ m	No. of measurements
LE_HBa0303I24(13)–LE_HBa025N15 (18)	3.39 $\pm$ 2.06	5	366	1.5	3
LE_HBa0303I24(13)–LE_HBa0209K17 (25)	4.46 $\pm$ 0.48	12	200	2.7	3
LE_HBa025N15(18)–LE_HBa0209K17(25)	2.38 $\pm$ 0.49	7	184	2.9	6
LE_HBa025N15(18)–LE_HBa0025A22 (28)	3.8	10	205	2.6	1
LE_HBa0025A22(28)–LE_HBa0155C04 (36.5)	2.31	8.5	146	3.7	1
LE_HBa0025A22(28)–LE_HBa0160F05 (38)	2.91	10	157	3.4	1
LE_HBa0025A22(28)–LE_HBa0072A04 (46)	4.71	18	141	3.8	1
LE_HBa0066C13(46)–LE_HBa0167J21 (72)	8.51 $\pm$ 1.16	26	177	3.1	6
LE_HBa0167J21(72)–LE_HBa0204D01 (73)	6.53 $\pm$ 5.5	1	3,526	0.1	3
SL_MboI0108P14(72)–LE_HBa0204D01 (73)	21.86 $\pm$ 0.44	1	11,806	0.0	5
LE_HBa0204D01(73)–LE_HBa0198A03 (76)	2.11 $\pm$ 0.2	3	381	1.4	3
LE_HBa0291P19(77.5)–LE_HBa0060J03 (83)	1.6 $\pm$ 0.35	5.5	157	3.4	14
LE_HBa0060J03(83)–LE_HBa0164H08 (89)	1.99 $\pm$ 0.27	6	179	3.0	6
LE_HBa0164H08(89)–LE_HBa0016A12 (92)	1.09 $\pm$ 0.31	3	196	2.8	3
LE_HBa0011A02(94)–SL_MboII014P22 (96)	0.86 $\pm$ 0.16	2	232	2.3	3
SL_MboII014P22(96)–LE_HBa0172G12 (106)	2.25 $\pm$ 0.52	10	121	4.4	15

FIGURE 4.—Comparison of linkage and physical distances of loci. Left: the tomato genetic linkage map 2; map positions are given in centimorgans (yellow). Right: the physical distance in base pairs (red) corresponding to 1 cM. Horizontal bars represent the analyzed loci (yellow, corresponding to the genetic linkage map 2); vertical bars indicate the regions analyzed using BAC-FISH.

BAC- and fiber-FISH used for the confirmation of “next” BAC candidates: After identifying the sequences of seed BAC clones, we selected “next” BAC clones using a BLASTN search of the BAC end-sequence (BES) database of the SGN (<http://www.sgn.cornell.edu/tools/blast/>). Of the next BAC candidate clones that matched seed BAC sequences, those having minimal overlap with seed BAC sequences were selected as next BAC clones. We also filtered repetitive BES to reduce the occurrence of false clone picks.

However, it is possible that seed BAC sequences and BES could contain additional repetitive sequences that we had not previously curated. Therefore, Dual-color BAC-FISH analyses using seed BAC clones and next BAC clones were used to confirm the selection of next BAC clones (Figure 6). For example, the digoxigenin-

FIGURE 5.—Determination of the centimorgan/micrometer relationships. (A) Chromosome diagram with genetic (left) and cytological (right) distances between analyzed loci. Red ellipses indicate the analyzed loci. (B) Comparison of the RN distribution and the centimorgan:micrometer ratio along the length of chromosome 2. The y-axis represents the long arm of chromosome 2. The top x-axis is the number of RNs in 0.1- μ m intervals along the chromosome. The bottom x-axis is the centimorgan:micrometer ratio along the chromosome. The red line indicates the general trend in the RN distribution, redrawn from SHERMAN and STACK (1995). The horizontal bars indicate the ratio of the genetic distance between analyzed loci (values to the left in B) to the cytological distance between the analyzed BACs (values to the right in B). The value of each bar is shown at the right of the bar.

labeled seed BAC clone LE_H168N10 overlapped with the biotin-labeled next BAC clone LE_M045L06 (Figure 6A). However, extended DNA fiber-FISH clearly resolved the two BAC clones (Figure 6B).

DISCUSSION

We defined the cytological architecture of tomato chromosome 2, including the location of the euchromatic block, and we integrated the recombination-based linkage map and the FISH-based physical map. This information is important in establishing a guide for genome sequencing and map-based gene cloning.

Microscopic observation of DAPI-stained pachytene chromosome 2 indicated a mean length of 70.22 μ m. Of this, 48.12 μ m was classified as a single euchromatic region. Taking 26 Mb as the total euchromatin of chromosome 2 gave a base pair/micrometer value for euchromatin of the pachytene chromosome of 540 kb/ μ m. Previous studies reported that the DNA compactness in euchromatin corresponds to 0.6 Mb/ μ m (ARUMUGANATHAN and EARLE 1991; PETERSON *et al.*

TABLE 3

BAC clones used for the selection of “seed” BAC clones

Marker	Position in linkage group 2	BAC	Hybridization pattern ^a
T1238	13	LE_HBa0303I24	1
T0888	13	LE_HBa0306D07	4
CT140	16	LE_HBa0163K16	1
T1706	18	LE_HBa0025N15	1
T0869	21	LE_HBa0280E02	1
SSR40	22	LE_HBa0107I05	4
SSR66	25	LE_HBa0209K17	1
cLED-19-B18	28	LE_HBa0025A22	1
T1768	31	LE_HBa0282E10	4
T1698	34	LE_HBa0320M09	1
T1361	34	LE_HBa0052L14	4
SSR96	36.5	LE_HBa0155H19	2
SSR104	37	LE_HBa0155C04	1
T1668	37	LE_HBa0168N10	1
T0683	38	LE_HBa0160F05	1
T1516	42	LE_HBa0060P24	2
T1516	42	LE_HBa0123G24	4
cLEC-27-M9	46	LE_HBa0066C13	1
T1654	46	LE_HBa0134A15	1
TG451	46	LE_HBa0168N18	4
cLPT1E8	47	LE_HBa0002C08	4
TG139	57	LE_HBa0139G01	4
T0266	66	LE_HBa0059M17	1
T1532	69	LE_HBa0273J19	1
T1438	70	LE_HBa0329G05	1
T1625	72.5	LE_HBa0323A14	1
TG154	72.5	LE_HBa0118P10	4
TM34	73	LE_HBa0204D01	1
TG131	73.4	LE_HBa0147E11	4
T1537	74.5	LE_HBa0320D04	1
T0702	76	LE_HBa0198A03	1
T0086	76	LE_HBa0105H04	4
SSR26	77.5	LE_HBa0291P19	1
TM20	78	LE_HBa0236H12	2
TM20	78	LE_HBa0221D04	4
cLET1A5	79	LE_HBa0238L13	4
TG373	79.5	LE_HBa0011A02	4
T0759	82	LE_HBa0060J03	1
T1671	82	LE_HBa0010B01	1
CT229	82.5	LE_HBa0121J22	4
TG583	83.2	LE_HBa0023K06	4
C2_At4g38630	83.5	LE_HBa0009K06	4
T1535	88	LE_HBa0164H08	1
T1555	88	LE_HBa0213A01	1
TG48	92	LE_HBa0016A12	1
TG373	94	LE_HBa0011A02	1
cLED-19B24	100	SL_EcoRI0010H16	4
T1480	106	LE_HBa0172G12	1
d	107	SL_EcoRI0092M23	1
P61	108	LE_HBa0046M08	1
cLEC-7-L24	108	LE_HBa0124N09	4
T1158	108	LE_HBa0167P17	4
T1158	109	LE_HBa0150M11	4
TG34	111	LE_HBa0046M08	4
cTOB-9-L18	112	LE_HBa0111M10	1
cLPT-1-A21	114	LE_HBa0210D10	1

(continued)

TABLE 3

(Continued)

Marker	Position in linkage group 2	BAC	Hybridization pattern ^a
T1744	117	LE_HBa0208N01	4
TG167	118	LE_HBa0266G08	4
T1400	119	LE_HBa0194N24	4
U153274	120	LE_HBa0073P13	1
T0706	128	LE_HBa0080H21	4
T0634	130	LE_HBa0155D20	1
CT24	140	LE_HBa0064B17	1
T1096	141	LE_HBa0194L19	1
T1202	142	LE_HBa0257H21	1
T1554	142	LE_HBa0177F12	1
T1566	142	LE_HBa0258N07	3
cLEF-2-A11	142	LE_HBa0219K15	4
SSR50	143	LE_HBa0256J01	4

^a 1, hybridized only on pachytene chromosome 2; 2, hybridized on multiple chromosomes; 3, hybridized on chromosomes other than chromosome 2; 4, no hybridization.

1996; BUDIMAN *et al.* 2004). Thus, although different chromosomes and independent chromosome-spreading techniques were used, similar DNA compactness was determined, indicating that the mean base pair/micrometer relationship of euchromatin of the tomato pachytene is 0.5–0.6 Mb/μm.

To assess whether molecular marker loci existed for the distal ends of the chromosome arms, BAC probes corresponding to the ends of linkage group arms were hybridized to pachytene bivalents, and the physical locations of the FISH signals were examined. Most of the BACs derived from markers located between 0.0 and 12 cM showed multiple FISH signals in the pericentromeric heterochromatic region of all chromosomes, including the NOR or the pericentromeric heterochromatin of chromosome 2, except for one BAC (LE_HBa0155E05, 2 cM), which showed a single FISH signal in the heterochromatic region of the short arm of chromosome 2 (data not shown). Both FISH and DAPI data indicate that this segment may be composed of clusters of repetitive sequences. This result forced us to ignore this segment of the euchromatic region when selecting BAC clones for further sequencing projects. Thus, we used the euchromatic block between 13 and 142 cM for this project.

Comparing the linkage map EXPEN 2000 with our BAC–FISH map revealed an inversion in chromosome 2 (Figure 3, A2). This inconsistency in the position of the loci may have been caused by variation among the strains examined. Such apparent inversions have been reported in maize and tomato (PETERSON *et al.* 1999). The order of the loci on the genetic and cytological maps was generally the same, as expected.

BAC–FISH maps sometimes allowed us to resolve the locations of markers that were not resolved on the

FIGURE 6.—Confirmation of “next” BAC clones. (A) After “seed” BAC clone sequencing, we performed a BLASTN search of BAC end sequences. The selected next BAC candidates were verified using BAC–FISH. Digoxigenin-labeled seed BAC clones LE_H168N10 (red) and biotin-labeled next BAC candidates LE_M045L06 (green) were hybridized with pachytene chromosome 2. Colocalized red and green signals indicate that the analyzed next BAC clones are located next to the seed BAC clones in the tomato genome. (B) Both clones were then hybridized with extended DNA fiber (DNA from interphase nuclei, which have been extended into individual fibers).

linkage map. For example, both LE-HBa0213A01 and LE-HBa0164H08 were located at 88 cM in the linkage map. However, the BAC–FISH signals were separable (Figure 3, A3). This inconsistency in the position of the loci implies a low rate of recombination in the interval between them. The higher resolution of FISH mapping revealed physical gaps that could be troublesome for sequencing projects. FISH has been used to estimate the positions and sizes of gaps in the physical maps of rice and Arabidopsis. The linkage map of tomato reported by TANKSLEY *et al.* (1992) contains more markers per centimorgan than any other plant linkage map. However, chromosome 2 still contains four gaps (*i.e.*, the absence of molecular marker-anchored BAC regions) that span >10 cM. BAC–FISH identified only one physical gap of 3.7–3.9 Mb, occurring between 46 and 66 cM, that is considered troublesome for further genome sequencing (Figure 3, A2).

Our data based on five BAC clones that spanned the entire euchromatin of chromosome 2 indicated that, on the average, 1 cM corresponds to 189 kb (range: 131–258 kb) to be compared with previous estimations of 330–1150 kb/cM (TANKSLEY *et al.* 1992; GIOVANNONI *et al.* 1995; ALPERT and TANKSLEY 1996; TOR *et al.* 2002). Thus, our mean value is 16–56% as large as previous estimates. However, previous studies estimated the base pair/centimorgan relationship of specific regions, rather than of the entire chromosome (TANKSLEY *et al.* 1992; GIOVANNONI *et al.* 1995; ALPERT and TANKSLEY 1996; TOR *et al.* 2002). Furthermore the genetic map they used was less saturated than the EXPEN 2000 map. The twofold differences in the base pair:centimorgan ratio between the G3 and G4 regions can be explained by higher recombination ratios in G4 than in G3. This is

also consistent with the RN map of tomato (RICHARDS and AUSUBEL 1988; SHERMAN and STACK 1995). To measure more localized base pair:centimorgan ratios of chromosome 2, we used 28 BAC clones that covered the entire chromosome 2 (Table 2 and Figure 3). Although most of the regions gave values close to the mean of 200 kb/cM, we observed a relatively hot spot (120–141 cM) and some cold spots (72–73, 106–108, and 141–142 cM) (Figure 4). Similar variation was observed in rice and Arabidopsis (UMEHARA *et al.* 1994; SCHMIDT *et al.* 1995).

Because RNs on synaptonemal complexes represent the site of crossovers (ANDERSON and STACK 2005) and are visible under transmission electron microscopy, we can estimate the accuracy of the integrated map of physical distance by comparing the centimorgan/micrometer relationship with the average number of RNs along the chromosome. SHERMAN and STACK (1995) measured the absolute positions of RNs on chromosome 2 and reported the number that occurred in each 0.1- μ m segment. In Figure 5B we compare the RN distribution with the local centimorgan:micrometer ratios and find the trends in the two data sets to be similar. The DNA at both ends of the euchromatin block showed less recombination frequency than in the central region.

This may have occurred because recombination is low near the large 45S rDNA loci or telomeric repeats. This integration map will be valuable for estimating the physical structure of chromosome 2 before the chromosome is sequenced completely.

In the initial stage of genome sequencing, the marker-anchored BAC clones should be selected and termed as seed BAC clones. Because plants generally have many repetitive sequences the selection of accurate seed BAC clones is important. We used BAC–FISH analyses to select chromosome-2-specific seed BACs. We screened 69 seed BAC candidates that were previously selected by the Giovannoni and Tanksley groups at Cornell University, using overgo hybridization. FISH analysis identified 37 BACs that exhibited single strong FISH signals on pachytene chromosome 2; these were selected as the seed BACs. However, 46% of BAC clones identified by overgo hybridization resided on chromosomes other than chromosome 2. Some BAC–FISH signals detected on multiple chromosomes may be explained by the presence of repetitive sequences, so we do not know which chromosome segment was represented by these BAC clones. These FISH analyses imply that identifying the physical location of chromosome-specific BAC clones is important for this type of sequencing project.

BAC-based fingerprint contig (FPC), iterative hybridization, and sequence tag contig have been used successfully in sequencing the human, rice, and Arabidopsis genomes (MARRA *et al.* 1997, 1998; MAYER *et al.* 1999; MOZO *et al.* 1999; MCPHERSON *et al.* 2001; CHEN *et al.* 2002; PAMPANWAR *et al.* 2005; SASAKI *et al.* 2005). A tomato FPC map was constructed using 88,640 BAC clones that covered the tomato genome 10 times. The

map comprises 4385 contigs and 22,945 singletons; 82% of contigs are composed of <25 contig members. The small number of contig members prohibits the application of the FPC map to the selection of next BAC clones. Alternatively, we used a BLASTN search of the SGN database for BES to select next BAC candidates that overlapped the sequences of seed BAC clones. Considering the complexity of plant genomes, a BLASTN search using <1 kb BESs has the potential to identify the wrong chromosomal segment. To overcome this problem, we used BAC- or fiber-FISH to confirm the accuracy of the BLASTN search results. Dual-color BAC-FISH using seed BAC clones and next BAC clones was successful (Figure 6A). Because fiber-FISH has higher resolution than BAC-FISH, it showed a clearer relationship between seed and next BAC clones (Figure 6B).

Compared to the three other techniques presently used for physical mapping, namely, DNA contigs (Mozo *et al.* 1999), cytogenetic stocks (KUNZEL *et al.* 2000), and *in situ* hybridization (CHENG *et al.* 2001a,b), the use of BAC-FISH for the integration of cytogenetic and genetic linkage maps, as in this study, is advantageous in terms of speed, accuracy, and applicability to a broad spectrum of organisms. Polyploidy and heterochromatin reduce the usefulness of contigs. For some species, the production of cytogenetic stocks is time consuming or impossible. Moreover, the resolution of the physical map obtained is comparatively low.

In situ hybridization techniques give varied resolutions depending upon such factors as genome size and ploidy. As shown in our study, a combination of cytogenetic and genetic methods can yield a high-resolution physical map.

We thank the Solanaceae Genome Network (Jim Giovannoni and Steve Tanksley) at Cornell University for providing all the tomato BAC resources. This work was supported by grants from the Crop Functional Genomics Center (CG1221) of the 21st Century Frontier Research Program funded by the Ministry of Education and Science of the Korean government.

LITERATURE CITED

- ALPERT, K. B., and S. D. TANKSLEY, 1996 High-resolution mapping and isolation of a yeast artificial chromosome contig containing fw2.2: a major fruit weight quantitative trait locus in tomato. *Proc. Natl. Acad. Sci. USA* **93**: 15503–15507.
- ANDERSON, L. K., and S. M. STACK, 2005 Recombination nodules in plants. *Cytogenet. Genome Res.* **109**: 198–204.
- ANDERSON, L. K., N. SALAMEH, H. W. BASS, L. C. HARPER, W. Z. CANDE *et al.*, 2004 Integrating genetic linkage maps with pachytene chromosome structure in maize. *Genetics* **166**: 1923–1933.
- ARUMUGANATHAN, K., and E. D. EARLE, 1991 Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep.* **9**: 208–218.
- BENNETT, M. D., and J. B. SMITH, 1976 Nuclear DNA amounts in angiosperms. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **274**: 227–274.
- BUDIMAN, M. A., S. B. CHANG, S. LEE, T. J. YANG, H. B. ZHANG *et al.*, 2004 Localization of jointless-2 gene in the centromeric region of tomato chromosome 12 based on high resolution genetic and physical mapping. *Theor. Appl. Genet.* **108**: 190–196.
- CHANG, S. B., 2004 Cytogenetic and molecular studies on tomato chromosomes using diploid tomato and tomato monosomic additions in tetraploid potato. Ph.D. Dissertation, Wageningen University, The Netherlands.
- CHANG, S. B., L. K. ANDERSON, J. D. SHERMAN, S. M. ROYER and S. M. STACK, 2007 Predicting and testing physical locations of genetically mapped loci on tomato pachytene chromosome 1. *Genetics* **176**: 2131–2138.
- CHEN, M., G. PRESTING, W. B. BARBAZUK, J. L. GOICOECHEA, B. BLACKMON *et al.*, 2002 An integrated physical and genetic map of the rice genome. *Plant Cell* **14**: 537–545.
- CHENG, Z., G. PRESTING, C. R. BUELL, R. A. WING and J. JIANG, 2001a High-resolution pachytene chromosome mapping of bacterial artificial chromosomes anchored by genetic markers reveals the centromere location and the distribution of genetic recombination along chromosome 10 of rice. *Genetics* **157**: 1749–1757.
- CHENG, Z., C. R. BUELL, R. A. WING, M. GU and J. JIANG, 2001b Toward a cytological characterization of the rice genome. *Genome Res.* **11**: 2133–2141.
- CHENG, Z., C. R. BUELL, R. A. WING and J. JIANG, 2002 Resolution of fluorescence *in-situ* hybridization mapping on rice mitotic prometaphase chromosomes, meiotic pachytene chromosomes and extended DNA fibers. *Chromosome Res.* **10**: 379–387.
- FRANSZ, P. F., S. ARMSTRONG, J. H. DE JONG, L. D. PARNELL, C. VAN DRUNEN *et al.*, 2000 Integrated cytogenetic map of chromosome arm 4S of *A. thaliana*: structural organization of heterochromatic knob and centromere region. *Cell* **100**: 367–376.
- GERLACH, O. L., and J. R. BEDROCK, 1979 Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucleic Acids Res.* **109**: 1346–1352.
- GIOVANNONI, J. J., E. N. NOENSIE, D. M. RUEZINSKY, X. LU, S. L. TRACY *et al.*, 1995 Molecular genetic analysis of the ripening-inhibitor and non-ripening loci of tomato: a first step in genetic map-based cloning of fruit ripening genes. *Mol. Gen. Genet.* **248**: 195–206.
- HAANSTRA, J. P. W., C. WYE, H. VERBAKEL, F. MEIJER-DEKENS, P. v. d. BERG *et al.*, 1999 An integrated high-density RFLP-AFLP map of tomato based on two *Lycopersicon esculentum* x *L. pennellii* F2 populations. *Theor. Appl. Genet.* **99**: 254–271.
- JACKSON, S. A., M. L. WANG, H. M. GOODMAN and J. JIANG, 1998 Application of fiber-FISH in physical mapping of *Arabidopsis thaliana*. *Genome* **41**: 566–572.
- KOO, D. H., P. PLAHA, Y. P. LIM, Y. HUR and J. W. BANG, 2004 A high-resolution karyotype of *Brassica rapa* ssp. *pekinensis* revealed by pachytene analysis and multicolor fluorescence *in situ* hybridization. *Theor. Appl. Genet.* **109**: 1346–1352.
- KUNZEL, G., L. KORZUN and A. MEISTER, 2000 Cytologically integrated physical restriction fragment length polymorphism maps for the barley genome based on translocation breakpoints. *Genetics* **154**: 397–412.
- MARRA, M. A., T. A. KUCABA, N. L. DIETRICH, E. D. GREEN, B. BROWNSTEIN *et al.*, 1997 High throughput fingerprint analysis of large-insert clones. *Genome Res.* **7**: 1072–1084.
- MARRA, M. A., L. HILLIER and R. H. WATERSTON, 1998 Expressed sequence tags-EST abolishing bridges between genomes. *Trends Genet.* **14**: 4–7.
- MAYER, K., C. SCHULLER, R. WAMBU, G. MURPHY, G. VOLCKAERT *et al.*, 1999 Sequence and analysis of chromosome 4 of the plant *Arabidopsis thaliana*. *Nature* **402**: 769–777.
- MCPHERSON, J. D., M. MARRA, L. HILLIER, R. H. WATERSTON, A. CHINWALLA *et al.*, 2001 A physical map of the human genome. *Nature* **409**: 934–941.
- MICHAELSON, M. J., H. J. PRICE, J. R. ELLISON and J. S. JOHNSTON, 1991 Comparison of plant DNA contents determined by Feulgen microspectrophotometry and laser flow cytometry. *Am. J. Bot.* **78**: 183–188.
- MOZO, T., K. DEWAR, P. DUNN, J. R. ECKER, S. FISCHER *et al.*, 1999 A complete BAC-based physical map of the *Arabidopsis thaliana* genome. *Nat. Genet.* **22**: 271–275.
- MUELLER, L. A., T. H. SOLOW, N. TAYLOR, B. SKWARECKI, R. BUELS *et al.*, 2005 The SOL Genomics Network: a comparative resource for Solanaceae biology and beyond. *Plant Physiol.* **138**: 1310–1317.
- PAMPANWAR, V., F. ENGLER, J. HATFIELD, S. BLUNDY, G. GUPTA *et al.*, 2005 FPC Web tools for rice, maize, and distribution. *Plant Physiol.* **138**: 116–126.
- PETERSON, D. F., H. J. PRICE, J. S. JOHNSTON and S. M. STACK, 1996 DNA content of heterochromatin and euchromatin in to-

- mato (*Lycopersicon esculentum*) pachytene chromosomes. *Genome* **39**: 77–82.
- PETERSON, D. G., N. L. LAPITAN and S. M. STACK, 1999 Localization of single- and low-copy sequences on tomato synaptonemal complex spreads using fluorescence *in situ* hybridization (FISH). *Genetics* **152**: 427–439.
- RICHARDS, E. J., and F. M. AUSUBEL, 1988 Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. *Cell* **53**: 127–136.
- RICK, C. M., and J. I. YODER, 1988 Classical and molecular genetics of tomato: highlights and perspectives. *Annu. Rev. Genet.* **22**: 281–300.
- SASAKI, T., T. MATSUMOTO, B. A. ANTONIO and Y. NAGAMURA, 2005 From mapping to sequencing, post-sequencing and beyond. *Plant Cell Physiol.* **46**: 3–13.
- SCHMIDT, R., J. WEST, K. LOVE, Z. LENEHAN, C. LISTER *et al.*, 1995 Physical map and organization of *Arabidopsis thaliana* chromosome 4. *Science* **270**: 480–483.
- SHERMAN, J. D., and S. M. STACK, 1992 Two-dimensional spreads of synaptonemal complexes from solanaceous plants. V. Tomato (*Lycopersicon esculentum*) karyotype and ideogram. *Genome* **35**: 354–359.
- SHERMAN, J. D., and S. M. STACK, 1995 Two-dimensional spreads of synaptonemal complexes from solanaceous plants. VI. High-resolution recombination nodule map for tomato (*Lycopersicon esculentum*). *Genetics* **141**: 683–708.
- TANKSLEY, S. D., M. W. GANAL, J. P. PRINCE, M. C. DE VICENTE, M. W. BONIERBALE *et al.*, 1992 High density molecular linkage maps of the tomato and potato genomes. *Genetics* **132**: 1141–1160.
- TOR, M., K. MANNING, G. J. KING, A. J. THOMPSON, G. H. JONES *et al.*, 2002 Genetic analysis and FISH mapping of the Colourless non-ripening locus of tomato. *Theor. Appl. Genet.* **104**: 165–170.
- UMEHARA, Y., A. INAGAKI, H. TANOUE, Y. YASUKOCHI, Y. NAGAMURA *et al.*, 1994 Construction and characterization of a rice YAC library for physical mapping. *Mol. Breed.* **1**: 78–89.
- VAN DER KNAAP, E., A. SANYAL, S. A. JACKSON and S. D. TANKSLEY, 2004 High-resolution fine mapping and fluorescence *in situ* hybridization analysis of sun, a locus controlling tomato fruit shape, reveals a region of the tomato genome prone to DNA rearrangements. *Genetics* **168**: 2127–2140.
- WANG, C. J., L. HARPER and W. Z. CANDE, 2006 High-resolution single-copy gene fluorescence *in situ* hybridization and its use in the construction of a cytogenetic map of maize chromosome 9. *Plant Cell* **18**: 529–544.

Communicating editor: F. W. STAHL