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ORIGINAL PAPER

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Localization of *jointless-2* gene in the centromeric region of tomato chromosome 12 based on high resolution genetic and physical mapping

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Abstract Abscission is a universal process whereby plants shed their organs, such as flowers, fruit and leaves. In tomato, the non-allelic mutations *jointless* and *jointless-2* have been discovered as recessive mutations that completely suppress the formation of pedicel abscission zones. A high resolution genetic map of *jointless-2* was constructed using 1,122 jointless F_2 plants. Restriction fragment length polymorphism (RFLP) marker RPD140 completely co-segregated with the *jointless-2* locus and mapped in a 2.4 cM interval between RFLP markers CD22 and TG618. To chromosome walk to *jointless-2*, all three markers were used to screen a bacterial artificial chromosome (BAC) library and contigs were developed. Intensive efforts to expand and merge the BAC contigs were unsuccessful because of the highly repetitive sequence content on the distal ends of each contig. To determine the physical distance between and the orientation of the three contigs, we used high resolution pachytene fluorescence in situ hybridization (FISH) mapping. The RPD140 contig was positioned in the centromeric region of chromosome 12 between two large pericentric heterochromatin blocks, about 50 Mb from the TG618 contig on the short arm and 10 Mb from the CD22 contig on the long arm, respectively. Based on high resolution genetic and physical mapping, we conclude that the *jointless-2* gene is located within or near the chromosome 12 centromere where 1 cM is approximately 25 Mb in length.

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Introduction

Abscission is an important developmental process whereby plants shed their organs. Abscission can be divided into three stages: (1) the development of an abscission zone; (2) cell separation followed by organ shedding at a fully developed abscission zone; and (3) formation of a protective layer on the proximal end of the abscission zone. Although stage 2 has been extensively characterized, little is known about the first and last stages of abscission. Our laboratory is interested in understanding how abscission zones develop in plants and is taking advantage of two recessive tomato mutants that completely suppress the formation of pedicel abscission zones; *jointless* (*j*) and *jointless-2* (*j-2*). The jointless mutants are not only important for studying abscission zone biology but have a practical application in that they can be used as an aid in mechanical harvesting of processing tomatoes.

jointless was isolated as a spontaneous mutation in a field of cultivated tomatoes by Butler in 1934 and was genetically mapped to chromosome 11 (Rick 1980; Wing et al. 1994). Our laboratory cloned *jointless* using a map-based cloning strategy (Mao et al. 2000) and determined that it was a MADS-box gene with sequence similarity to those genes expressed primarily in vegetative tissue. We are now extensively characterizing *jointless* at the molecular, genetic and biochemical levels.

jointless-2 was discovered by Rick (1956) in a wild species of tomato, *Lycopersicon cheesmanii* (LA166), found on the Galapagos Islands. *jointless-2* was introgressed into cultivated tomato and is still extensively used in the processing tomato industry.

To better understand abscission zone development, we are now attempting to clone *jointless-2* using a map-based cloning approach. Map-based cloning involves precise linkage mapping of the trait of interest, followed by establishment of the relationship between genetic and physical distance, chromosome walking and finally gene identification through genetic complementation. Previously, we mapped *jointless-2* within a 3.0 cM interval between TG618 and CD22 on chromosome 12 (Zhang et al.2000). In this study we have developed a high resolution genetic map around the *jointless-2* locus, used fluorescence in situ hybridization (FISH) on pachytene chromosomes to determine the relationship between genetic and physical distance in the *jointless-2* region, and initiated a chromosome walk using a tomato bacterial artificial chromosome (BAC) library (Budiman et al.2000).

Materials and methods

Plant materials

An F_2 population was developed from a cross between *Lycopersicon esculentum* Heinz 1706 (*J-2*/*J-2*) and *Lycopersicon cheesmanii* LA166 (*j-2*/*j-2*), the same parental lines used previously (Zhang et al.2000). Tomato Heinz 1706 and LA166 were kindly provided by Dr. J. Philouze and by Dr. C. M. Rick (Tomato Genetic Stock Center, University of California at Davis, California, USA), respectively. The population was grown in the summer of 1995 at Texas A&M University and approximately 5,000 F_2 progenies were selected for the absence of pedicel abscission zones resulting in 1,122 phenotypically jointless plants.

Pooled genomic DNA extraction and high resolution mapping

Tomato genomic DNA was extracted from pooled and individual samples for restriction fragment length polymorphism (RFLP) analysis following the method of Bernatzky and Tanksley (1986). Approximately 5 g of ground leaf samples originating from five jointless (*j-2*/*j-2*) plants was used for each pool (Zhang et al. 2000) and RFLP analysis was performed as previously described (Tanksley et al. 1992). The genomic DNA of individual plants from the pool containing any recombinant DNA was re-analyzed to identify individual recombinants.

Chromosome walking using a tomato BAC library

RFLP clones TG618 and CD22 were provided by S.D. Tanksley, Cornell University, USA (Tanksley et al. 1992). RPD140 was generated by randomly amplified polymorphic DNA (RAPD) analysis and converted to a co-dominant RFLP as previously described (Zhang et al. 2000). The tomato BAC library representing 15 genome equivalents on seven full size filters (22.5 × 22.5 cm, 129,024 clones) was screened following the method of Budiman et al. (2000). All identified BAC clones were purified using alkaline lysis (Sambrook and Maniatis 1989), sequenced on both ends (Budiman et al. 2000), and fingerprinted with *Hin*dIII digestion (Marra et al. 1997; Chen et al. 2002). For expanding the initial BAC contigs, overgoes (http://genome.wustl.edu/tools) derived from unique BAC end sequence from the ends of contig were hybridized to the tomato BAC library filters essentially as described in Chen et al. (2002).

FISH mapping

Chromosome preparations

L. esculentum cv. Cherry (tomato $2n=2x=24$) was used in all FISH experiments. Young anthers of approximately 2 mm containing microsporocytes at meiotic prophase I were selected for chromosome preparation. We squashed one anther per flower in 2% aceto-carmine and monitored the stage of development using a phase contrast microscope. Anthers containing late pachytene microsporocytes were fixed in freshly prepared ice-cold 96% ethanol: glacial acetic acid (3:1) for at least 30 min. Anthers were then rinsed in distilled water three times, and in 10 mM citrate buffer, pH 4.5. Cell walls were digested in a pectolytic enzyme mixture containing 0.3% (w/v) cellulase RS, 0.3% (w/v) pectolyase Y23 and 0.3% (w/v) cytohelicase in citrate buffer for 2 h at 37°C. After two washes in water, we carefully transferred each anther to a grease-free slide. The material was dissected with fine needles, covered with acetic acid (60%) and baked on a hot plate at 45°C to fully release individual cells from the anther tissue. Cells were spread with ice-cold acetic acid fixative and left to air-dry for at least 2 h. Further treatments of the slides followed the protocol of Zhong et al. (1996).

Cot-100 DNA

Cot-100 fraction of tomato genomic DNA was prepared according to Zwick et al. (1997) with some modifications. Total genomic DNA was isolated according to the CTAB method and sonicated to a fragment length of 100–1,000 bp. We denatured 0.5 μg/µl sonicated DNA in 0.3 M NaCl at 95°C for 10 min, and then transferred it to an incubator at 62.4°C (Peterson et al.1998) for single strand DNA molecule re-association over the following 19 h. The remaining ssDNA was digested with S1 endonuclease (Fermentas, final concentration 1 U/μg) for 90 min at 37°C. The DNA was finally purified by ethanol precipitation.

Fluorescence in situ hybridization

Six BAC clones were chosen for FISH analysis: four BACs from the distal regions of the RPD140 contig and the CD22 contig (two from each contig); one from the TG618 contig; one from an independent separate contig which was identified from a BAC end of the RPD140 contig. Each BAC DNA (1–2 μg) was labeled with either biotin-16-dUTP or digoxigenin-11-dUTP by nick translation using the manufacturer's protocol (Roche) and FISH was performed according to Zhong et al. (1996). Chromosomes were counterstained in 5 μg /ml DAPI in Vectashield anti-fade (Vector Laboratories). Slides were examined with a Zeiss Axioplan 2 Photomicroscope equipped with epifluorescence illumination, filter sets for DAPI, FITC and rhodamine fluorescence and a Photometrics 1,400×1,000 pixel CCD camera. Selected images were captured and processed with Genus Image Analysis Workstation software (Applied Imaging Corporation). DAPI images were separately sharpened with a 7×7 Hi-Gauss high pass spatial filter to accentuate minor details and heterochromatin banding of the chromosomes. All fluorescence images were pseudo-colored and improved for optimal brightness and contrast.

Results

High resolution mapping of *jointless-2*

As a first step for the map-based cloning of the *jointless-2* gene, we constructed a high resolution map around the *jointless-2* locus based on the previous genetic map of 151 F2 segregants (Zhang et al.2000). Approximately 5,000 F_2 plants from a cross between a jointed *L. esculentum* (Heinz 1706) and jointless *L. cheesmanii* (LA166) were planted in the field and 1,122 jointless plants were identified. To avoid any possible error in scoring the jointless phenotype, we scored the phenotype four times independently for each plant. To identify rare recombinants between jointless and closely linked RFLP markers we followed a modified pooled sampling mapping approach (Churchill et al.1993). After a first round of screening against the DNA pools, DNA from each plant in the pools showing recombinants was analyzed to identify recombinant individuals. The recombination fraction between each marker and *jointless-2* is shown in Fig. 1. Among the 1,122 jointless F_2 plants, no recombinants were detected between RPD140 and *jointless-2* indicating that RPD140 was tightly linked to *jointless-2*. One recombinant was identified between *jointless-2* and three co-segregating markers, CD22, TG112 and CT189. More recombinants, (2, 19, and 101) were identified for TG318, TG565, and TG111, respectively. On the opposite side of *jointless-2*, 21 and 52 recombinants were identified for TG618 and TG360, respectively. The high resolution map (Fig. 1) represented the same order of markers with slight changes of genetic distances compared to the previous map by Zhang et al. (2000).

Chromosome walking and long range physical mapping

To initiate a chromosome walk to *jointless-2*, tomato BAC library filters representing 15 genome equivalents from a wild-type jointed cultivar (Heinz 1706) were screened with three RFLP markers, RPD140, CD22 and TG618, flanking the *jointless-2* locus. All positive clones were fingerprinted with *Hin*dIII and assembled into three non-overlapping BAC contigs using FPC (Soderlund et al.2000) (shaded clones of each contig in Fig. 2). To extend each contig we developed overgo probes from unique BAC-end sequences located on the ends of the contigs. These probes were then hybridized to the BAC library and positively hybridizing clones were fingerprinted and assembled into FPC contigs. Each contig was expanded but could never be merged even after several rounds of chromosome walking. One serious limitation to further contig expansion was the presence of repetitive BAC-end sequences where unique overgo probes could not be designed. The final contig sizes were about 300 kb, 500 kb and 200 kb, and hybridized with RPD140, CD22 and TG618 respectively.

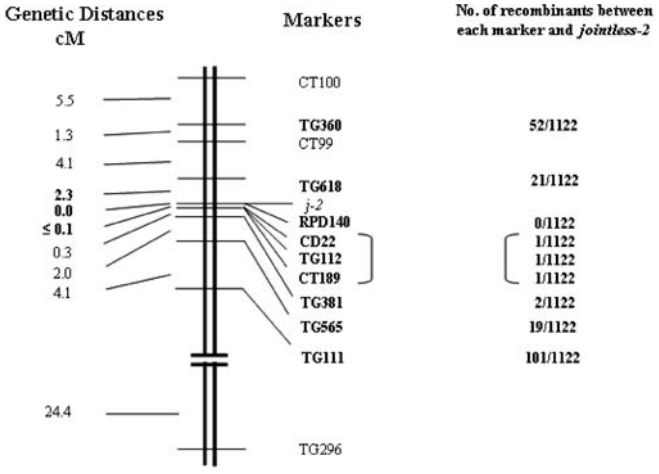

Fig. 1 High resolution mapping of the *jointless-2* locus on chromosome 12. A larger population containing about 5,000 F_2 progenies from *Lycopersicon esculentum* Heinz 1706 *J-2*/*J-2* and *L. cheesmanii* LA166 *j-2*/*j-2* was used to further resolve the order of clustered markers on the high resolution map. Recombination fraction between each marker and *jointless-2* (*j-2*) were determined based on the number of recombinants. RPD140 co-segregated with *jointless-2*, CD22 has one recombination on one side and TG618 has 21 recombinations on the opposite side. Three markers, CD22, TG112 and CT189 co-segregated

Because the RPD140 and CD22 contigs were only 0.1 cM apart and could not be merged, we attempted to determine the relationship between genetic and physical distance using long-range restriction mapping based pulsed field gel electrophoresis (PFGE) as previously described by Wing et al. (1994). Unfortunately we were unable to detect common bands between RPD140, and CD22, even though both markers hybridized as unique bands at 50 kb up to 1,000 kb in size for megabase DNA blots digested with seven rare-cutting restriction enzymes (data not shown). This result suggested that even though RPD140 and CD22 are genetically very close, their physical distances could be quite far apart.

Centromeric localization of BAC contigs on chromosome 12 by FISH

We selected five BAC clones from the three contigs previously identified to determine the physical distances among them and the linearity of each contig using high resolution pachytene FISH: 49G18 from TG618 contig; 120H06 and 121H12 from RPD140 contig; 229H19 and 129H18 from CD22 contig (circled clones on Fig. 2). Another BAC clone, 58M09, from a 9 BACs contig that hybridized with a probe from one end of the RPD140 contig was tested to determine the orientation of the contig relative to RPD140, TG618 and CD22. FISH revealed that all five BAC clones were assigned to chromosome 12 with the expected order, including huge physical gaps between the contigs. The BAC clone

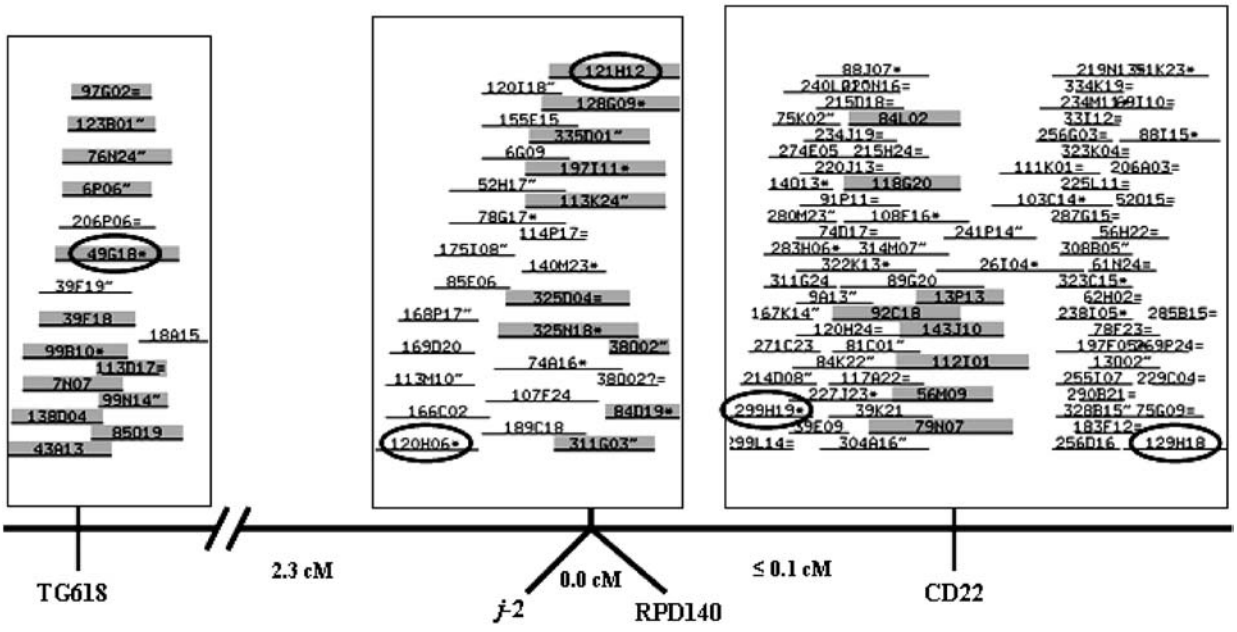

Fig. 2 BAC contigs from the *jointless-2* region. Primary contigs were constructed from BAC clones (*shaded* in each contig) identified with three RFLP markers, *TG618*, *RPD140*, and *CD22*. Each BAC contig was expanded by three or four rounds of serial STC-based overgo hybridization and fingerprinting. BAC clones *circled* in the contigs (49G18, 120H06, 121H12, 229H19, and 129H18) were used for confirmation of the physical distances and the locations among three contigs as well as their linearity on chromosome 12 using FISH. Contig assembly was done using a tolerance level of seven and a cutoff of 1e-09

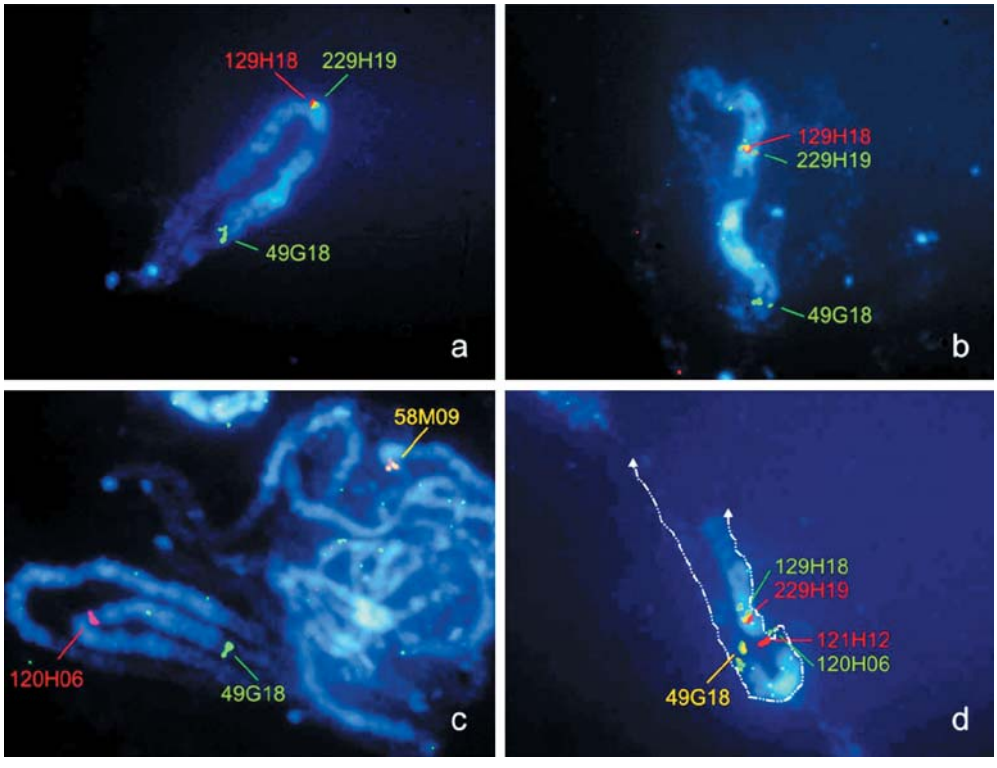

Fig. 3a–d FISH mapping of six BAC clones on chromosome 12. Each BAC clone was chosen from Fig. 2. **a** BAC 49G18 from contig TG618 was located in the euchromatin of the short arm at the border of a pericentromeric heterochromatin block. **b** BACs 229H19 and 129H18 from contig CD22 were located in a gap of the long arm pericentromeric heterochromatin. **c** BAC 58M09 was localized to a different unidentified chromosome. **d** BACs 120H06 and 121H12 from contig RPD140 were located in the centromeric region

49G18, from the TG618 contig, was found in the euchromatin of the short arm, just at the border of the pericentromeric heterochromatin block. BACs 120H06 and 121H12 from the RDP140 contig were located in the centromere region while BACs 229H19 and 129H18 from the CD22 contig were located in the pericentromeric heterochromatin, in a small weakly fluorescing gap of the long arm (Figs. 3a–d). BAC 58M09, however, hybridized

to the border of a heterochromatin region on a different unidentified chromosome (Fig. 3c).

The physical distances between TG618 and RPD140, and RPD140 and CD22 are 7.9 μm and 1.6 μm, respectively, which correspond to about 50 Mb and 10 Mb, respectively, using ratio values of 6.3 Mb/μm for heterochromatin and 0.6 Mb/μm for euchromatin (Zhong et al.1998). These results confirmed our suspicion that RPD140 and CD22 are physically far apart.

Discussion

High resolution mapping of the *jointless-2* locus

Previously, the *jointless-2* locus was mapped to a 3.0 cM interval flanked by RPD140 and TG618 on chromosome 12 using a population of 151 F_2 plants near the centromere of chromosome 12 (Zhang et al. 2000).

In this study we used pooled sample mapping to identify rare recombinants between closely linked markers and *jointless-2*. We were unable to identify any recombinants between RPD140 and *jointless-2* but were able to identify one recombinant between RPD140 and RFLP markers CD22, TG112 and CT189. We also identified 21 additional recombinants between *jointless-2* and TG618. This study confirms our previous low resolution map (Zhang et al. 2000) except that RPD140 completely co-segregates with the jointless phenotype. Zhang et al. reported a single recombinant between *jointless-2* and RPD140 and no recombinants between RPD140 and CD22 using 151 F_2 plants from the identical cross used in the present study. If this finding was correct, assuming the same level of recombination, then one would expect to identity approximately31 recombinants between *jointless-2* and RPD140 in this expanded F_2 population. We now believe that, based on our new high resolution genetic map, the jointless phenotype of the single recombinant between *jointless-2* and RPD140 was scored incorrectly. However, we can not rule out the possibility that the single recombinant previously identified was a true recombinant. Unfortunately, this plant or its progeny no longer exist which could be used to determine between the two possibilities.

Centromeric localization of the *jointless-2* gene by FISH

Chromosome 12 measures about 30.3 μm on average at the pachytene stage, which is 6% of the cell complement. Chromosome 12 is relatively rich in heterochromatin with large characteristic pericentromeric blocks on both arms interrupted with a variable number of small weakly fluorescing gaps (Ramanna et al. 1967; Sherman et al. 1992; Peterson et al. 1996, 1999; Zhong et al. 1998). The centromere itself is 1.2 μm and fluoresces even more weakly than the distal euchromatin segments. Both arms have small heterochromatin knobs at the distal ends containing the sub-telomeric TGR1 repeat (Zhong et al. 1996). Chromosome 12 is the shortest of the cell complement with a symmetrical heterochromatin pattern and is easily distinguishable from all other chromosomes except for chromosome 5 which measures 20% longer and has slightly less heterochromatin (Fig. 3a–d).

To determine the relationship between genetic and physical distance in the *jointless-2* region, we used FISH with BAC clones that hybridized with the *jointless-2* linked RFLP markers. FISH is an extremely powerful and visual method to physically map genes, markers and low copy sequences including BAC clones to chromosomes (Hanson et al. 1995; Lapitan et al. 1997). We applied Cot 100 fraction DNA to block off the repetitive sequence in the heterochromatin block and thus obtain clear localization of each BAC as shown in Fig. 3. The Cot 100 fraction gave high resolution by suppressing the signal from repetitive sequence. We confirmed the assignment of five BAC clones on chromosome 12 and positioned them with respect to centromere, pericentromeric heterochromatin and distal euchromatin. Measurements of the FISH signals gave chromosomal distances between the markers linked to the *jointless-2* gene, which could be converted into molecular length on the physical map. We compared linkage distances between the molecular markers and estimated physical map lengths (Fig. 4).

The markers TG618-RPD140 on the short arm have a genetic map length of 2.3 cM, whereas their BACs 49G18 and 120H06 flanking the pericentromeric block of the short arm have a chromosome distance of 7.9 μm, which corresponds with a physical length of approximately 50 Mb. For markers RPD140 and CD22, 0.1 cM apart, their corresponding BACs were positioned on the long arm of chromosome 12 in the pericentromeric heterochromatin, 1.6 μm apart, a distance that corresponds to approximately10 Mb.

Extreme suppression of recombination at the centromere on chromosome 12

The ratio of physical and genetic distance in the *jointless-2* region is represented in Table 1. On average, a genetic distance of 1 cM on the tomato molecular map corresponds to approximately 750 kb (Tanksley et al. 1992). Our data revealed 21.74 Mb/cM (29 times higher than average) between TG618 and RPD140 on the pericentromeric region of the short arm of tomato chromosome 12 and 100 Mb/cM (134 times higher than average) between RPD140 and CD22 on the pericentromeric region of the long arm. The skewed genetic / physical map ratios can be explained by the suppression of recombination in the pericentromeric regions of the tomato chromosomes (Sherman et al. 1995). In *Arabidopsis*, recombination rates at centromeric regions were 10 – 30 times below the genomic average (Copenhaver et al.1999). Physical mapping near the centromere of tomato chromosome 9 revealed that a genetic distance of 1 cM is at least 4 Mb of DNA (Ganal et al. 1989). FISH mapping of *Mi-1* and *Aps-1* on chromosome 6 revealed that 1 cM is

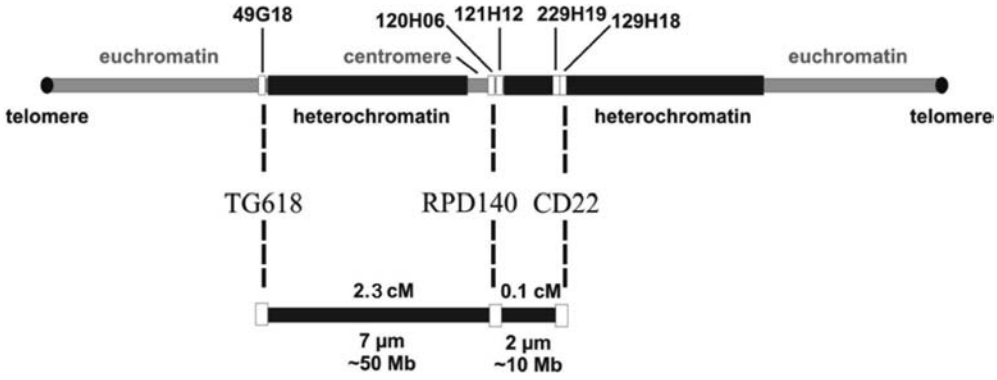

Fig. 4 Comparison of physical and genetic distances between molecular markers TG618, RPD140 and CD22 and an estimation of their physical distances. The distances between TG618 and RPD140, and RPD140 and CD22 are 7.9 μm and 1.6 μm, which correspond to about 50 Mb and 10 Mb, respectively, based on ratio values of 6.3 Mb/μm for heterochromatin and 0.6 Mb/μm for euchromatin

Table 1 Comparison of Mb/cM ratio values for the pericentromeric chromosome 12 region

	Physical length (Mb)	Genetic length (cM)	Mb/cM	Comparison
TG618-RPD140	50	2.3	21.74	29.2
RPD140-CD22	10	0.1	100.00	134.3
Overall average[a]	950	1,276.0	0.74	1.0

[a] Based on Tanksley et al. 1992

40 Mb which is 50-fold higher than an average (Zhong et al. 1999). Frary et al. (1996) defined the centromere region using approximate 2,300 segregating plants, but they could not find recombinants between many of centromeric markers on chromosomes 7 and 9. On the other hand, Mao et al. (2001) reported higher recombination, less than 50 kb/cM, at the *jointless* region. Our data revealed that the recombination suppression on the centromeric region of chromosome 12 is much higher than in the other reports described above. One reason for this finding may be the special feature of chromosome 12 having an excessive amount of large pericentromeric blocks. The interspecific cross used for mapping in this study may have also contributed to a suppression of recombination.

Genes present in centromeric regions

It is interesting to compare the location of the two genes, *jointless* and *jointless-2*, which govern the same phenotype. *Jointless* is a spontaneous mutant, and was cloned from a 119 kb BAC clone, 240K04, which has a high gene density and high recombination ratio (less than 50 kb/cM) on a euchromatic region of chromosome 11 (Mao et al. 2001). On the other hand, *jointless-2*, discovered from a wild tomato species, *L. cheesmanii* LA166, was found to be located in the centromeric region of chromosome 12 and may represent a possible function of *jointless-2* related to the evolution of the tomato. In *Arabidopsis*, many unknown genes have been found in the centromeric region. Interestingly, gene density on the centromeric region of chromosome 4 is higher than on chromosome 2. The average number of predicted genes per 100 kb is 25. Five and 12 predicted genes per 100 kb were found for the centromeres of chromosome 2 and 4, respectively. Based on cDNA database searches, 6 and 21 predicted genes were found on the centromeric regions of *Arabidopsis* chromosomes 2 and 4, respectively (Copenhaver et al. 1999). Further, 15 predicted genes were found within the centromeric sequence of rice chromosome 4 (Feng et al. 2002). One evolutionally important gene, *S-RNase*, was found in the centromeric region of *Petunia hybrida* (Entani et al. 1999). *S-RNase* gene is an S-allele-specific styler determinant for the self-incompatibility response in Solanaceae. Centromeres are the most prominent domains in eukaryotic chromosome. The centromere may harbor numerous expressed genes, such as the *jointless-2* gene, in addition to their essential role for maintaining chromosome structure. Positional cloning of *jointless-2* is more challenging because of the technical difficulty and lack of knowledge about the centromere. However, obtaining the sequence of a centromere which is recombination-deficit and repeat-rich will provide an understanding of genome evolution as well as aid in the cloning of novel important genes.

Positional cloning of *jointless-2*

Because of the location of *jointless-2* near the chromosome 12 centromere and the relationship between genetic and physical distance it appears it will continue to be difficult to positionally clone *jointless-2* by chromosome walking. One important tool that is missing is a closer marker than TG618 which is physically located opposite

to a heterochromatic block approximately 50 Mb from *jointless-2*. An additional round of BSA screening will be required to develop more closely linked markers to narrow this interval.

To aid in chromosome walking we are currently developing a genome-wide physical map of tomato Heinz1706 by fingerprinting the entire *Hin*dIII BAC library and then assembling the fingerprints into contigs using FPC (Soderlund et al. 2000). We are hopeful that this contig map will provide additional contigs around the *jointless-2* locus that will allow us to construct a contiguous contig in the region. Such a region could then be draft sequenced to identify *jointless-2* candidate genes. Such maps have been useful to sequence through centromeric regions as recently demonstrated for rice chromosome 4 (Feng et al. 2002) using the rice contig map constructed in our laboratory (Chen et al. 2002).

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