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Map-based cloning in crop plants. Tomato as a model system: I. Genetic and physical mapping of *jointless*

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Abstract. A map-based cloning scheme is being used to isolate the *jointless* (*j*) gene of tomato. The *jointless* locus is defined by a single recessive mutation that completely suppresses the formation of the fruit and flower pedicel and peduncle abscission zone. *jointless* was mapped in an F_2 population of an interspecific cross between *Lycopersicon esculentum* and *Lycopersicon pennellii* to a 7.1 cM interval between two restriction fragment length polymorphism (RFLP) markers TG523 and TG194. Isogenic DNA pools were then constructed from a subset of the mapping population and screened with 800 random decamers for random amplification of polymorphic DNA (RAPD) polymorphisms. Five new RAPD markers were isolated and mapped to chromosome 11, two of which were mapped within the targeted interval. One marker, RPD158, was mapped 1.5 cM to the opposite side of *jointless* relative to TG523 and thus narrowed the interval between the closest flanking markers to 3.0 cM. Physical mapping by pulse-field gel electrophoresis using TG523 and RPD158 as probes demonstrated that both markers hybridize to a common 600 kb *Sma*I restriction fragment. This provided an estimate of 200 kb/cM for the relationship between physical and genetic distances in the region of chromosome 11 containing the *j* locus. The combined results provide evidence for the feasibility of the next step toward isolation of the *jointless* gene by map-based cloning – a chromosome walk or jump to *jointless*.

Key words: *Lycopersicon esculentum* – Map-based cloning – RFLP/RAPD mapping – Physical mapping – Abscission

Introduction

Map-based cloning is becoming widely accepted as an important method for the isolation of genes for which only the phenotype and the map position are known (for reviews see Wicking and Williamson 1991; Paterson and Wing 1993). The first step toward successful map-based cloning is the mapping of the target gene in a segregating population. Once markers are found linked to the target gene, this region can be saturated with DNA markers, using a variety of methods, to obtain more closely linked markers. The next step is to establish the relationship between genetic and physical distance by physical mapping of the most closely linked markers. This step is crucial to the success of a map-based cloning effort because the correspondence between genetic and physical distances can vary over 100-fold in different regions of a genome (e.g. tomato, Ganal et al. 1989; Segal et al. 1992). The third step is to use the most closely linked markers as starting points for chromosome walking or jumping toward the target gene. Chromosome walking is continued until a genomic clone is isolated that can be determined genetically to contain the target gene. Finally, once a candidate clone is isolated, target gene identity must be determined to prove that the gene has been isolated (e.g. phenotypic complementation in transgenic plants).

This general strategy has been successfully employed in mammalian systems, most notably for the cystic fibrosis gene (Rommens et al. 1989). Map-based cloning in plants has only recently been demonstrated in the model plant *Arabidopsis thaliana* by isolation of the *ABI3* gene (Giraudat et al. 1992) and the omega-3 fatty acid desaturase gene (Arondel et al. 1992). Although several important plant genes can potentially be isolated by map-based cloning and studied in *A. thaliana*, crop plants contain many well characterized and agronomically important genes for which no *A. thaliana* homolog has yet been identified or even exists. Thus it is of significance to develop reliable map-based cloning systems for specific crop plants to isolate agriculturally important genes that may be directly applicable to crop improvement, and can serve as models for analysis of biological processes that are not readily accessible in *A. thaliana*.

Tomato (*Lycopersicon esculentum*) is an ideal crop plant for the establishment of such a map-based gene

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cloning system because all of the elements described above are currently available. (1) Tomato has well developed classical (Rick and Yoder 1988) and molecular genetic maps (Tanksley et al. 1992) and methods have been developed to isolate markers efficiently in specific regions of the tomato genome (Martin et al. 1991; Giovannoni et al. 1991; Michelmore et al. 1991). (2) Physical mapping tools have been developed for the establishment of the relationship between genetic and physical distance (Ganal et al. 1989; Wing et al. 1993). (3) A complete yeast artificial chromosome (YAC) library has been constructed, which is essential for chromosome walking across large distances (Martin et al. 1992). (4) Verification of target gene isolation, by genetic complementation or antisense suppression, can be facilitated because tomato transformation is routine (McCormick et al. 1986).

In this study we chose as our target gene *jointless,* a single-locus, recessive, developmental mutation that completely suppresses the formation of the flower and fruit pedicel and peduncle abscission zones (Butler 1936). Abscission zones are morphologically distinct regions of plants where organs such as fruits, flowers, and leaves are shed in a developmentally regulated manner. In the flower pedicels of tomato, abscission zones are formed in the middle of the pedicel, which can be visualized by the presence of an indentation called the "joint". The joint consists of five to ten tiers of unexpanded cells that traverse the pedicel (Roberts et al. 1984). Addicott (1982) describes these abscission zone cells as being less developed, i.e., they are not completely differentiated or meristematic.

The mechanism regulating the development of this as well as other abscission zones (e.g., leaves) is not understood (see reviews by Addicott 1982; Sexton and Roberts 1982). A major problem in studying the development of abscission zones is that they are easy to identify only after they have differentiated. Thus, it has been very difficult to identify the cells that will differentiate into abscission zone cells and determine how they differ from surrounding cells.

We chose *jointless* because it is one of a very limited number of mutations that affect abscission zone development and its isolation may provide clues as to the underlying mechanism regulating this process. In addition, by isolating a gene that directly affects abscission zone development in tomato, it may be possible to use this gene to control abscission in both tomato and other crop plants.

In this paper we report the first and second steps of our map-based cloning of the tomato *jointless* gene, genetic and physical mapping, and discuss the feasibility of using this information for the initiation of a chromosome walk or jump to *jointless.*

Materials and methods

Plant materials and mapping population. L. esculentum cv. VFNT Cherry, LA624 (*jointless* homozygote *jlj*) and *Lycopersicon pennellii* LA716 (jointed homozygote *J*/*J*) were kindly provided by Dr. C. Rick, Tomato Genetics Stock Center, University of California at Davis. The F_1 between LA624 (female) and LA716 (male) and the corresponding F_2 progeny were established by R.A.W. at the USDA/ARS Plant Gene Expression Center, Albany, Calif., under the guidance of Dr. S. McCormick.

DNA isolations. Plant DNA for restriction fragment length polymorphism (RFLP) analysis was isolated as previously described (Bernatzky and Tanksley 1986). High molecular weight (megabased sized) DNA was isolated from cv. VFNT Cherry protoplasts as previously described (Wing et al. 1993).

RFLP and linkage analysis. RFLP analysis was performed as previously described (Tanksley et al. 1992). Linkage data was analyzed using the Mapmaker (version 1.0) program for Macintosh computers using the Kosambi mapping function (Kosambi 1944) with an LOD score of 3.0. Standard errors for recombination frequency were determined according to Allard (1956).

Random amplification of polymorphic DNA (RAPD) screening. Isolation of additional markers in the *jointless* region by RAPD screening was as previously described (Giovannoni et al. 1991) except that the polymerase chain reaction (PCR) volume was reduced from 25 to 10 µl. RAPD decamer primers were purchased from Dr. John Carlson, University of British Colombia, Vancouver, Canada. The nucleotide sequences for the *jointless*-specific RAPDs are primer 158, 5′-TAGCCGTGGC-3′, and primer 443, 5′-TGATTGCTCG-3′.

The reproducible RAPD bands that were polymorphic between the two isogenic DNA pools were excised from an agarose gel, radiolabeled and hybridized to Southern filters containing DNAs from both mapping parents digested with various restriction enzymes in order to identify codominate RFLPs for mapping. The polymorphic bands generated from primers 158 and 443 that were mapped between TG194 and *jointless* were subsequently cloned into the plasmid pCRII using the TA Cloning Kit (InVitrogen).

Physical mapping. Megabase sized DNA was digested with various rare cutting restriction enzymes followed by pulse-field gel electrophoresis (Chu et al. 1986). The DNAs fractionated in the gel were nicked with 60 mJ of ultra-violet light (254 nm) using the GS Gene Linker (Bio-Rad), and blotted with 0.4 N NaOH and 1.5 M NaCl (Southern 1975; Reed and Mann 1985) to Hybond-N+ (Amersham). Southern blots were hybridized with radioactively labeled (Feinberg and Vogelstein 1984) gel-purified DNA inserts of the clones. After autoradiography, the filter was stripped of probe using three 10 min washes of: (1) 0.1 N NaOH; (2) 0.1 M TRIS-HCl, pH 7.5, 0.1% SDS, 0.1 × SSC; (3) 0.1% SDS, 0.1 × SSC. After stripping, the filter was autoradiographed for 2 days to ensure that no labeled probe remained on the filter.

Results

Construction and phenotypic analysis of an interspecific F_2 population segregating for jointless

In order to map the *jointless* (*j*) locus using RFLP markers, an F_2 mapping population was generated, segregating for *jointless,* from an interspecific F_1 hybrid *(L. esculentum j/j × L. pennellii J/J)*. One hundred F_2 plants were analyzed for segregation of the *jointless* phenotype to determine the penetrance of *jointless* in such a cross by scoring the presence or absence of a pedicel abscission zone. Sixteen *jointless* and 84 jointed plants were detected. Figure 1 shows examples of the jointed and *jointless* phenotypes scored in the F_2 population. The *jointless* versus jointed phenotype is clearly scorable in this cross. Jointed plants shed their flowers at abscission zones, which form either at the base of the pedicel as in *L. pennellii* (Fig. 1A), or in the middle of the pedicel, as in *L. esculentum*. The *jointless* F_2 plants, on the other hand, never shed their flowers. Usually *jointless* flowers senesce and completely shrivel up on the plant.

TG523 is closely linked to jointless

jointless was previously mapped to chromosome 11 between *gametophytic factor* (*x*) and *elegans* (*ele*) on the morphological map (Fig. 2A, and see Rick and Yoder 1988). Unfortunately, when this mapping study was initiated, the morphological and RFLP maps had not been interdigitated. Only a single RFLP marker (TG105) was shown to be linked to a gene on chromosome 11, the *Fusarium oxysporum* disease resistance gene *I2* (Sarfatti et al. 1989). Based on this information, several RFLP markers on the opposite arm of chromosome 11 were tested for linkage to *jointless* using DNA isolated from the 16 *jointless* F_2 segregants. Figure 3 shows an autoradiograph of a Southern blot of the 16 *jointless* F_2 plants hybridized with TG523. Of the 16 *jointless* F_2 segregants (*j/j*), 15 have the *L. esculentum* (*jointless*) RFLP pattern (lanes 5–19, plants 2–16), and one plant has a heterozygous RFLP pattern (lane 4, plant 1). This result indicated that TG523 is closely linked to *jointless*. The presence of an F_2 homozygous *jointless* plant showing a heterozygous RFLP pattern for TG523 (Fig. 3, plant 1) indicated that at least one crossover had occurred between TG523 and *jointless*.

High resolution mapping of jointless

Since TG523 is linked to *jointless,* all the available RFLP markers closely linked to TG523 were mapped in an F_2 population consisting of the original 16 *jointless* F_2 plants and 84 additional F_2 plants from the same cross (13 *jointless,* 68 jointed and 3 not scored). Figure 2B shows the resulting RFLP map of the *jointless* region. The *jointless* gene was mapped to a 7.1 (+/− 1.8) cM interval between TG194 and TG523. TG523 is still the most closely linked marker, mapping 1.5 (+/− 2.0) cM from *jointless*.

To isolate more markers in the 7.1 cM interval defined by TG523 and TG194, two isogenic DNA pools homozygous for either *L. esculentum* (pool I, 7 F_2 plants) or *L. pennellii* (pool II, 7 F_2 plants) in this interval, were constructed as previously described (Giovannoni et al. 1991) and screened for polymorphisms with 800 random decamer primers with PCR. Thirty-five reproducible polymorphic bands were detected. Hybridization analysis, to detect co-dominant RFLPs, was performed with all 35 of the RAPD bands and revealed that 15 were low-copy-number sequences and the remaining 20 contained repetitive elements. Of the 15 low-copy-number probes, 5 were RFLP mapped to chromosome 11, 9 were RFLP mapped to other chromosomes, and 1 was not mapped because no RFLP was detected between the two parents. Two of the 5 RAPDs mapping to chromosome 11, designated RPD158 and RPD443, were mapped between *jointless* and TG194, thus narrowing the interval defined by TG523 and TG194 flanking the *jointless* locus to 3.0 (+/− 1.9) cM. Figure 2B shows the map position of RPD158 and RPD443 on chromosome 11. RPD158 is the most closely linked marker to *jointless* opposite to TG523, mapping 1.5 (+/− 2.0) cM from *jointless*.

Fig. 1A, B. Examples of jointed (**A**) and *jointless* (**B**) phenotypes scored in the F_2 mapping population

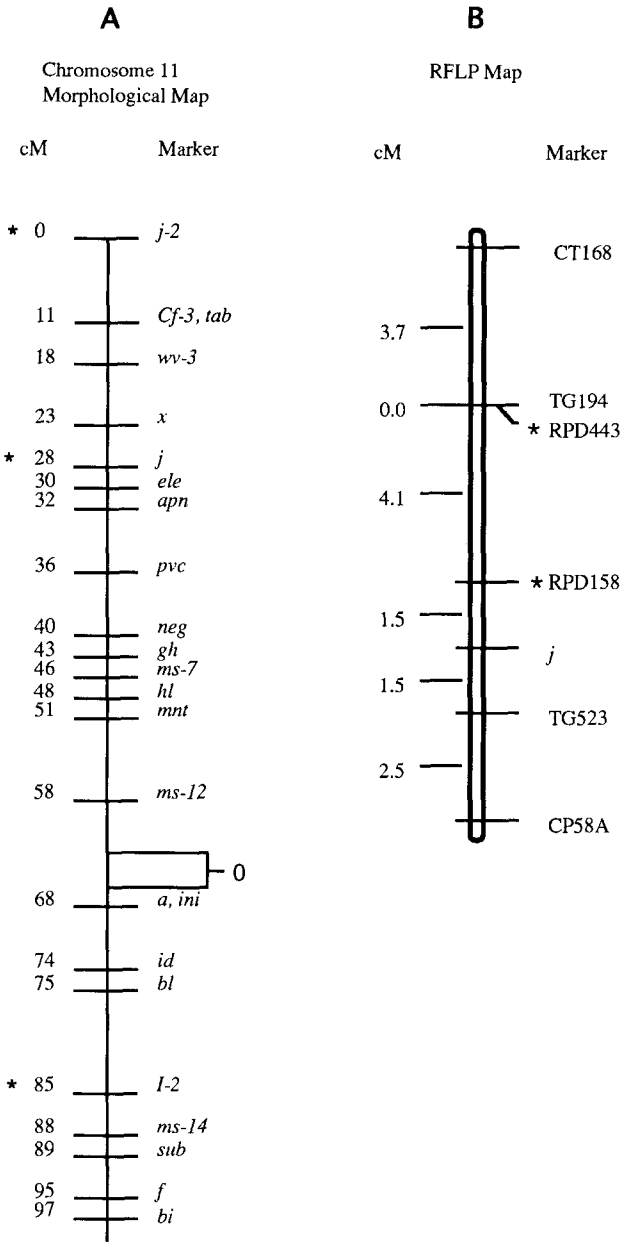

Fig. 2. A Morphological genetic map of tomato chromosome 11 (taken from Rick and Yoder 1988). The *asterisks* mark the *jointless* mutation and the *Fusarium oxysporum* resistance gene discussed in the text. **B** Molecular restriction fragment length polymorphism (RFLP) genetic map of the *jointless* region incorporating random amplification of polymorphic DNA (RAPD) markers RPD443 and RPD158 (indicated by *asterisks*). All genetic distances are in centimorgans (cM)

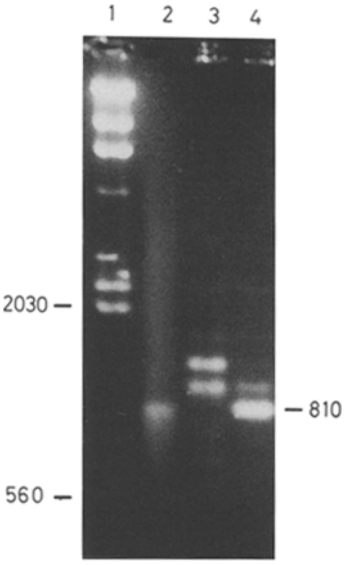

Fig. 4. Ethidium bromide-stained 1.5% agarose gel used to resolve polymerase chain reaction (PCR) amplification products with decamer primer UBC158. Lanes 3 and 4, PCR amplification of near-isogenic DNA pools I and II, respectively, with decamer primer UBC158. Lane 2, aliquot of a PCR amplification with decamer primer UBC158 using the 810 bp polymorphic band in lane 4 as the template DNA source. Lane 1, molecular weight marker, lambda DNA digested with *Hin*dIII

Figure 4 (lanes 3 and 4) shows the RAPD polymorphism for primer 158 between the two DNA pools. Surprisingly this primer resulted in a co-dominant marker between the pools, however, only the 810 bp polymorphic band derived from pool II could be RFLP mapped because it represented a low-copy-number sequence. The polymorphic band derived from pool I showed a repetitive hybridization pattern (data not shown).

Physical mapping

To determine the relationship between genetic and physical distances in the 3.0 (+/− 1.9) cM interval between TG523 and RPD158, megabase sized DNA was isolated from VFNT Cherry protoplasts embedded in agarose microbeads (Wing et al. 1993) and digested with restriction enzymes recognizing either 8 or 6 bp nucleotide sequences. The DNA fragments were then fractionated by pulse-field gel electrophoresis (CHEF, Chu et al.

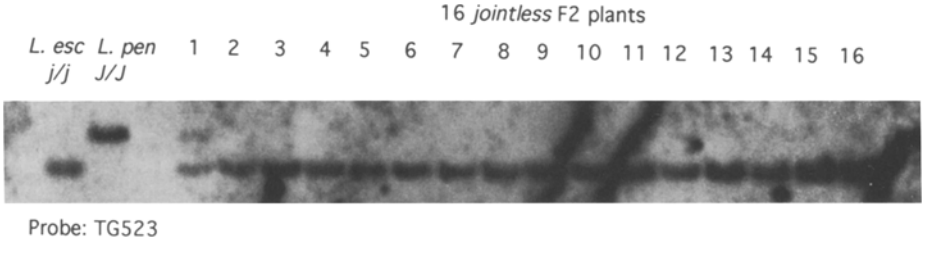

Fig. 3. Autoradiograph of a Southern filter hybridized with radioactively labeled TG523. DNAs (2 µg) from the parents and 16 *jointless* F_2 plants were digested with *Hae*III and electrophoresed through a 0.8% agarose gel. *L. esc, Lycopersicon esculentum* parent LA624, *jointless* homozygote. *L. pen, Lycopersicon pennellii* parent LA716, jointed homozygote

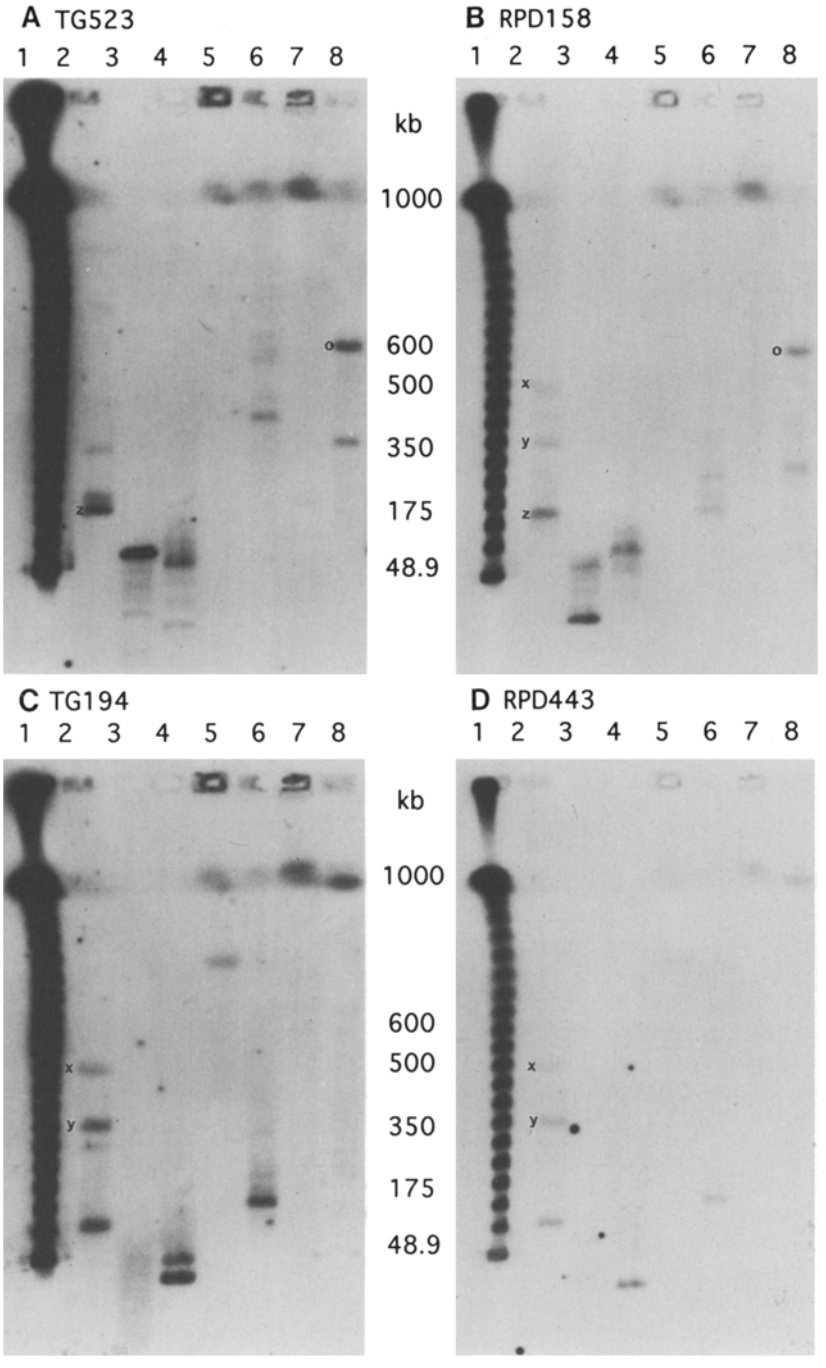

Fig. 5A–D. Physical mapping of the *jointless* region. Autoradiographs of a single Southern filter hybridized sequentially with radioactively labeled DNA probes: **A** TG523; **B** RPD158; **C** TG194; and **D** RPD443. Tomato VFNT Cherry megabase sized DNA embedded in agarose microbeads was digested with: lane 2, *Mlu*I; lane 3, *Nco*I; lane 4, *Nhe*I; lane 5, *Not*I; lane 6, *Sal*I; lane 7, *Sfi*I; lane 8, *Sma*I. Lane 1, lambda concatemer (FMC, USA). Contour damped homogeneous electric field (CHEF) gel pulse conditions set with MJ Research Programmable Power Inverter: A, 100 s; B, −0.05 s; C, 100 s; D, −0.05 s; E, 175 times; F, −0.01 s; G, −0.01 s. The gel was run for 40 h with 150 V at 11° C

1986), blotted and hybridized sequentially with radioactively labeled TG523, TG194, RPD158, and RPD443. All hybridizations were performed on two independent gels to ensure that the hybridization bands were reproducible (Fig. 5). The results for TG523 and TG194 show that both probes do not hybridize to any common restriction fragments (Fig. 5A and C) indicating that they are not closely linked. Hybridization with RPD443 shows a hybridization pattern nearly identical to that for TG194 (Fig. 5D), as anticipated from the linkage data indicating cosegregation (Fig. 2B), implying that the two markers are physically very tightly linked. TG523 and RPD158 hybridize to a common 600 kb *Sma*I fragment and a common 175 kb *Mlu*I fragment(s) (Fig. 5A and B; bands "o" and "z", respectively). These results suggested that TG523 and RPD158 are physically tightly linked within 175 kb to 600 kb. The estimation for genetic and physical distances in the interval between TG523 and RPD158 is approximately 58 to 200 kb/cM.

To verify this relationship, the same Southern filter was hybridized with the end of a 280 kb YAC that was mapped between markers TG523 and RPD158 (unpublished). This YAC end hybridized to the 600 kb *Sma*I fragment but not to the 175 kb *Mlu*I fragment(s). These results demonstrate that the apparently common 175 kb *Mlu*I fragment shared between TG523 and RPD158

must in fact represent two independent, comigrating restriction fragments and thereby eliminates the lower limit of 58 kb/cM. However, the hybridization of three independent markers to a common 600 kb *Sma*I fragment strongly supports the upper limit of 200 kb/cM.

The marker RPD158 also provides a physical link with TG523 to RPD443 and TG194. Figure 5 shows that markers RPD158, RPD443, and TG194 hybridize to two common *Mlu*I restriction fragments of 350 and 500 kb (Fig. 5B, C, and D, bands "y" and "x", respectively). This result demonstrates the physical linkage among all four DNA markers, TG523, RPD158, RPD443, and TG194, within 950 kb (600+350 kb), which corresponds to 130 kb/cM over the entire 7.1 (+/− 1.8) cM interval.

Discussion

To initiate a chromosome walk or jump aimed at the isolation of the *jointless* gene, two essential requirements must be met. First, *jointless* must be mapped and a sufficient number of molecular markers must be identified that are closely linked and flank both sides of the target gene. Secondly, the relationship between genetic and physical distance must be established to determine the feasibility of a chromosome walk or jump.

Penetrance of jointless *in the interspecific cross*

Accurate genetic mapping relies heavily on the ability to score unambiguously the phenotype of the target gene. The *jointless* mutation is very easy to score in segregating populations within *L. esculentum* (Butler 1936) but nothing was known as to its expressivity in a cross with the wild relative *L. pennellii*. Our results demonstrated that the *jointless* and jointed phenotypes were easily scorable in such an interspecific cross, as shown in Fig. 1. The penetrance for *jointless* appears to be near 100% in this cross.

This result is in sharp contrast to a similar interspecific cross with *jointless-2* (*L. esculentum j-2/j-2* × *L. pennellii*) where approximately 1000 F_2 plants were scored and in which no plant could be unambiguously scored as being *jointless* (unpublished data). *jointless-2* (Fig. 2A) is another single recessive mutation, non-allelic to *jointless*, that completely suppresses the formation of the pedicel abscission zone (Rick 1956). This result may indicate that *j-2* regulates a different step in the development of the abscission zone than *j*, and *L. pennellii* is not responsive to the *j-2* allele. Alternatively, *L. pennellii* may possess some modifier(s) that affect the expression of *j-2*.

Effectiveness of RAPDs for high resolution mapping

We initially mapped *jointless* to a 7.1 (+/− 1.8) cM interval between two RFLP markers on chromosome 11. To saturate this region with markers, we used a DNA pooling strategy and RAPDs to obtain two additional markers within this interval. One marker, RPD443 was not very useful because it cosegregated with TG194 and thus did not provide any additional genetic information. However, RPD158 mapped 1.5 (+/− 2.0) cM to the opposite side of *jointless*, relative to TG523, and thus reduced the 7.1 cM interval containing the *jointless* locus down to 3.0 (+/− 1.9) cM. This result clearly demonstrated that DNA pooling and RAPD techniques are effective for the isolation of additional markers for the purposes of physical mapping and eventual chromosome walking.

The frequency with which new markers were obtained by RAPD screening seems lower than expected since our previous studies with the same isogenic DNA pools permitted the isolation of one linked marker using 100 random decamer primers (Giovannoni et al. 1991). It is likely that some of the unmapped polymorphic bands containing repetitive elements may reside in the targeted interval. These polymorphisms are being verified by testing segregation of the polymorphic bands in our F_2 population. Such data could result in a greater number of new markers in the targeted interval.

Lower ratio of physical and genetic distance in the jointless *region*

The haploid DNA content of tomato is estimated to be approximately 950 Mb (Arumuganathan and Earle 1991) and the total genetic distance is 1276 map units (Tanksley et al. 1992). This means that on average 1 cM is equivalent to 750 kb. Physical mapping in the *jointless* region, comprising 3.0 (+/− 1.9) cM between markers TG523 and RPD158, however, provided an estimation for genetic and physical distance of approximately 200 kb/cM. This 3.5-fold reduction in genetic versus physical distance may reflect the fact that in this region of chromosome 11, there is less or no suppression of recombination. Recombination suppression has been suggested as a possible explanation of the approximate 6-fold increase in genetic and physical distance for the *Tm2a* region in tomato where 1 cM was estimated to be approximately 4000 kb (Ganal et al. 1989). The *Tm2a* gene was mapped to a heterochromatic region of chromosome 9 near the centromere. Heterochromatic and centromeric regions of tomato chromosomes often show recombination suppression (Rick 1971). The classical map position of *jointless* places it approximately 30 cM from the chromosome 11 centromere (Fig. 2A) indicating that the centromere is not likely to be involved in suppressing recombination around *jointless*. Further study by in situ hybridization mapping with cosmid (Trask et al. 1993) or YAC clones (Charlieu et al. 1993), isolated by hybridization to TG523 or RPD158, would permit a detailed analysis of the regional organization of the chromosome with respect to euchromatic, heterochromatic and centromeric DNA relative to the *jointless* locus. The significant variation in the correspondence of genetic and physical distance in different chromosomal regions further demonstrates the importance of physical mapping when using a map-based cloning strategy to isolate a gene.

Physical mapping with RPD158 also allowed for the linkage of markers TG194 and RPD443 with TG523 based on hybridization to two common bands in the *Mlu*I digest. *Mlu*I is a methylation sensitive enzyme and the multiple bands probably reflect incomplete methylation of the *Mlu*I sites. The *Mlu*I hybridization pattern seen for each probe was identical on two independent gels using different DNA preparations, which should eliminate any potential artifacts. In addition, the estimation of genetic and physical distance of approximately 130 kb in the entire 7.1 (+/−1.8) cM region is well within the estimation for that between TG523 and RPD158 or 200 kb/cM.

Chromosome walking – the next step

These genetic and physical mapping results provide the foundation for the next step toward the isolation of *jointless* by chromosome walking or jumping. The combined results demonstrate that *jointless* lies between TG523 and RPD158, which are genetically 3.0 (+/−1.9) cM apart, and physically a maximum of 600 kb apart. Recently, a complete YAC library has been constructed from tomato cultivars VFNT Cherry (jointed) and Rio Grande Pto *(jointless-2)* lines and has an average insert size of 150 kb (Martin et al. 1992). The most closely linked markers TG523 and RPD158 are currently being used to screen this YAC library. Preliminary evidence with TG523 has resulted in the isolation of seven positive YACs, which are currently being characterized (unpublished). Based on our physical mapping data and the average insert size of the tomato YAC library (Martin et al. 1992) we should be able to walk to *jointless* in two to four steps.

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