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Macrostructure of the Tomato Telomeres

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The macrostructure of the tomato telomeres has been investigated by in situ hybridization, genomic sequencing, and pulsed-field gel electrophoresis. In situ hybridizations with a cloned telomeric sequence from *Arabidopsis thaliana* indicated that the telomeric repeat of tomato cross-hybridizes with that of *Arabidopsis* and is located at all telomeres. Bal31 digestion kinetics confirmed that the tomato telomeric repeat represents the outermost DNA sequence of each tomato chromosome. Genomic sequencing of enriched tomato telomeric sequences, using primers derived from the *Arabidopsis* sequence, revealed that the consensus sequence of the tomato telomeric repeat is TT(T/A)AGGG compared with the *Arabidopsis* consensus sequence of TTTAGGG. Furthermore, as shown by pulsed-field gel electrophoresis, the telomeric repeat of tomato is separated by not more than a few hundred kilobases from a previously described 162-base pair satellite DNA repeat of tomato (TGR I) at 20 of the 24 telomeres. Together, these sequences are found in the heterochromatic terminal knob observed in pachytene chromosomes. Therefore, these two repeats determine the structure of 20 of the 24 tomato chromosome ends over approximately 2% of the total chromosome length.

INTRODUCTION

The recent cloning and characterization of the telomeres of two higher eukaryotes, *Arabidopsis* and human beings (Moyzis et al., 1988; Richards and Ausubel, 1988), have revealed that the structure and DNA sequence of most eukaryotic telomeres are very similar and widespread (Meyne et al., 1989). In most eukaryotic organisms studied to date, the telomere consists of many tandemly repeated copies of a basic oligonucleotide of the sequence $(T/A)_{1-4}$ G_{1-8}. As a result of this, there is strong inequality in the base composition of the two DNA strands because one is always G-rich and the other C-rich (Blackburn, 1984; Zakian, 1989). Telomeres show other unique characteristics apparently related to their function in maintaining the integrity and structure of eukaryotic chromosomes. One is their dynamic structure (increase and decrease in length over generations), which is a peculiar feature of telomeric replication because the telomeres are not able to replicate completely by the usual mechanisms of DNA replication (Bernards et al., 1983; Larson et al., 1987; Shippen-Lentz and Blackburn, 1990). Other telomeric features are the occurrence of terminal fold-back structures creating intramolecular guanine-guanine base pairing (Henderson et al., 1987) and single-stranded discontinuities (Blackburn and Challoner, 1984).

In many eukaryotes, telomeres are cytologically associated with other tandemly repeated DNA sequences. These sequences, called satellite DNAs, are often the most abundant DNA sequences with respect to copy number in a eukaryotic genome (Flavell, 1986; Miklos, 1986). Satellite DNA consists of long tandem arrays of individual repeat units ranging from a few base pairs to several kilobases in length and greatly varying in sequence from species to species (Flavell, 1986; Miklos, 1986; Schweizer et al., 1988). By in situ hybridization, it has been shown that satellite DNA is most often localized at or near telomeres (Bedbrook et al., 1980; Young et al., 1983; Barnes et al., 1985). However, it is not clear how close these sequences are to the absolute end of the chromosomes or whether they are part of the telomeric structure itself because in situ hybridization provides only limited resolution.

In tomato, we have recently shown that a 162-bp satellite DNA sequence (TGR I) is very tightly associated with the telomeres of 20 of the 24 chromosome arms (Ganal et al., 1988; Schweizer et al., 1988). By in situ hybridization, large clusters of TGR I ranging from 100 kb to 1000 kb in length are found to be indistinguishable from the chromosome ends (Lapitan et al., 1989). We have now characterized the DNA sequence of the tomato telomeres and used pulsed-field gel electrophoresis (PFGE) and a cloned telomere repeat from *Arabidopsis* (Richards and Ausubel, 1988) to investigate further the macrostructure and physical proximity of satellite DNA and telomeres in tomato.

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RESULTS

The observation that the telomeric repeat $(TTTAGGG)_n$ of *Arabidopsis* shows cross-hybridization to tomato DNA (Richards and Ausubel, 1988) was used to illuminate further the structure of the tomato chromosome ends. As shown in Figure 1, when in situ hybridizations of the TGR I repeat (Ganal et al., 1988; Lapitan et al., 1989) are compared with those of the telomeric repeat, two distinct observations can be made. First, the telomeric repeat hybridizes to all 24 chromosome ends. The signal, however, varies considerably between the ends of individual chromosome pairs. For example, the lower end of the smallest chromosome shows only a very weak signal that is difficult to see in most preparations, and the same is true for at least one weak interstitial site (see next section). The fact that this heterologous probe hybridizes with the ends of tomato chromosomes is consistent with previous evidence suggesting a high degree of sequence conservation in eukaryotic telomeres (Meyne et al., 1989). Second, the TGR I repeat hybridizes to only 20 chromosome ends; however, at these 20 ends, the position of the hybridization signal from TGR I is virtually indistinguishable from the position of the telomeric repeat.

Furthermore, the hybridization of pachytene chromosomes of tomato (Figure 1C) with the telomeric clone of *Arabidopsis* reveals that the telomere is localized in the terminal knobs of chromosome ends. TGR I also hybridized to most of the terminal knobs (not shown).

Bal31 Digestions Indicate That the Telomeric Repeat Represents the Outermost DNA Sequence of Each Tomato Chromosome

Although the in situ hybridizations indicate that the telomeric repeat of *Arabidopsis* hybridizes with a repeat near the ends of all tomato chromosomes, direct evidence that this sequence is at the outermost end of the tomato chromosomes comes from susceptibility of this repeat to digestion by the exonuclease Bal31. Because all other chromosomal sequences are not at the ends of chromosomes, they should not be affected by the Bal31 digest. High molecular weight DNA isolated from protoplasts (size range approximately 2 million bp to more than 5 million bp) was digested for different lengths of time with Bal31, cut with HaeIII, and separated on pulsed-field gels. When this DNA was probed with the telomeric repeat from *Arabidopsis*, a clear shift toward the lower molecular weight was observed with increasing periods of incubation, as shown in Figure 2A. No such shift was observed when the same gel was probed with an interstitial single copy probe (Figure 2B). We also observed at least one band (approximately 16 kb) that hybridized to the *Arabidopsis* telomere but was not affected by the Bal31 digestions. This band is most likely generated by an interstitial site confirming in situ hybridization data that indicate that there is at least one weak interstitial site. These results, combined with the in situ hybridization shown earlier, indicate strongly that the tomato telomeres cross-hybridize with the telomeric repeat

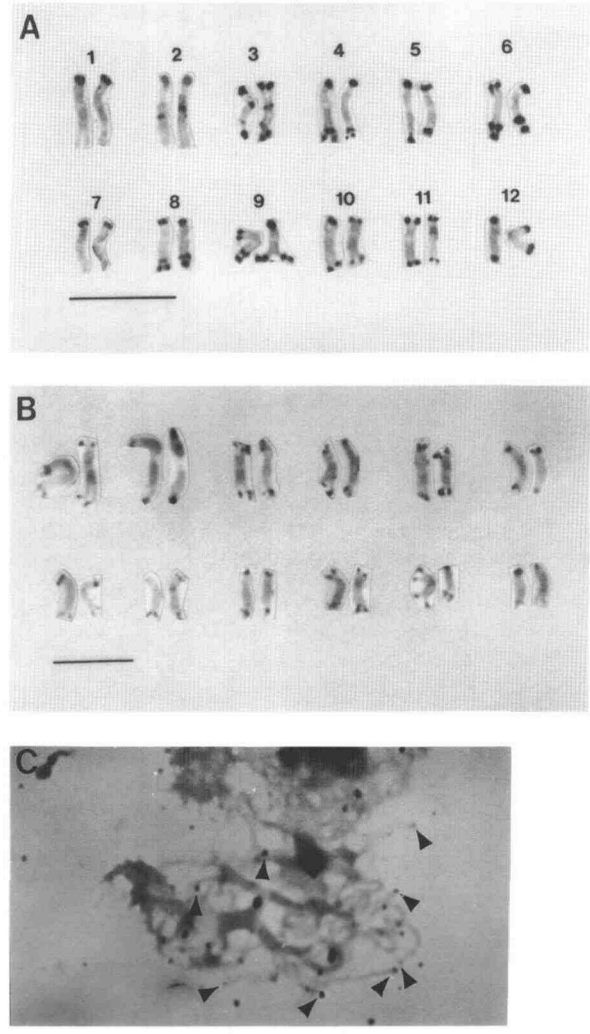

Figure 1. In Situ Hybridization of Tomato Metaphase and Pachytene Chromosomes with TGR I and pAtT4.

Biotin-labeled inserts of **(A)** TGR I and **(B)** pAtT4 were hybridized to mitotic metaphase chromosomes of tomato.

(A) Chromosomes are shown as published in Lapitan et al. (1989) according to chromosome number, previously determined using trisomic lines.

(B) Chromosomes are arranged according to size (except chromosomes 1 and 2, which can be identified unambiguously by their morphology).

(C) Hybridization of pAtT4 to meiotic pachytene chromosome spreads of tomato. Arrowheads indicate some hybridization sites at the telomeric knobs.

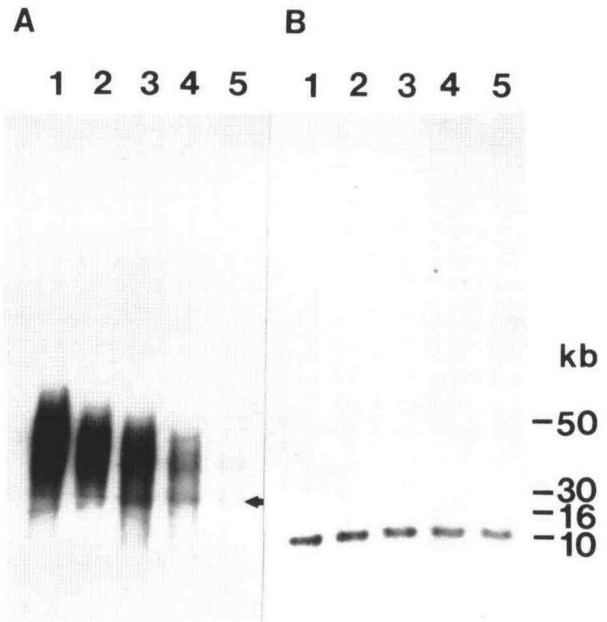

Figure 2. Bal31 Digestion Kinetics of Tomato DNA.

Tomato DNA was digested with Bal31 for 0 min (1), 20 min (2), 40 min (3), 60 min (4), and 80 min (5). The DNA was then cut with HaeIII and separated on a CHEF gel. The gel was then blotted and hybridized as shown in **(A)** and **(B)**.
(A) Gel hybridized with the telomeric repeat of pAtT4.
(B) Gel hybridized with the interstitial single-copy clone ZP 439.
The indicated size markers are in kilobases. The arrow indicates an interstitial fragment that is not affected by the Bal31 digestion and, therefore, serves as an additional internal control.

of *Arabidopsis* and comprise the most terminal sequences of the tomato chromosomes.

When tomato DNA is digested with any restriction enzyme (until now we have used at least 20 different enzymes) and probed with the telomeric repeat from *Arabidopsis*, the vast majority of the hybridization signal is more than 30 kb. For example, DNA that is digested with HaeIII and separated on high resolution pulsed-field gels shows a clear signal ranging from approximately 30 kb to 60 kb in size, as shown in Figure 2. As in other organisms, the terminal restriction fragments appear as heterodispersed bands (de Lange et al., 1990), suggesting that the length of individual telomeric fragments varies considerably between different tomato chromosome ends. This also confirms the in situ hybridization data that show a very strong signal at some chromosome ends and a weak signal at others.

Another important point is that after 60 min to 80 min of Bal31 digestion, most of the hybridization signal from the telomeric repeat is lost (Figure 2A). However, after the same period of Bal31 digestion, the average size of the fragments to which the telomeric repeat hybridizes drops only from approximately 40 kb to 30 kb. If these telomeric fragments were composed entirely of sequences homologous to the *Arabidopsis* telomeric repeat, one would expect them to continue hybridizing until the fragments were entirely degraded. Therefore, these results suggest that the tomato telomeric fragments contain only approximately 10 kb of TTTAGGG homologous repeats. The remainder (approximately 30 kb) is likely to be composed of other sequences in tandem arrays. These putative additional repeats must be of very limited complexity because we have been unable to find a restriction enzyme that can separate them from the tomato telomeric repeat. Similar results and conclusions have also been made recently for human telomeres (Allshire et al., 1989; de Lange et al., 1990).

Genomic Sequencing of the Tomato Telomeric Repeat

The abundance of the telomeric repeat combined with the absence of restriction enzyme sites in the telomeric DNA fragment made it possible to isolate genomic DNA highly enriched for this sequence. High molecular weight DNA was cut with EcoRI and separated on 0.7% agarose gels. DNA >50 kb was eluted and found to be highly enriched for the telomeric repeat along with the TGR I repeat, both of which have no EcoRI site (Schweizer et al., 1988). Genomic sequencing was performed on this enriched fraction using primers (see Methods) derived from the *Arabidopsis* sequence and resulted in a consensus sequence for the tomato telomeres. Figure 3 shows a comparison of the sequences obtained from genomic sequencing of the tomato telomeres as well as the insert of pAtT4 of *Arabidopsis* (Richards and Ausubel, 1988), which was used as a control. The entire telomeric repeat of tomato and *Arabidopsis* was sequenced from one strand. For the other strand, only 4 bases out of the 7-bp repeat could be determined unambiguously because the Klenow DNA polymerase was not able to copy accurately that strand as well in tomato as in *Arabidopsis*. However, the information from this strand confirms the results from the other strand. Other enzymes tested also failed to copy this strand. The combined data, however, show clearly that the sequence of tomato, $[TT(T/A)GGG]_n$, is nearly identical to the *Arabidopsis* consensus sequence $(TTTAGGG)_n$.

Determination of the Physical Proximity of the TGR I Repeat and the Telomeric Repeat by PFGE

In situ hybridizations provide only approximate physical locations of sequences on chromosomes. Usually, a separation of several million base pairs is necessary to discriminate the localization of two different probes (Saiga and Edström, 1985). In contrast, the technique of PFGE

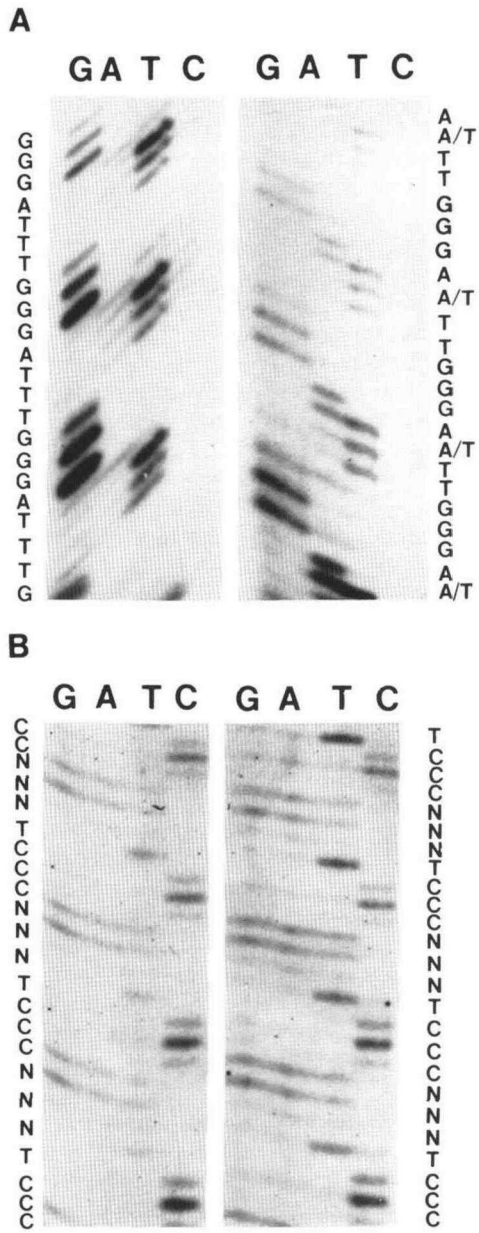

Figure 3. Genomic Sequencing of the Tomato Telomeres.

DNA enriched for telomeric repeats from tomato (right panel) was sequenced directly, resulting in a consensus sequence. Simultaneously, the same procedure was applied to the insert of the *Arabidopsis* clone pAtT4 as a control (left panel).

(A) *The G-rich strand.*

(B) The C-rich strand, which was not completely analyzable by this technique because of incorrect termination.

(Schwartz and Cantor, 1984; Carle et al., 1986) allows the resolution of DNA fragments up to several million base pairs, thus making it possible to investigate the structure and juxtaposition of sequences like TGR I and the telomeric repeat. Considering this, high molecular weight DNA, isolated from protoplasts, was cut with a number of restriction enzymes for which no sites exist in the telomeric repeat or the TGR I repeat. The digested DNA was subsequently separated on PFGE gels, blotted, and successively probed with the TGR I repeat and the cloned *Arabidopsis* telomeric repeat. The results are shown in Figure 4.

When DNA was cut with EcoRV or BglII, most clusters of TGR I, ranging from 25 kb to 1000 kb in size, were separable from fragments containing the telomeric repeat. The majority of the fragments containing the telomeres were found in the size range of from 50 kb to 150 kb. Because the average fragment size of tomato DNA cut with BglII and EcoRV is approximately 10 kb (J. Miller, P. Broun, and S.D. Tanksley, unpublished results), this indicates that in the majority of the observed cases the telomeric repeat clusters and the TGR I clusters are separated by more than that distance. Only four BglII fragments and seven EcoRV fragments hybridized both to the telomeric repeat and the TGR I repeat, indicating that they are physically tightly linked to each other. The possibility of accidental concurrence can be excluded for that size range because BglII and EcoRV cut most tomato DNA into

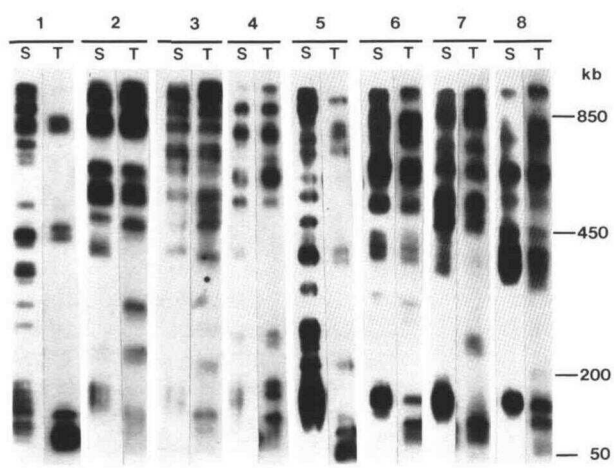

Figure 4. Hybridization of Filters from Pulsed-Field Gels with the TGR I Repeat and the Telomeric Repeat from *Arabidopsis*.

High molecular weight DNA from tomato was digested with BglIII (1), MluI (2), ClaI (3), SalI (4), EcoRV (5), PvuII (6), SmaI (7), and SfiI (8). The DNA was then separated on field inversion gels for a size range of up to 1 million bp, blotted, and subsequently probed with the TGR I satellite repeat (S), and the telomeric repeat (T) from pAtT4.

very small fragments, and when the pulsed-field gel is overloaded, individual bands can be seen in the size range of 300 kb to 1000 kb on ethidium bromide-stained gels (data not shown). In addition, the four BglII fragments that showed hybridization to both repeats could not be further dissected in double digestions with BglII and EcoRV, indicating a very tight association of the telomeric repeat and the TGR I arrays on these fragments.

The use of restriction enzymes like SalI, ClaI, MluI, PvuII, SfiI, and SmaI, which generate much larger genomic tomato fragments (Ganal and Tanksley, 1989), provided an estimate for the distance between the TGR I repeat and the telomere. These enzymes are not able to separate the telomeric repeat from the TGR I repeat in most cases. Only a few bands, especially in the MluI, SmaI, and PvuII digestions, showed exclusive hybridization to either the telomeric or the TGR I repeat. However, this was expected because four chromosome ends do not contain a TGR I repeat and the TGR I repeat is also found at a few interstitial sites away from the telomeres. Together, these data indicated that in these cases the distance between the telomeric repeat and the TGR I repeat is not more than several hundred kilobases.

DISCUSSION

Until recently, studies on the macrostructure of eukaryotic chromosomes have been limited to the techniques of cytogenetics. Although such studies have provided valuable insight into chromosome behavior, they have not produced a much-needed molecular description of chromosome organization. Until such a picture emerges, it is unlikely that significant progress will be made in understanding chromosomal architecture and function. The advent of molecular biological techniques, including PFGE, now offers the opportunity to study chromosome organization with a degree of resolution previously not possible.

In this report, we provide a molecular description of the ends of tomato chromosomes. Based on in situ hybridization, it is concluded that both the species-specific 162-bp TGR I repeat and the 7-bp telomeric repeat are located at the ends of most tomato chromosomes. Bal31 digestion kinetics showed that of these two repeats, the telomeric repeat is the outermost of all 24 chromosome arms. The sequence of the telomeric repeat (derived from genomic sequencing) is very similar to telomeric sequences published for animals and nearly identical to that of *Arabidopsis*. The only difference between the tomato repeat and the *Arabidopsis* repeat was detected at the fifth position. In tomato, this position can be occupied by either an A or T $[TT(A/T)GGG]_n$, whereas in *Arabidopsis*, the consensus sequence is reported to be TTTAGGG. The reason for the heterogeneity in this position in tomato is unknown. It is possible that within a cluster (at a single telomere) all of the repeats are identical and that the difference in the fifth position represents differences between chromosomes or chromosome arms. On the other hand, we cannot rule out that the two different variants of this repeat are interspersed within the same cluster. It is worth noting that the *Arabidopsis* sequence was derived from sequencing a single telomeric clone and also showed minor sequence heterogeneity (Richards and Ausubel, 1988), whereas the template for genomic sequencing in tomato represented a population of repeats from all of the tomato telomeres.

Although the sequence of the tomato telomeric repeat is very similar to that of other animal and plant species, the length of the terminal restriction fragment is dramatically different from most other organisms. Digestion with many restriction enzymes (including numerous 4-bp recognition site enzymes) yields fragments of at least 30 kb to 60 kb in size, suggesting that simple sequences with short repeated motifs occupy most of that stretch of DNA. This is much longer than the several kilobases for *Arabidopsis* (Richards and Ausubel, 1988). The Bal31 digestion kinetics suggest that the tomato telomeric repeat represents only a portion of that 30-kb to 60-kb region and that the remainder is occupied by other sequences. This is in agreement with data from a number of other organisms (Zakian, 1989). At the moment, it is not clear whether these sequences are variations of the telomeric motif (Allshire et al., 1989) and/or other sequences (de Lange et al., 1990).

The TGR I repeat is located at 20 of the 24 telomeres and is very tightly associated with the telomeres. This repeat of 162 bp itself represents 1.75% of the tomato genome (Ganal et al., 1988). By use of PFGE, we have been able to deduce the long-range physical structure and juxtaposition of these two elements. The telomere represents the outermost DNA element of a tomato chromosome arm, and at 20 of the 24 arms, this repeat is separated by not more than several hundred kilobases of DNA from the TGR I repeat, which itself extends up to 1000 kb at each location.

At the level of in situ hybridization and banding techniques, association of telomeres with satellite DNA has been observed in a large number of species (Flavell, 1986; MacGregor and Sessions, 1986; Miklos, 1986). The reasons for the frequent associations between satellite DNA and telomeres are not clear and have been the subject of much speculation. The telomeric position of these sequences might favor recombination between satellite arrays on nonhomologous chromosomes and lead to interchromosomal homogenization of the chromosome ends (Dover, 1982; Flavell, 1986). Interchromosomal recombination would be permitted at the ends of chromosomes because it would not lead to deleterious products in the progeny of such a recombination event and would result in sequence homogeneity among chromosome ends. In

support of this, the TGR I repeat is very homogeneous, and it has not been possible to identify chromosome-specific variants, as reported for the centromeric human α satellite (Miklos, 1986; Waye et al., 1987).

Finally, the close physical association of the tomato telomeres with the TGR I satellite repeat is very similar to some telomeric structures in lower eukaryotes (Zakian, 1989). For example, at most chromosome ends of *Plasmodium*, a telomeric complex has been identified (Corcoran et al., 1988). In this case, the telomeric repeat is also in association with a species-specific repeated DNA sequence, rep20, at most but not all of the chromosome ends, and it is assumed that there is homologous recombination between the individual clusters creating chromosome length polymorphisms and deletions of rep20 that result in chromosome ends lacking this sequence. Therefore, it is possible that the structure of the tomato telomeres described here is a general and widespread feature in eukaryotes.

The molecular description of the structure of most tomato telomeres may allow the development of techniques for the cloning and mapping of DNA sequences at or very close to the telomeres, an accomplishment necessary to complete the restriction fragment length polymorphism map of the tomato genome. This can be done, for example, by utilizing the demonstrated enrichment procedure for tomato telomeres and subsequent cloning of the telomeres and their flanking sequences in yeast artificial chromosomes (Riethmann et al., 1989) or by direct mapping of the TGR I clusters adjacent to the tomato telomeres using segregation analysis on PFGE gels (M.W. Ganal, K. Wu, P. Broun, and S.D. Tanksley, manuscript in preparation).

METHODS

Plant Material and Clones

Lycopersicon esculentum cv VFNT cherry was used for all experiments in the characterization of the tomato telomeres. Tomato nuclear DNA was isolated as previously described (Bernatzky and Tanksley, 1986). The clone pAtT4 (Richards and Ausubel, 1988) containing the telomeric repeat from *Arabidopsis thaliana* was used as a probe for the tomato telomeres. The tomato satellite repeat TGR I was detected by a clone containing one repeat (Schweizer et al., 1988). ZP 439 is an anonymous cDNA clone that was selected for its large HaeIII fragment out of approximately 500 single-copy clones.

Chromosome Preparation and in Situ Hybridization of Tomato Metaphase and Pachytene Chromosomes

Metaphase chromosomes were prepared from root tips as described previously (Ganal et al., 1988; Tanksley et al., 1988). Pachytene chromosome spreads were prepared from immature flower buds fixed in 3:1 ethanol:glacial acetic acid. Inserts of the TGR I repeat and the telomeric clone pAtT4 were labeled with biotin-dUTP by random hexamer labeling as previously described (Feinberg and Vogelstein, 1983; Rayburn and Gill, 1985) and used for in situ hybridizations. The pAtT4 insert was additionally labeled with biotin-dCTP to achieve labeling of both strands due to the asymmetry of telomeric sequences. Enzymatic detection of the hybridization signal was as described (Tanksley et al., 1988). Photographs were taken with an Olympus Vanox AHBT photomicroscope using Kodak Tech Pan 2415 film. For each experiment, approximately 10 metaphase or pachytene spreads were analyzed in detail.

DNA Isolation and Bal31 Digestion Kinetics

High molecular weight DNA was isolated from protoplasts. The DNA in agarose blocks was then washed as described (Ganal and Tanksley, 1989) and subsequently equilibrated in 100 mM NaCl, 10 mM EDTA, pH 7.5. After melting the blocks at 65°C for 10 min, the solution was cooled to 37°C and incubated with 50 units of agarase (Calbiochem) per milliliter of solution. After 12 hr at 37°C, another 25 units of agarase per milliliter of solution were added and the incubation continued for 6 hr. Then the DNA in solution was gently phenol extracted and dialyzed against 10 mM Tris-HCl, pH 8, 1 mM EDTA (Anand et al., 1989). This DNA was found to be approximately 2 million bp to more than 5 million bp in length on pulsed-field gels. For digestion kinetics, the DNA was incubated at 37°C for 0 min, 20 min, 40 min, 60 min, and 80 min using 10 units of Bal31 (Boehringer Mannheim) in a volume of 30 μL. The reaction was stopped by the addition of EGTA to a final concentration of 20 mM. After gentle extraction with phenol/chloroform, the DNA was dialyzed and used for restriction enzyme digestions. The DNA was separated on 1% agarose gels using a CHEF system (Chu et al., 1986) and a switch time of 10 sec and transferred to GeneScreen *Plus* (Du Pont-New England Nuclear), as described elsewhere (Ganal et al., 1988).

Isolation and Genomic Sequencing of the Tomato Telomeres

Two hundred fifty micrograms of total tomato DNA were digested with EcoRI and separated on 0.7% low melting agarose (Bethesda Research Laboratories) gels. A strip of the gel was stained with ethidium bromide and then the region containing fragments of more than 30 kb was excised from the unstained gel. After equilibration in 100 mM NaCl, 10 mM EDTA, pH 8, the gel piece was melted, treated with agarase, and extracted with phenol/chloroform, as described above. After that, it was dialyzed against 10 mM Tris-HCl, pH 8, 1 mM EDTA and finally precipitated with ethanol. The genomic sequencing was performed using the double-strand sequencing methods described for plasmids using the Klenow fragment of DNA polymerase (Sambrook et al., 1989). As a control, isolated insert of pAtT4 was used in the same way. As primers, we used GGTTTAGGGTTTAG for the G-rich strand and CCTAAACCCTAAAC for the C-rich strand, although other primers with different 3′ ends resulted in the same sequencing pattern. In most cases, the primers were end labeled with γ-^{32}P-ATP because of the asymmetric structure of the telomeres. Separation was on standard sequencing gels.

Pulsed-Field Gel Electrophoresis

Very high molecular weight DNA (average size more than 5 million bp to 10 million bp) was isolated from protoplasts and digested

with restriction enzymes (Ganal and Tanksley, 1989). Separation of large DNA fragments was performed on a field inversion gel system (Carle et al., 1986), as described earlier (Lapitan et al., 1989), or on a CHEF system (Chu et al., 1986). Blotting and hybridization procedures have been described in detail by Ganal and Tanksley (1989).

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