Restriction Enzyme-Resistant High Molecular Weight Telomeric DNA Fragments in Tobacco

Katsunori Suzuki, Yoko Yamagiwa, Tsunehiro Matsui, and Kazuo Yoshida

Department of Biological Science, Faculty of Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima 724, Japan

(Received 22 April 1994)

Abstract

Restriction endonuclease-resistant high-molecular-weight (HMW) DNA fragments were isolated from nuclear DNA fragments in tobacco. The size of the fragments produced by EcoRI, HindIII, AfaI, and HaeIII ranged from 20 kb to over 166 kb. The kinetics of digestion by Bal31 nuclease showed that most of the HMW fragments are chromosome ends. The consensus sequence for tobacco telomere repeats was determined to be CCCTAAA by genomic sequencing using the HMW fragments and by sequencing after cloning. Besides the telomere sequence, 9 tandem repeats of a 45-bp sequence were identified, in which a 35-bp unit sequence (AGTCAGCATTAGGGTTTTAAACCCTAAACTGAACT) formed a stem structure. The front of the stem is composed of a palindrome of the telomere repeats. This highly conserved unit is surrounded by less conserved internal sequences that are around 10-11 bp in size and contain a TTTT stretch. The internal sequences resemble the 10-11 bp consensus for the scaffold attachment regions found in yeast and drosophila. The characteristic 45-bp sequence was abundant on the ends of chromosomes. The shortest distance between the repeats containing telomeric stem and the telomere was less than 20 kb. This architecture of the tobacco chromosome end region resembles the end region of yeast chromosomes in which autonomous replication sequences are present frequently.

Key words: telomere; telomere associated sequence; plant chromosome; palindrome of telomere sequence; Nicotiana tabacum

1. Introduction

Telomeres are the outermost ends of eukaryotic chromosomes; there are G-rich terminal repeat sequences at the very end of chromosomes. So far, much of our understanding about the function and synthesis of telomeres and telomere-associated sequences has come from research in ciliates and yeast Saccharomyces cerevisiae. The biochemical aspects of human telomeres have also been clarified. In contrast, the current knowledge of higher plant telomeres is limited although the importance of telomeres has been well recognized cytogenetically in plants since the early work by McClintock.

Among higher plants, telomere sequences were cloned in Arabidopsis thaliana by Richards and Ausubel. The repeat unit is CCCTAAA in the plant. Although in situ studies revealed that there are homologs of the repeat in telomeric regions in several plants, the exact sequences have not yet been reported.

In general, simple telomere repeats contained no recognition site for restriction endonucleases. It was reported that human telomeres tend to resist enzymatic digestion possibly by hyper-methylation. According to Wagner and Capesius, tobacco genomic DNA is rich in methylated nucleotides. Thus, it seems likely that restriction endonuclease-resistant long sequences are present in tobacco telomeres. This study was performed to purify telomere fragments, and to examine telomeres biochemically.

During the analysis of tobacco DNA, we found a satellite signal in endonuclease digests of the DNA. We report here the isolation and partial characterization of telomeric DNA fragments from tobacco nuclear DNA cleaved with common restriction endonucleases.

2. Materials and Methods

2.1. Plant DNA preparation

Tobacco nuclear DNA was isolated from young leaves of Nicotiana tabacum c.v. SR-1. Nuclei were prepared...
from 15 g of leaves as described previously. The nuclei were suspended and then lysed by adding a solution of sarkosyl. Into 28 ml of the lysed nuclei solution, 30 g of powdered CsCl was added and dissolved. After ethidium bromide (EtBr) was added at low concentration (final 10 μg/ml) into the CsCl solution, ultracentrifugation at 40,000 rpm for 3 hr was undertaken for banding DNA. The resultant DNA band was transferred to a new tube and then the CsCl isopycnic centrifugation was repeated. To minimize shearing damage, the standard purification methods using CsCl centrifugation were modified as follows. The powdered CsCl was prepared by grinding CsCl with a mortar and pestle as finely as possible. Not only the EtBr concentration limited in the above processes but EtBr extraction after centrifugation was performed as gently as possible: a layer of n-butanol was gently pipetted on top of the DNA solution in a beaker and then stood for half a day at room temperature to extract EtBr. The extraction with butanol was repeated 3–4 times until EtBr was not detected visibly. CsCl in the solution was removed by dialysis at 4°C.

2.2. Electrophoresis

Standard agarose electrophoresis was performed essentially as described previously. Pulsed-field (CHEF) gel electrophoresis was accomplished using a Pulser system (Pharmacia, Uppsala, Sweden) with 1% agarose gel in 0.5x TBE buffer [45 mM Tris/45 mM boric acid/1 mM EDTA (pH 8.3)] at 12°C and 30 sec switching at 200 V.

2.3. Southern hybridization

DNA in agarose gel was transferred to Hybond N+ membrane (Amersham, Buckinghamshire, UK). Alkaline transfer of DNA from gel to the membrane was done essentially as previously reported using a vacuum blotting apparatus with 0.4 N NaOH as a transfer medium after acid depurination. Filter hybridization was carried out essentially according to the method of Maniatis et al. Washing conditions were as follows: 0.1 × SSC/0.1% SDS at 60°C with probes labeled by the random priming method, 0.5 × SSC/0.1% SDS at 40°C with end-labeled oligonucleotide probes, and 6 M urea/0.1 × SSC/0.4% SDS at 42°C with probes prepared by chemical crosslinking with peroxidase.

2.4. Exonuclease treatment

Tobacco DNA was incubated at 30°C in 500 μl of a reaction mixture containing 14 units of Bal31 nuclease (Takara, Kyoto, Japan), 20 mM Tris-HCl (pH 8.0), 12 mM MgCl₂, 12 mM CaCl₂, 0.6 M NaCl, 1 mM EDTA, 100 mg/ml BSA and substrate DNA. To stop the enzyme reaction aliquots were removed and transferred to ice-chilled tubes with EGTA, then treated with phenol and chloroform to inactivate the enzyme.

2.5. DNA cloning

Enrichment of telomeric DNA and cloning processes are outlined in Fig. 4. Isolated plant DNA fragments were sonicated for 5 sec at low energy setting and the resulting fragmented DNA was made flush-ended by using T4 DNA polymerase according to the manufacturer’s instructions (Takara). The flush-ended short DNA was ligated with SmaI-cleaved dephosphorylated vector pUC19. An E. coli strain SURE (λ⁻, mcrA, Δ(mcrBC-hsdRMS-mrr)171, supE44, thi-1, gyrA96, relA1, recB, recJ, sbcC, umuC::Tn5(kan'), uvrC, Δlac, [F, proAB, lacIqZAM15, Tn10(tet')], Strategene, La Jolla, CA, USA) was used as a transformation host strain. Phagemid vectors pEMBL18 and pEMBL19, and an E. coli strain XL-1Blue (λ⁻, endA1, hsdR17, supE44, thi-1, relA1, gryA96, relA1, Δlac, [F, proAB, lacIqZAM15, Tn10(tet')], Strategene) were used for subcloning. Plasmid transformation was done according to the method of Inoue et al.

2.6. DNA sequencing

Sequencing was carried out essentially by the dideoxy chain termination method. For genomic sequencing of the tobacco telomere, primer oligonucleotides were end-labeled by T4 polynucleotide kinase with [γ-32P]ATP. The primers used were GGTTTAGGGTTTAGGG for sequencing the G-rich strand and (CCCTAAA)₃ for sequencing the C-rich telomere sequences. After AflII digestion, the HMW DNA fragments were isolated and then denatured by boiling, and the DNA was used as a template. Extension and termination by Bac BEST DNA polymerase was done according to the manufacturer’s instructions (Takara). The resultant reaction products were separated on a standard denaturing 8% acrylamide gel and analysed by autoradiography. Fluorescently labeled universal primers were used for sequencing of plasmids, and the resultant reaction products were analyzed by an automatic DNA sequencer 373A (Applied Biosystems, Foster City, CA, USA).

3. Results

3.1. Restriction enzyme-resistant high-molecular-weight DNA fragments were detected in tobacco nuclear DNA

A slowly migrating substance was visible in gel electrophoresis of restriction endonuclease digests of tobacco nuclear DNA (Fig. 1A). When the EcoRI digest of tobacco nuclear DNA was applied to the standard agarose
gel electrophoresis, a weak but distinct signal was detected near the 23-kb marker DNA, as a minor signal slightly above the major signal. In HindIII-digested nuclear DNA, similar signal was also detected. The signal at around 23 kb remained and was more clearly visible when using 4-base-recognition endonucleases such as Afal and HaeIII because almost all tobacco nuclear DNA was digested by the enzymes into short fragments less than 6 kb in size.

In general, long DNA is very sensitive to shearing force. The HMW substance disappeared when Afal-digested DNA was treated by short-time sonication (data not shown). The HMW substance in Afal-digested nuclear DNA was extracted from gel bands of agarose electrophoresis. The isolated HMW substance acted as a template for a DNA polymerase. These results indicate that the HMW substance is long DNA. To determine the size, the HMW substance was applied to the pulsed-field gel electrophoresis. As shown in Fig. 1B, the HMW DNA fragments ranged from 20 kb to over 166 kb.

Using isolated HMW DNA fragments, Southern blot hybridization experiments were carried out. HMW DNA fragments isolated from Afal digests were sonicated, labeled and then used as a hybridization probe. In the Afal digest (lane 1 in Fig. 2), most of the signal was positioned at the HMW fragments. In the smaller molecular weight region, periodical ladders of signal were detected. The HaeIII digest (lane 2 in Fig. 2), showed a signal around 0.3 kb and at the HMW fragments, but other signals were not detected.

Figure 1. Agarose gel electrophoresis of tobacco nuclear DNA digested with endonucleases. Tobacco DNA was digested with endonucleases (lane 1: EcoRI, lane 2: HindIII, lane 3: Afal, lane 4: HaeIII). The digests were size-fractionated by electrophoresis. (A) Standard agarose gel electrophoresis and (B) CHEF electrophoresis followed by ethidium bromide (EtBr) staining.

Figure 2. Genomic southern hybridization with high-molecular-weight (HMW) DNA fragments as a probe. Tobacco nuclear DNA was digested with Afal (lane 1) and HaeIII (lane 2). The digests were size-fractionated by the standard agarose gel electrophoresis and subsequently transferred to a nylon membrane. The membrane was probed with isolated Afal HMW fragments labeled with peroxidase.
were scarce. This suggests that HaeIII-cleavable sites are present only near the \textit{AfaI}-cleaved end in most of the \textit{AfaI} HMW fragments.

3.2. Telomere sequences are situated on \textit{Bal31} hypersensitive HMW DNA fragments

The \textit{Bal31} nuclease acts as an exonuclease for double-stranded DNA. Nuclear DNA was treated with \textit{Bal31} and then digested with \textit{AfaI}. As shown in Fig. 3, the 10-min \textit{Bal31} treatment reduced the molecular length much but enhanced the EtBr fluorescence signal of the HMW fragments. During 10–100 min, the size and intensity of the signal decreased with the reaction time. This hypersensitivity indicates that the HMW substance is DNA and suggests that most of them are situated on chromosome ends. This idea was supported by the following experiments that are outlined in Fig. 4.

To determine whether the HMW fragments are really situated at chromosome ends, tobacco telomere sequences were examined. The telomeric sequence of \textit{Arabidopsis} was used to synthesize an oligonucleotide probe (CCCTAAA)\textsubscript{3} for screening tobacco telomeric DNA. The Southern blot signal was clearly detected at the HMW fragment position. When the nuclear DNA was pretreated with \textit{Bal31} nuclease, the signal by the telomere sequence was decreased with the reaction time (Fig. 5B). After 10 min of incubation, the size was decreased considerably, but the signal intensity was enhanced. Thereafter, the size gradually decreased with time. These phenomena during the 40-min treatment are consistent with the results obtained by the EtBr-induced fluorescence signal data in Fig. 3. During 10–100 min of treatment, the intensity of the telomere signal decreased rapidly. In these incubation periods, the size and intensity of the rDNA signal was not much affected (Fig. 5A).

The size of the HMW fragments was from 20 kb to over 166 kb. If telomere repeats are found in almost all of the sizes of the HMW fragments, the hybridization signal should extend to shorter DNA region as a result of \textit{Bal31} treatment; however, telomere signals were scarcely visible in the region less than 20 kb. Therefore, the large part of the HMW fragment contains other sequences.

3.3. Telomere repeat unit is CCCTAAA

To identify what segments are localized at chromosome ends and homologous with the \textit{Arabidopsis} telomere repeat sequence, two approaches were undertaken: 1) cloning and sequencing the chromosome end sequences and 2) genomic sequencing.

Tobacco chromosome end DNA fragments are highly concentrated at the HMW fragments. At first, isolated HMW fragments were sonicated to reduce the fragment length. The sonicated DNA was treated with T4 DNA polymerase to make them flush-ended. Subsequently, they were ligated with \textit{SmaI}-cleaved plasmid vector DNAs. The ligation products were transformed into an \textit{E. coli} strain that is defective in recombination, restriction and repair to protect recombinants against rearrangement. DNA preparations at respective steps were used as templates, probes and substrates.
Figure 5. Bal31 digestion kinetics of tobacco telomere sequence. Tobacco nuclear DNA was treated with Bal31 nuclease (lane 1: 0 min, lane 2: 10 min, lane 3: 20 min, lane 4: 40 min, lane 5: 70 min, lane 6: 100 min). (A) The Bal31-treated DNAs were digested with EcoRV, and then size-fractionated by standard electrophoresis. (B) The Bal31-treated DNAs were digested with Afal, then size-fractionated by CHEF electrophoresis. The DNAs blotted to membranes were probed with labeled rice rDNA plasmid pRR217 (a gift from Dr. F. Takaiwa, see Takaiwa et al.27) (A) and oligonucleotide (CCCTAAA)₉ (B).

(A) pTtell

CTAACCCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAAAACCCTAA

Figure 6. Nucleotide sequence of tobacco telomere DNA clones. Tobacco sequences were described as strands extending from an universal primer (TGTAAGACGGCCAGCCAGT) in pUC19 vector. The nucleotide sequence in pTtell (A) and nucleotide sequences in four plasmids written in an abbreviated form (B).

(B)

| Plasmid | Sequence               |
|---------|------------------------|
| pTtell1 | CTAAACCCCTAAAACCCTAAA  |
| pTtell2 | GGG(TTGGG)₅₆TTGG       |
| pTtell3 | CCTAAACCCCTAAAACCCTAAA |
| pTtell4 | TAAATCCCTAAA₃₃ACCCTAAA |

above. Five positive clones were isolated, and four of the plasmids were sequenced. As shown in Fig. 6, inserts of all 4 clones composed entirely of tandem repeats of CCCTAAA.

The telomere sequences were also examined directly using isolated HMW fragments as template DNA. The primers used were ³²P-end labeled oligonucleotides synthesized based on the sequence of Arabidopsis telomeres.⁶
Figure 7. Genomic sequencing of tobacco telomere sequence. Tobacco telomeres were sequenced with labeled oligonucleotides as primers. IsolatedAfal HMW fragments were used as a template (A, C). Telomere plasmid pTtel2 (see Fig. 6) as template (B, D, and E) and a universal sequencing primer (CCCAGTCACGACGTTGT) (E) were also used as control experiments. (A, B) C-rich strand and (C, D) G-rich strand.

Using a primer for the C-rich strand, the tobacco telomere sequence was shown to be tandem repeats of CCCTAAA. The nucleotide signals other than C were also seen at the second position in the repeat (Fig. 7A), but the second nucleotide was thought to be C because the C signal was the strongest and ambiguous signals were also seen at the same position in the control template pTtel2 that contains the CCCTAAA repeats (see Fig. 6). Using a primer for the G-rich complementary strand, the tobacco telomere sequence was found to be tandem repeats of NTTAGGN (Fig. 7C). This datum has ambiguity at 2 nucleotides in the heptanucleotide repeat unit. This is not simply due to the polymerase used for the reaction, because the control template containing TTAGGG repeats (pTtel2) was exactly sequenced by using the same primer (Fig. 7D and E). There are many possible explanations for this including: (1) similar sequence not at the telomere but at telomere-associated regions in HMW fragments is recognized by the G-rich primer; (2) nucleotide modifications at the telomere cause the polymerase to incorporate incorrect dideoxynucleotides when reading the C-rich template. At present, we could not determine the cause of the ambiguity. Regardless, the latter datum supports heptanucleotide repeats of the former datum. These data along with the data from plasmid clones indicate that the consensus sequence for tobacco telomeres is tandem repeats of CCCTAAA.
3.4. Palindromes of telomeric sequences are present in HMW DNA fragments

The kinetics of Bal31 digestion shows that DNA sequences other than the tandem CCCTAAA repeats should exist on the HMW fragments. We tried to isolate the DNA segments. After short fragments derived from sonicated A\textit{f}al HMW fragments were cloned into \textit{E. coli}, 14 of the resulting recombinants were picked up randomly. One recombinant was characterized. The insert of the plasmid hybridized well with the HMW fragments (Fig. 8). This probe hybridized with the HMW fragments with greater specificity than the probe made from the A\textit{f}al HMW fragments (see Fig. 2). The difference between the A\textit{f}al and \textit{HaeIII} digest lanes was less significant by the probe than the HMW probe. By the HMW probe a distinct signal around 0.3 Kb was shown in the \textit{HaeIII} lane but on the other hand periodicals of signal (1–6 Kb) were remarkable in the A\textit{f}al lane (Fig. 2), while the intensity and size of signals of such small molecular weight were almost equal between the two lanes by the RETS probe (Fig. 8). This suggests that the sequence is at distal to the A\textit{f}al and \textit{HaeIII} sites in most of the A\textit{f}al HMW fragments.

On sequencing the plasmid clone, 9 tandem repeats of a 45-bp sequence were detected (Fig. 9A). Each of the repeat unit sequences has a palindrome, which may form a stem (Fig. 9B). All of these stems have a nearly identical (99%) 35bp sequences. The stems were separated by 10- to 11-bp sequences (10, the mean value; 11, the most frequent value). These internal sequences all have...
T stretches and are rich in pyrimidines (88%). A TTTT stretch is conserved in all the internal sequences. This structure is similar to the TTTT motif of the scaffold attachment regions (SAR) found in yeast *S. cerevisiae* and *drosophila.* Like the tobacco spacer sequence, the SAR consensus of the yeast and the fly is 10–11 bp in length. In the conserved 35 bp sequence, a palindrome of telomeric repeat units exists, which is the front of the putative stem. These peculiar characteristics suggest the close relationship between telomeres and the repeats. We have termed these repeats RETS (repeats containing telomeric stem).

To examine the positional relationship in tobacco genomic DNA, nuclear DNA digests were analyzed by hybridization using telomere sequences and RETS as probes (Fig. 10B and C). With the RETS probe, most of the signals were observed in the HMW DNA region. Although a signal was observed also in low molecular weight DNA region, the ratio was very low because most of the genomic DNA fragments were positioned in this low molecular weight region (Fig. 10A). In the HMW DNA region, a cluster of telomere probe signals corresponded to that of the RETS probe. In the broad signal, faint ladders were observed. The signal intensity varied and the signal ratio between the RETS probe and the telomere probe differed depending on the respective ladders. However, the ladders by the RETS probe corresponded to those by the telomere probe. These results indicate that RETS sequences are rich in chromosome ends while the amount of RETS per end are variable, and the shortest distance between RETS and telomere repeats is less than 20 kb.
4. Discussion

Among plants, tobacco has high efficiency of transformation by chimeric DNA and that of regeneration from protoplast and callus. Many genes from various plant species were introduced into tobacco cells to study gene function and expression. Furthermore, protoplast works and biochemical techniques are easy in tobacco. Unfortunately, however, little is known in tobacco about its chromosome system molecularly.

In this report, we found and isolated HMW DNA fragments from Afal digests of tobacco nuclear DNA. Because this portion of the nuclear DNA was hypersensitive to Bal31 nuclease digestion, much of the HMW fragments were thought to be situated at chromosome ends. This notion was supported by the facts that the telomere sequence oligonucleotide hybridized to the HMW fragments, the telomere signal was preferentially reduced by Bal31 nuclease and the kinetics profile of the degradation at early stages were similar between the DNA signal and the telomere signal.

The HMW fragments were easily isolated as highly discrete bands from other genomic fragments. Consequently, the isolated HMW fragments will help us to examine telomere regions biochemically. Genomic sequencing using the HMW DNA fragments revealed that the tobacco telomere consensus sequence is CCCTAAA. Sequencing of four plasmid clones derived from the HMW fragments supported the genomic sequencing.

Besides the telomere sequence, we found a repeat sequence containing RETS in the HMW fragments. The smallest telomere fragments which contained RETS were 20 kb in size. When the length of telomeres and RETS is subtracted from the size, the shortest distance between RETS and telomere repeats is less than 20 kb. The highly conserved 35-bp portion of RETS may form a stem structure, the front of which is a palindrome of telomere repeat sequence oligonucleotide hybridized to the HMW fragments supported the genomic sequencing.

The telomeric regions of the yeast chromosomes contain autonomous replicating sequences (ARS). The telomeric regions of the yeast chromosomes contain autonomously replicating sequences (ARS) and drosophila. The telomeric regions of the yeast chromosomes contain autonomously replicating sequences (ARS). The telomeric regions of the yeast chromosomes contain autonomously replicating sequences (ARS). A number of telomeric Y′ ARS and X ARS have been reported in the yeast. It is an attractive idea to suppose that RETS is the functional homolog as well as the structural homolog of the yeast telomeric ARSs. Regardless, these characteristics suggest close relationship between telomeres and the repeats. According to the data obtained in this study, we propose a model for tobacco chromosome end DNA regions (Fig. 11). The isolated HMW fragments from tobacco will further help us to search telomeric regions by biochemical and molecular genetic methods.

Acknowledgments: Authors are grateful to Dr. F. Takaia (National Institute of Agrobiological Resources, Tsukuba) for generously providing us the rice rDNA plasmid and to anonymous referees for advice. The plant was cultivated at the conservatory of our faculty. Characterization of nucleotide sequences was carried out partly using the computer systems in the National Institute of Genetics (Mishima) and the Institute of Physical and Chemical Research (Tsukuba). This work was supported by a grant from the Cosmetology Research Foundation (Tokyo) and by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan to K.S.

References

1. Zakian, V. A. 1989, Structure and function of telomeres, Annu. Rev. Genet., 23, 579-604.
2. Morin, G. B. 1989, The human telomerase transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats, Cell, 59, 521-529.
3. Wang, S., Lapitan, N. L. V., Roder, M., and Tsuichiya, T. 1992, Characterization of telomeres in Hordeum vulgare chromosomes by in situ hybridization, Genome, 35, 975-980.
4. Tsujimoto, H. 1993, Molecular cytological evidence for gradual telomere synthesis at the broken chromosome ends in wheat, J. Plant Res., 106, 239-244.
5. McClintock, B. 1941, The stability of broken ends of chromosome in Zea mays, Genetics, 26, 234-282.
6. Richards, E. J. and Ausubel, F. M. 1988, Isolation of a higher eukaryotic telomere from Arabidopsis thaliana, Cell, 53, 127-136.
7. Kanal, M. W., Lapitan, N. L. V., and Tanksley, S. D. 1991, Macrostructure of the tomato telomeres, Plant Cell, 3, 87-94.
8. Rawlins, D. J., Higbett, M. I., and Shaw, P. J. 1991, Localization of telomeres in plant interphase nuclei by in situ hybridization and 3D confocal microscopy, Chromosoma, 100, 424-431.
9. Schwarztser, T. and Heslop-Harrison, J. S. 1991, In situ hybridization to plant telomeres using synthetic oligomers, Genome, 34, 317-323.
10. Crosson, S., Lidsay, J., Fantes, J., McKay, S., McGill, N., and Cooke, A. H. 1990, The structure of a subterminal sequence present on many human chromosomes, Nucl. Acids Res., 18, 6649-6657.
11. Wagner, I. and Cepkies, I. 1981, Determination of 5-methylcytosine from plant DNA by high-performance liquid chromatography, Biochim. Biophys. Acta, 654, 52-56.
12. Jofuku, K. D. and Goldberg, R. B. 1988, Analysis of plant gene structure, In: Shaw, C. H. (ed) Plant molecular biology: a practical approach. IRL press, Washington DC, pp. 37-66.
13. Suzuki, K. and Yoshida, K. 1986, Stepwise transformation in Saccharomyces cerevisiae yeast: construction of strains for transformation and subsequent cytoductive transfer of plasmid DNA with mitochondria, Plant Cell Physiol., 27, 801-808.
14. Chiu, G., Vollrath, D., and Davis, R. W. 1986, Separ-
tion of large DNA molecules by contour-clamped homogeneous electric fields, *Science*, **234**, 1582-1585.

15. Suzuki, K., Ichikawa, K., and Jigami, Y. 1989, Yeast mutants with enhanced ability to secrete human lysozyme: isolation and identification of a protease-deficient mutant, *Mol. Gen. Genet.*, **219**, 58-64.

16. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982, Molecular cloning: a laboratory manual, Cold spring harbor laboratory, Cold spring harbor, New York.

17. Feinberg, A. P. and Vogelstein, B. 1983, A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity, *Anal. Biochem.*, **132**, 6-13.

18. Renz, M. and Kurz, C. 1984, A colorimetric method for DNA hybridization, *Nucl. Acids Res.*, **12**, 3435-3444.

19. Yanisch-Perron, C., Vieira, J., and Messing, J. 1985, Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors, *Gene*, **33**, 103-119.

20. Dente, L. and Cortese, R. 1987, pEMBL: A new family of single-stranded plasmids for sequencing DNA, *Methods Enzymol.*, **155**, 111-119.

21. Inoue, H., Nojima, H., and Okayama, H. 1990, High efficiency transformation of *Escherichia coli* with plasmids, *Gene*, **96**, 23-28.

22. Sanger, F., Nicklen, S., and Coulson, A. R. 1977, DNA sequencing with chain-terminating inhibitors, *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.

23. Amati, B. B. and Gasser, S. M. 1988, Chromosomal ARS and CEN elements bind specifically to the yeast nuclear scaffold, *Cell*, **54**, 967-978.

24. Shillito, R. D. and Saul, M. W. 1988, Protoplast isolation and transformation, In: Shaw, C. H. (ed) Plant molecular biology: a practical approach, IRL press, Washington DC, pp. 161-186.

25. Chan, C. M. and Tye, B. 1983, A family of *Saccharomyces cerevisiae* repetitive autonomously replicating sequences that have very similar genomic environments, *J. Mol. Biol.*, **168**, 505-523.

26. Chan, C. M. and Tye, B. 1983, Organization of DNA sequences and replication origins at yeast telomeres, *Cell*, **33**, 563-573.

27. Takaia, F., Oono, K., and Sugiu, M. 1984, The complete nucleotide sequence of a rice 17s rRNA gene, *Nucl. Acids Res.*, **12**, 5441-5448.