RFLP and physical mapping with an rDNA-specific endonuclease reveals that nucleolus organizer regions of Arabidopsis thaliana adjoin the telomeres on chromosomes 2 and 4

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Summary
Ribosomal RNA genes are organized in tandem arrays called nucleolus organizer regions (NORs). In a prior study, RFLP mapping on pulsed-field gels placed NOR2 at the northern tip of Arabidopsis thaliana chromosome 2. New polymorphisms have allowed the other NOR, NOR4, to be mapped to the northern tip of chromosome 4. To map NOR-associated loci, rDNA-specific cleavage by I-Ppol, an endonuclease with a 15 nucleotide recognition sequence involved in rDNA-homing of a mobile, self-splicing Group I intron in Physarum was exploited. I-Ppol digestion of A. thaliana genomic DNA liberated two telomere-containing fragments no larger than 13 kbp, and telomere polymorphisms identified using I-Ppol co-segregated with NOR2 and NOR4. Restriction mapping suggested that telomere-proximal rRNA genes are oriented with their 5' ends nearest the chromosome ends and their 3' ends nearest the centromere. This orientation was confirmed using the polymerase chain reaction to clone one of the telomere–rDNA junctions, most likely the junction on chromosome 4. The telomeric repeats join the terminal rRNA gene downstream of its promoter, suggesting that this first gene is inactive. Subtelomeric repetitive DNAs are absent at the telomere–rDNA junction. Localization of NOR2, NOR4 and their associated telomeres, TEL2N and TEL4N, respectively, provides end points for the genetic and physical maps of chromosomes 2 and 4.

Introduction
Ribosomal RNA genes (rRNA genes or rDNA) in eukaryotes encode the 18S, 5.8S and 25S rRNAs. These rRNAs are processed from a large primary transcript produced by RNA polymerase I (Flavell, 1986b; Gerbi, 1985; Moss and Stefanovsky, 1995; Paule, 1994; Reeder, 1992; Rogers and Bendich, 1987). In all eukaryotes, rRNA genes are present in hundreds, to thousands, of copies arranged in long tandem arrays, with plants having some of the highest gene copy numbers (reviewed in Flavell, 1986a, 1986b; Ingles et al., 1975; Rogers and Bendich, 1987). The rDNA repeats tandemly arrayed at a single locus comprise a nucleolus organizer region (NOR), so named because the nucleolus, the site of ribosome synthesis, is organized around the rRNA genes during interphase (McClintock, 1934; Phillips, 1978; Phillips et al., 1971).

In A. thaliana, approximately 570–750 rRNA gene repeats, each approximately 10.0–10.5 kbp in size, are present in each haploid genome (Copenhaver and Pikaard, 1996; Gruendler et al., 1989, 1991; Pruitt and Meyerowitz, 1986). Cytogenetic studies first suggested that the rDNA is located at two NORs, one on chromosome 2 and the other on chromosome 4 (Albini, 1994; Ambros and Schweizer, 1976; Bawens et al., 1991; Koornneef et al., 1983; Maluszynska and Heslop-Harrison, 1991; Sears and Lee-Chen, 1970). In situ hybridization with fluorescent rDNA gene probes suggested that the two NORs contain roughly equal numbers of genes (Albini, 1994; Maluszynska and Heslop-Harrison, 1991). Electron micrographs of synaptonemal complexes from pollen mother cells showed that the two nucleoli are organized near the tips of the two shortest chromosomes, presumably chromosomes 2 and 4 (Albini, 1994). However, no knowledge of the location of the NORs relative to other genes or molecular markers has come from cytogenetic analyses.

Though there are approximately 1200–1500 rRNA genes in an Arabidopsis diploid, they are virtually identical in sequence complexity. The rRNA genes of different Arabidopsis strains are also similar, an obstacle for conventional RFLP mapping. Of necessity, we have turned to RFLP mapping on a large scale using restriction endonucleases which cleave infrequently within a NOR, generating large fragments in the range of 0.1–1.0 Mbp. These fragments are resolved by Contour-clamped Homogeneous Electric Field (CHEF) gel electrophoresis (Chu et al., 1986) and visualized by Southern blotting. Despite the similar sequence complexity (including similar types of variants) of rRNA genes in different strains of A. thaliana, we have found that the interspersion pattern of variants can be strain-specific (Copenhaver et al., 1995). Consequently, NOR digestion profiles (sets of co-segregating polymorph-
isms) on CHEF gels can be used for RFLP mapping. In a previous study, we showed that strain-specific NOR digestion profiles revealed with HindIII segregated among a recombinant inbred mapping population (Lister and Dean, 1993), allowing us to map NOR2 as the most distal marker on the northern arm of chromosome 2 (Copenhaver et al., 1995). Here, we report a new set of polymorphisms that have allowed us to map the other NOR, NOR4, to the northern tip of chromosome 4.

In Physarum polycephalum, many rRNA genes contain a group I self-splicing intron that can spread to the identical location in other rRNA genes that lack the intron. This phenomenon, known as intron-homing (Dujon, 1988; Dujon et al., 1989; Perlman and Butow, 1989), involves mobilization of the element via the action of an endonuclease, l-Ppol (intron-encoded Physarum polycephalum endonuclease I), encoded by an open reading frame located within the intron itself (Muscarella and Vogt, 1989). l-Ppol catalyzes a double-stranded cleavage of rRNA genes which lack the intron at the exact site where the intron is located in intron-bearing genes. The cleaved ends are thought to potentiate a gene conversion event via strand invasion of an intron-bearing rRNA gene. Importantly for our study, l-Ppol has the 15 nt recognition sequence CTCTCTTAAGGTAGTC (Lowery et al., 1992; Muscarella et al., 1990), a sequence that is highly conserved within the 25–28S (depending on species) rRNA coding sequence of eukaryotes. We showed previously that l-Ppol cleaves once within every rRNA gene in Arabidopsis, yielding unit gene length (10 kb) rDNA fragments due to their simple head-to-tail tandem arrangement (Copenhaver et al., 1995). The specificity of l-Ppol for rDNA is best appreciated by considering that an enzyme with a 15 nt recognition sequence is expected to cleave random-sequence DNA approximately once every $10^9$ bp (probability = $(1/4)^{15}$). The likelihood that l-Ppol cleaves outside of the rDNA is very low given that the haploid genome size in Arabidopsis is only approximately $10^6$ bp (Meyerowitz, 1992).

We reasoned that an essentially rDNA-specific endonuclease such as l-Ppol should be useful for mapping rDNA-associated traits. Specifically, we questioned whether the NORs were physically close to the telomeres, the specialized DNA sequences located at the ends of eukaryotic chromosomes (for reviews see Blackburn, 1990, 1991; Zakian, 1989), consistent with their distal locations on chromosomes 2 and 4. Using l-Ppol and other restriction endonucleases in conjunction with telomere-specific probes, we present physical and genetic evidence that NOR2 and NOR4 are telomeric, which we have named TEL2N and TEL4N, at the northern tips of chromosomes 2 and 4. TEL2N and TEL4N represent end points for the NORs and for the genetic and physical maps of chromosomes 2 and 4.

Figure 1. BstEII digestion reveals rDNA polymorphisms among different A. thaliana strains.

A agarose-embedded genomic DNA from A. thaliana strains Columbia, Col-4 (labeled 'C', lane 1); Wassilewskija (W, lane 2); Turkalle (T, lane 3); RLD (R, lane 4); Kendalville (K, lane 5); and Landsberg erecta, Ler-0 L, lane 6) was subjected to digestion with 100 units of BstEII. Digests were then subjected to CHEF gel electrophoresis and gels were blotted and hybridized to a probe representing 185–205 rDNA coding regions. The autoradiogram of the blot shows that BstEII digestion profiles are unique in several strains, including Columbia and Landsberg, the parental strains of the Lister/Dean recombinant inbred mapping population.

Results

BstEII digestion reveals strain-specific NOR polymorphisms that define the locus, NOR4

To find useful restriction endonucleases for NOR mapping, we searched published A. thaliana rRNA gene sequences (Gruendler et al., 1989, 1991; Unfried and Gruendler, 1990; Unfried et al., 1989) for recognition sites for methylation-insensitive restriction enzymes predicted to cut once, or not at all. Because sequenced genes represent only one or a few isolates of the 1200–1500 rDNA genes in a diploid, some rRNA genes can lack restriction sites identified in cloned genes. Conversely, sites absent from a cloned gene can be present in other rRNA genes. Therefore, we systematically tested restriction enzymes predicted to be single-cutters or non-cutters and examined the resulting rDNA digestion patterns on CHEF gels.

Digestion of genomic DNA with the enzyme BstEII (a predicted non-cutter of rDNA), CHEF electrophoresis, Southern blotting and hybridization to an rDNA coding region probe yielded unique patterns in a number of A. thaliana strains (Figure 1). A point mutation at any of approximately 20 sites within the rDNA genes sequenced could generate a BstEII site (data not shown). Importantly, the BstEII digestion profiles were different in the Arabidopsis strains Columbia and Landsberg, the parental strains of a widely used recombinant inbred population used for RFLP mapping (Lister and Dean, 1993). Columbia rDNA appeared to be uncut by BstEII (Figure 1, lane 1) but Landsberg rDNA was cut to yield six
fragments ranging in size from approximately 60 to approximately 1300 kbp (lane 6).

Segregation of the Columbia and Landsberg BstEII NOR profiles was examined among the Lister/Dean recombinant inbred (RI) mapping population, using the same subset of RI lines used to map NOR2 (Copenhaver et al., 1995). A Landsberg-like BstEII NOR profile was observed in 29 of the lines, whereas 16 lines displayed a Columbia-like profile (Figure 2, Table 1). In those lines yielding a Landsberg-like NOR profile, the BstEII fragments co-segregated, suggesting that they mapped to the same locus. Occasional size changes of BstEII fragments were observed in the RI lines (denoted by arrowheads in Figure 2), as was observed previously for NOR2 HindIII fragments (Copenhaver et al., 1995). Size changes in NOR fragments are most likely due to intrachromosomal deletions caused by recombination within the rDNA array or by unequal crossing over between NORs of sister chromosomes (Adam, 1992; Butler and
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L, Landsberg-like; C, Columbia-like; U, unknown.

Metzenberg, 1989, 1990; Chindamporn et al., 1993; Copenhagen et al., 1995; Pasero and Marilley, 1993; Pukkila and Skrzynia, 1993; Zolan et al., 1994). The BstEII profiles segregated among the RIs in a manner distinct from NOR2, and mapped as the most distal marker on the northern tip of chromosome 4, approximately 7.4 centimorgans from the marker BIO217 (Table 1; summarized in Figure 3). We suggest the name NOR4 for this rDNA locus. Localization of the NOR to chromosome 4 agrees with cytogenetic evidence (Albini, 1994; Ambros and Schweizer, 1976; Bauwens et al., 1991; Maluszynska and Heslop-Harrison, 1991; Sears and Lee-Chen, 1970).

I-Ppol digestion of A. thaliana genomic DNA liberates telomere fragments

Because there are five chromosomes in A. thaliana, most restriction endonucleases should liberate a maximum of 10 telomere fragments provided that the enzymes do not cut within the telomeres themselves. In contrast, we reasoned that I-Ppol digestion should yield only two telomere fragments if I-Ppol was specific for the rDNA, as predicted, and if the distal locations of NOR2 and NOR4 placed them close enough to the telomeres for NOR-telomere distances to be determined. To test these predictions, we compared the digestion of genomic DNA from the strains Landsberg erecta and Columbia using I-Ppol, Stf1 and NdeI (enzymes which cut once within every rRNA gene; see diagram at the top of Figure 4). Nodl (a non-cutter of rRNA genes, data not shown), HindII and BstEII. The latter two enzymes were chosen because they cut some rRNA genes, but not others, revealing the polymorphisms used to map NOR2 and NOR4, respectively (Copenhagen et al., 1995; this study). Following CHEF gel electrophoresis and Southern blotting, blots were hybridized to a probe corresponding to the terminal repeats of a cloned Arabi-
dopsis telomere (Richards and Ausubel, 1988) and were visualized by autoradiography.

Digestion of Landsberg and Columbia DNA with SfiI yielded similar patterns with at least seven telomere-containing fragments of 14–720 kbp as well as hybridizing material at approximately 1.5 Mbp, the limit of resolution on the gel shown (Figure 4, lanes 3 and 6). The large average size of the fragments was consistent with the fact that SfiI is a relatively rare cutting endonuclease with an 8 bp recognition sequence. Digestion with NotI, another enzyme with an 8 bp recognition sequence, but one that does not cut within the rDNA, also yielded five to six distinct telomeric DNA fragments in Landsberg erecta and Columbia (data not shown). NdeI (an enzyme with a 6 bp recognition sequence) yielded numerous fragments whose average size was smaller than those of SfiI, as expected, and no hybridizing material remained at the top of the gel, consistent with cutting near (or within) all 10 telomeres (lanes 4 and 7). A number of telomere polymorphisms are revealed using NdeI. In contrast to the numerous bands detected with SfiI, NdeI or NotI, L-Ppol digestion yielded a simple pattern. Using Landsberg genomic DNA, only one diffuse band of approximately 8 kbp was detected (Figure 4, lane 2). In Columbia, two bands were detected, one near 13 kbp and the other near 8 kbp (Figure 4, lane 5). In both strains, the majority of the DNA hybridizing to the telomere probe following L-Ppol digestion remained at the top of the gel, and aside from the 8 and 13 kb bands, the L-Ppol and uncut DNA control lanes looked very similar (compare lane 1 with lanes 2 and 5). Therefore, the L-Ppol digestion pattern was consistent with cutting at two telomeres (lane 5), presumably the telomeres associated with the two NORs. Quantitation by phosphor-imaging revealed that the diffuse 8 kbp band in Landsberg (lane 2) contained as much radioactivity as the 8 and 13 kb bands of Columbia (lane 5) combined. This suggested that the diffuse 8 kb band in Landsberg might represent two co-migrating telomere bands, one of which might be the homolog of the 13 kb Columbia band. Note that the diffuse nature of the telomere bands is not unusual because telomere length varies in
among the Lister–Dean recombinant inbred (RI) lines examined previously to map NOR2 and NOR4. Twenty-two of the 46 lines we scored were Columbia-like and 24 were Landsberg-like with respect to the I-Ppol-telomere polymorphism (Figure 5, Table 1). Importantly, all recombinant inbred lines previously shown to have a Columbia-like NOR2 profile displayed the Columbia-like 13 kbp I-Ppol telomere polymorphism (a representative group of such RIIs are shown in Figure 5, lanes 3–9). Likewise, those RI lines in which the 13 kbp band was absent, as in the strain Landsberg erecta (Figure 5, lanes 1 and 10), were previously shown to have a Landsberg-like NOR2 profile (representative RI lines are shown in Figure 5, lanes 12–13). Co-segregation of the 13 kbp telomere polymorphism with NOR2, with no recombination detected between these two markers, suggests that TEL2N, the telomere at the northern tip of chromosome 2, maps to the same site as NOR2. Importantly, one cannot determine the order of the TEL2N and NOR2 loci relative to one another or to other markers on chromosome 2 based solely on the RFLP mapping data. However, we place TEL2N as the distal marker on chromosome 2 (Figure 3a) based on our knowledge of telomere function and localization at chromosomal termini.

HindIII and BstEII telomere fragments susceptible to I-Ppol cleavage reveal additional telomere polymorphisms

1-Ppol digestion alone did not reveal a useful polymorphism with which to genetically map the telomere putatively attached to NOR4. To generate additional NOR-associated telomere polymorphisms we used endonucleases which reveal RFLPs unique to NOR2 and NOR4, exploiting differences between the strains Columbia and Landsberg. At NOR2, HindIII sites are distributed differently in Columbia and Landsberg, yielding strain-specific RFLP profiles (Copenhaver et al., 1995). However, every rRNA gene (at both NOR2 and NOR4) appears to have a site for I-Ppol (Copenhaver et al., 1995). We reasoned that the most distal rRNA gene bearing a HindIII site at NOR2 might be located far enough from the end of the chromosome such that other rRNA genes lacking HindIII sites would be located on its telomere-proximal side. If so, the terminal, telomere-containing, HindIII fragment of NOR2 would be susceptible to I-Ppol cleavage. Likewise, we have shown that at NOR4 of Landsberg, infrequent BstEII sites are distributed throughout the locus, whereas BstEII sites are absent from NOR2 of Columbia or Landsberg and from NOR4 of Columbia (this study). Therefore, we reasoned that the terminal BstEII fragment of Landsberg NOR4 might be susceptible to cleavage with I-Ppol. Telomere fragments liberated from other chromosomes with HindIII or BstEII would be unlikely to be cleaved by I-Ppol due to the absence of rDNA at these sites. These predictions were

different cells, reflecting the presence of variable numbers of terminal repeats due to the action of telomerase, the self-templated reverse transcriptase responsible for telomere maintenance (for review see Blackburn, 1990).

The telomere polymorphism revealed upon I-Ppol digestion maps to the same site as NOR2

Genomic DNA digested with I-Ppol and hybridized to a telomere probe revealed a useful polymorphism between the strains Columbia and Landsberg, namely, the presence or absence of the 13 kbp band (Figure 4). This polymorphism was used to estimate the position of the corresponding telomere on the genetic map by examining its segregation
supported by analysis of HindIII and HindIII/I-Ppol digests as well as BstEII and BstEII/I-Ppol digests of Columbia and Landsberg genomic DNA (Figure 6).

Genomic DNA cleaved with HindIII revealed numerous telomere fragments, most smaller than about 14 kbp in both strains (Figure 6, lanes 1 and 3). In Landsberg, a 72 kbp (±12 kbp) telomere-containing fragment (lane 3) was susceptible to cleavage with I-Ppol (lane 4). Other HindIII telomere fragments were unchanged in their mobility. In Columbia, the HindIII telomere fragments tended to be small such that I-Ppol did not have as dramatic an effect on their mobility. However, the two largest fragments, of roughly 14 kbp and 9 kbp, were trimmed approximately 1 kbp by I-Ppol (Figure 6, compare lanes 1 and 2), consistent with the sizes of the 13 and 8 kbp telomere fragments liberated by I-Ppol alone (Figure 4, lane 5). Because the 13 kbp I-Ppol fragment co-segregates with the Columbia NOR2 locus (Figure 5), these results suggest that at NOR2, the first HindIII site is 14 kbp from the end of the chromosome whereas the first I-Ppol site is 13 kbp from the end of the chromosome. Likewise, at NOR4, the first HindIII site is deduced to be 9 kbp from the end of the chromosome whereas the first I-Ppol site is 8 kbp from the end. Consistent with these interpretations, shown in Figure 3, sequence data show that in an rDNA gene variant bearing a HindIII site, this site is located about 1 kbp 3′ of the I-Ppol site (Gruendler et al., 1991). Therefore, the HindIII and HindIII/I-Ppol double digestion data of Figure 6 suggest that the most telomere-proximal rRNA genes at both NOR2 and NOR4 are oriented with their 5′ ends telomere-proximal and their 3′ ends centromere-proximal. This interpretation is supported by the telomere–NOR junction clone we have obtained (see below).

BstEII digestion revealed eight to nine telomere fragments in Landsberg and Columbia, respectively (Figure 6, lanes 5 and 7). Upon double digestion of Columbia genomic DNA with BstEII and I-Ppol, a novel 8 kbp telomere-containing I-Ppol fragment was generated (lane 6). Presumably, this telomere band was derived from a fragment that was at the top of the gel following digestion with only BstEII because none of the 12–775 kbp BstEII telomere-containing fragments were affected by double digestion with I-Ppol. Because no BstEII sites appear to be present within either NOR of Columbia (Figure 1), these results were expected. In Landsberg, BstEII does not cut within NOR2, but cleaves NOR4 to generate the fragments used to map the locus (Figure 2). We predicted that one of these NOR4 fragments might include the telomere and multiple rRNA genes, each containing an I-Ppol site. As expected, one of the Landsberg BstEII fragments was susceptible to I-Ppol cleavage (Figure 6, compare lanes 7 and 8). This telomere-containing BstEII fragment is approximately 640 kbp, the same size as one of the polymorphic NOR4 BstEII fragments that hybridized to the rDNA probe (Figures 1 and 2).

HindIII and BstEII telomere fragments susceptible to I-Ppol cleavage reveal a second TEL2N polymorphism and a polymorphism that defines the NOR4-associated telomere TEL4N

The data of Figure 6 showed that HindIII and BstEII fragments susceptible to I-Ppol digestion could be identified
Figure 6. Additional telomere polymorphisms are revealed by double digestion with I-Ppol and endonucleases which yield NOR-specific rDNA fragments. HindIII (H), which is useful for revealing strain-specific polymorphisms at NOR2, and BstEII (B), which cuts NOR4 of Landsberg but not either of the two Columbia NORs, nor Landsberg NOR2, were tested alone and in double-digests with I-Ppol (I). Following digestion and CHEF gel electrophoresis, Southern blots were hybridized to a telomere probe. Note that a 72-kbp HindIII fragment of Landsberg (lane 3) was susceptible to I-Ppol digestion (compare lanes 3 and 4). Similarly, a 640-kbp BstEII fragment (lane 7), also apparent in Landsberg, was susceptible to I-Ppol digestion (compare lanes 7 and 8). These large I-Ppol susceptible Landsberg HindIII and BstEII fragments were not found in Columbia and proved useful for mapping NOR-associated telomeres (see Figures 7 and 8).

and that the fragments were polymorphic between Columbia and Landsberg. Susceptibility to I-Ppol suggested that the 72-kbp HindIII and 640-kbp BstEII fragments of Landsberg, and the 14-kbp and 9-kbp HindIII fragments of Columbia, corresponded to telomeres associated with NOR2 and NOR4, respectively. If so, we predicted that the 72-kbp HindIII telomere fragment should co-segregate with the Landsberg NOR2 locus and the 14-kbp HindIII fragment should co-segregate with the Columbia NOR2 locus. Likewise, the 640-kbp BstEII fragment should co-segregate with the Landsberg NOR4 locus. Consistent with these predictions, the 72 and 14-kbp telomere-containing HindIII fragments co-segregated with NOR2 of Landsberg and Columbia, respectively, among the recombinant inbred lines (Figure 7 and data not shown). Therefore, the 72 and 14-kbp HindIII fragments represent additional polymorphisms that define the TEL2N locus, the first being the 13-kbp I-Ppol fragment detected only in Columbia (Figures 4 and 5). These data suggest that at the northern end of chromosome 2 in Landsberg, the first HindIII site within NOR2 is approximately 72 kbp from the end of the chromosome, whereas in Columbia the first HindIII site is located 14 kbp from the end of the chromosome (summarized in Figure 3).

Because the 640-kbp telomere-containing BstEII fragment was polymorphic between Columbia and Landsberg (Figure 6, compare lanes 5 and 7), we also examined the segregation of this polymorphism among the recombinant inbred lines (Figure 8). The 640-kbp telomere-containing band co-segregated with the Landsberg-like NOR4 locus among all the lines examined (Figure 8, Table 1) and defines the locus we have named TEL4N. As with NOR2 and TEL2N, which mapped to the top of chromosome 2, NOR4 and TEL4N map to the northern tip of chromosome 4 (summarized in Figure 3). These data suggest that the most telomere-proximal BstEII site within NOR4 of Landsberg is approximately 640 kbp from the northern tip of chromosome 4. Note that in Figure 8, additional polymorphic BstEII fragments segregated among the recombinant inbred lines. These include a 775-kbp band, not susceptible to I-Ppol digestion (Figure 6, compare lanes 5 and 6), that mapped to chromosome 1 (data not shown).

Cloning an rDNA-telomere junction

To test the hypothesis that the rRNA genes are oriented with their 5' ends telomere-proximal and their 3' ends centromere-proximal, we designed oligodeoxynucleotide primers to amplify the telomere-rDNA junction via the polymerase chain reaction (PCR). A successful primer pair was a telomere-specific oligo (28 mer (5'-CCCTAAA-3')4) complementary to four T3AG3 heptamer repeats (Richards and Ausubel, 1988), and an rDNA-specific oligo (5'-GCATATGACTACTGGCAG-3') complementary to the 5' end of the 18S rRNA coding sequence, as shown at the top of
Figure 8. The 640 kbp telomere-containing BstEII fragment co-segregates with the Landsberg N0R4 locus among the recombinant inbred lines and defines the telomere locus  TEL4N.

Recombinant inbred lines known to have a Columbia-like (lanes 3–9) or Landsberg-like (lanes 12–19) N0R4 locus were tested by subjecting genomic DNA to digestion with BstEII and hybridization to a telomere probe. Two representative gels are shown. Columbia and Landsberg genomic DNA controls were run in lanes 1, 2, 10 and 11. All recombinant inbred lines previously shown to have a Landsberg-like N0R4 locus also displayed the 640 kbp telomere polymorphism (lanes 12–19), whereas the absence of the 640 kb fragment was observed in all RI lines with a Columbia-like N0R4 locus (lanes 3–9). Segregation analysis of all lines tested (see Table 1) shows that the telomere polymorphism maps to the same genetic location as N0R4 and defines the telomere locus  TEL4N.

Figure 9. When used to amplify genomic DNA isolated from the strain Columbia, a specific product of approximately 450 bp was generated. This was true using several different genomic DNA preparations and several different PCR conditions. The PCR products were cloned and several were completely sequenced. Reading from the 18S rRNA towards the telomeric DNA, the clones matched the rRNA intergenic spacer sequence to a site approximately 1400 nt downstream of the promoter, where the rRNA gene sequences join the telomere repeats (Figure 9). A 13 nt duplication of a sequence normally found only once per rRNA gene is located at the junction with the T4AG3 telomere repeats, but no extensive subtelomeric repetitive DNAs are present.

Mapping TEL2N sheds light on rDNA rearrangements at the N0R2 locus

In our previous study, seven of the RI lines (RI lines 5, 54, 161, 194, 321, 390 and 394) had a N0R2 pattern so different from either parent that their N0R2 loci could not be scored as being either Landsberg-like or Columbia-like (Copenhaver et al., 1995; see Table 1). We speculated that the altered N0R2 HindIII-digestion profiles might be due to internal deletions or rearrangements within the N0R2 locus (i.e. intrachromosomal events). Alternatively, we suggested that the rearrangements might be a consequence of recombination between Landsberg and Columbia N0R2s (i.e. interchromosomal exchanges). To reconsider the nature of the rearrangements in the N0R2 ‘unknowns’, we asked whether rearrangements at N0R2 were associated with changes in linkage to the nearest known markers flanking the N0R on either side (Figure 3; Table 1).

Examination of Table 1 shows that all RI lines with a Columbia or Landsberg-like N0R2 profile also have a TEL2N pattern of the same parental type, as discussed previously. Likewise, N0R4 and TEL4N display 100% linkage in the lines we examined. In only five of the 37 lines (14%) in which N0R2 and ve012 could be scored as being clearly Columbia or Landsberg-like (C or L) were TEL2N and ve012 alleles derived from different parents (RI lines 62, 188, 253, 264, 397). Likewise, on chromosome 4, N0R4 differed in parental type from its nearest centromere-proximal marker, BIO217 in only 18% of the lines tested. However, in those lines in which N0R2 could not be scored as resembling the N0R2 of either parental strain (RI lines 5, 54, 161, 194, 390 and 394; scored ‘U’ for unknown), four out of six had the TEL2N polymorphism of one parent and the ve012 polymorphism of the other parental strain (lines 5, 194, 390 and 394). These data suggest that at least one recombination event occurred between ve012 and TEL2N in two-thirds (67%) of the lines that show drastically rearranged N0R2 loci. Given the scope of the rearrangements (Copenhaver et al., 1995), it is likely that the recombination event(s) that altered the N0R2 HindIII profiles occurred within the N0R2 locus itself.
a.

![Diagram of telomere-rDNA junction}

(b) The sequence at the junction of the telomere repeats and rDNA is shown. The gel at left shows the dideoxynucleotide sequencing ladder derived from a primer on the telomere-proximal side of the clone. By convention, the sequence at the right is given in the direction of the telomere, such that the gel should be read top to bottom as labeled (T, A, C, G). Nucleotides in bold match the T3AG3 telomeric repeat consensus; those in parentheses are derived from the PCR primer. The underlined nucleotides shown in bold type represent an incomplete telomeric repeat present at the junction. Nucleotides in italics are sequences usually present only once per rRNA gene. A 13 bp sequence is duplicated at the junction as a direct repeat. The telomere-proximal 13 bp repeat overlaps the incomplete telomere repeat and the beginning of the sequences normally found only once per gene repeat.

Discussion

The rDNA abuts the telomeres on A. thaliana chromosomes 2 and 4.

NORs are multimegabase loci composed of virtually identical tandemly repeated rRNA genes. Their size, combined with the lack of internal landmarks, has made it difficult to characterize these important chromosomal loci in higher eukaryotes. RFLPs can be found within the NORs if one examines the loci on a large scale (greater than 100 kbp), and we have used this approach to map the positions of NOR2 and NOR4. In turn, knowledge of the map positions for the NORs, the restriction endonucleases that cleave the NORs, and the strains in which these digestion patterns are informative has allowed us to map the NORs physically relative to the ends of their respective chromosomes. In so doing we have defined NOR-associated telomere polymorphisms that genetically define the loci TEL2N and TEL4N. These NOR-associated telomeres represent end points for the genetic and physical maps of chromosomes 2 and 4.

The rDNA-homing endonuclease of Physarum, I-Ppol, has been invaluable in our studies because its 15 bp recognition sequence makes it an essentially rDNA-specific endonuclease. In yeast, whose genome is approximately fivefold smaller than Arabidopsis, I-Ppol appears to cleave only within the rDNA on chromosome XII, having no effect on the mobility of other chromosomes on pulsed-field gels (Link and Olson, 1991). Likewise, in Arabidopsis, when genomic DNA cleaved with I-Ppol is run on a CHEF gel, and is stained with ethidium bromide, a prominent band
at 10 kb is visible (the rDNA), but otherwise the DNA appears to be uncut and remains at the top of the gel. In contrast, restriction endonucleases with 8 bp recognition sequences, such as NotI and SfiI, generate a smear of genomic DNA due to cutting at many hundreds of sites (data not shown). We expect that I-Ppol will also be useful for mapping the centromere-proximal junctions between the NORs and the flanking non-rDNA and for mapping other NOR-associated traits. Such knowledge may have practical uses beyond the completion of genetic and physical maps of complex genomes. For instance, in crop plants such as maize and barley, beneficial quantitative traits have been positively correlated with variation in the rDNA (Powell et al., 1992; ROCHEFORD et al., 1990). I-Ppol should prove useful for the physical and genetic mapping of NORs and their flanking genes in these species, allowing more accurate evaluations of correlations between NORs and complex traits.

Using telomere-specific and 18S rRNA-specific primers, we were able to amplify and clone NOR-telomere junctions using PCR and genomic DNA from the A. thaliana strain Columbia. Multiple clones of the same junction were obtained. Arabidopsis telomeres are thought to average approximately 3 kb in size (Richards and Ausubel, 1988; Richards et al., 1992). The distance from the telomere-rDNA junction in our clone to the I-Ppol site of the terminal rRNA gene should be 5.1 kb, provided that the gene continues to be normal downstream of the 18S sequence present in our junction clone. Therefore, the distance from the end of the chromosome to the I-Ppol site of this terminal rRNA gene should be approximately 8 kb. Recall that in Columbia, the telomere fragments generated by I-Ppol were 8 and 13 kb in size and the larger fragment maps to TEL2N (see Figure 4). Therefore, our clones are most likely derived from the NOR4-TEL4N junction.

These clones confirm that the telomere-proximal rRNA gene is oriented with its 5' end nearest the end of the chromosome and its 3' end nearest the centromere, as was first suggested for both NORs by Southern blotting using HindIII and HindIII/I-Ppol digestion and a telomere probe (Figure 6). Because of the head-to-tail tandem arrangement of A. thaliana rRNA genes (COPENHAVER et al., 1995), it is likely that the orientation of the terminal rRNA genes will reflect the orientation of the entire rRNA gene arrays at both NOR2 and NOR4.

The absence of subtelomeric repetitive DNAs in our NOR-telomere junction clones, including degenerate copies of the T3AG3 repeats, suggests that the telomere capping the NOR is relatively new. A similar situation has been described in Neurospora, Giardia and Tetrahymena where telomeric repeats abruptly join the rDNA (ADAM et al., 1991; BUTLER, 1992; YU and BLACKBURN, 1991). Telomerase is capable of adding consensus telomere repeats de novo to broken chromosome ends, a process known as chromo-some healing (reviewed in Blackburn, 1991). De novo addition of telomeric repeats to Tetrahymena rDNA has been demonstrated experimentally (Yu and Blackburn, 1991). Consequently, it has been suggested that the abrupt transition from rDNA to telomeric repeats in Neurospora and Giardia is also due to de novo addition of a telomere to a chromosome break occurring within the rDNA. Duplication of DNA sequences at the telomere junction, as we have observed at the NOR-telomere junction, has not been described in these other species. The 13 bp duplication we observe may have been caused by inaccurate recombination between a newly healed and a sister chromatin at the junction region. However, we have insufficient information to distinguish this from other possible models.

Possible significance of organizing the rRNA genes near the telomeres

In plants, a generalization is that active genes are probably separated from telomeres by tens of kbp of spacer DNA which is typically composed of repetitive satellite DNA (BEDBROOK et al., 1980) (for reviews, see KOLCHINSKY and GRESSHOF, 1994; LAPITAN, 1992). However, A. thaliana does not appear to conform to this pattern of organization. In fact, Richards and colleagues showed that unique sequence DNA could be found within several kbp of the telomeric repeats of cloned Arabidopsis chromosome ends (RICHARDS et al., 1992). Our finding that the rRNA gene abut the telomeres is consistent with these results. However, at the junction we cloned, probably the TEL4N-NOR4 junction, the terminal rRNA gene lacks a promoter and is presumed to be inactive. This inactive gene is predicted to provide approximately 8.5 kb of spacing between the telomeres and the promoter of the adjacent rRNA gene. At NOR2, there is an additional 5 kb of spacing between the first I-Ppol site and the end of the chromosome relative to the situation at NOR4 (13 kb instead of 8 kb; see Figures 4 and 5). If so, the NOR-telomere junction should occur in the 25S rRNA coding sequence, approximately 3.5 kb upstream of the first full gene's promoter.

In yeast, protein-coding genes (transcribed by RNA polymerase II) inserted near telomeres can be repressed and the degree to which a gene is silenced depends on its distance from the telomere, to a maximum distance of 6–20 kb (GOTTSCHLING et al., 1990). Silencing in yeast is apparently related to the formation of a chromatin structure that is not unique to telomeres (for review, see LAURENSON and RINE, 1992). Mutations which cause derepression of genes situated near telomeres also de-repress other negatively regulated chromosomal sites, such as silent mating-type loci (APARICIO et al., 1991). Telomere-proximal silencing in yeast may share mechanistic similarities to the phenomenon of position effect variegation (PEV) in Drosophila (EISENBERG, 1989; TARTOF et al., 1989). In flies, most active
genes are organized in euchromatin, with heterochromatic regions tending to be transcriptionally inactive. PEV occurs primarily when a gene is inserted near the centromeric heterochromatin. However, it is interesting that in Drosophila, the rRNA genes are associated with the centromeric heterochromatin despite the fact that the rRNA genes are very active transcriptionally. Therefore, if there is any mechanistic connection between position effects in yeast due to proximity to a telomere and PEV due to proximity to heterochromatin in Drosophila, it is possible that the rRNA genes might not be susceptible to such position effects. It is noteworthy that in Trypanosomes, the only eukaryotes known to use RNA polymerase I for both rRNA transcription and synthesis of protein-coding mRNAs (Rudenko et al., 1991), a telomere-proximal promoter recognized by RNA polymerase I is fully functional on a mini-chromosome and might explain transcription of telomeric repeats in an alpha-amanitin-resistant manner (Zomerdijk and Borst, 1992). Therefore, rather than having a negative effect, it is possible that organizing rRNA genes near the telomeres in Arabidopsis, or near the centromeric heterochromatin in Drosophila, is advantageous if the functions of rRNA genes, telomeres and centromeres are suited to a similar chromatin environment. In this regard it is interesting that in A. thaliana, telomeric DNA, centromeric DNA and rDNA are among the most heavily methylated sequences in the genome (Vongs et al., 1993).

**Experimental procedures**

**Plant material**

Recombinant inbred lines of A. thaliana (Columbia × Landsberg erecta) and the parental A. thaliana strains Columbia (Col-4, stock number N933) and Landsberg erecta (Ler-0, stock number NW20) were obtained from the Nottingham Arabidopsis Stock Centre (UK).

**Preparation and digestion of agarose-embedded protoplast DNA**

Preparation of protoplasts for enzymatic digestion and CHEF gel electrophoresis were exactly as described (Copenhaver et al., 1995). Briefly, seeds were sterilized, sown and grown for 2 weeks in sterile conditions on semi-solid germination medium (Valvekens et al., 1988). The 2-week-old plantlets were harvested and protoplasts were generated using macerozyme and cellulase according to our published methods for preparing Arabidopsis protoplasts for transient expression (Doelling and Pikaard, 1993; Doelling et al., 1993). The protoplasts were washed and resuspended at a concentration of approximately 1.5 × 10^6 protoplasts ml^-1 (determined by counting in a hemocytometer), mixed with an equal volume of 2% low-melting temperature agarose, and then poured into plug molds and processed according to Anand and Southern (1990). Briefly, this involved treatment with proteases and detergent (in the presence of EDTA to inhibit DNase) to remove proteins and cell membranes. The plugs were then digested with a 25- to 100-fold excess of appropriate restriction endonucleases (100 units; I-PpoI and other enzymes were purchased from Promega) and subjected to electrophoresis through 1% agarose gels (Bio-Rad Pulsed-field certified ultra pure DNA grade agarose) in a Bio-Rad CHEF gel apparatus (von Daelen and Zabel, 1992). A two step electrophoretic regime was used. Gels were run at 200 V using a switching time of 60 sec for the first 14 h followed by a switching time of 90 sec for the final 8 h. The buffer was 50 mM TBE and was circulated continuously. The gels were run in a 4°C cold room without additional cooling. CHEF gels were stained with ethidium bromide and photographed to provide a record of the migration of size markers composed of yeast chromosomes and HindIII-digested bacteriophage lambda DNA. The DNA within the gels was then subjected to UV nicking, denaturation and neutralization prior to transfer to nitrocellulose filters using standard Southern blotting procedures (Sambrook et al., 1989). Following Southern transfer, blots were rinsed and the DNA was covalently cross-linked to the membrane by exposure to ultraviolet light. Membranes were prehybridized prior to addition of high specific-activity probes (approximately 10^9 c.p.m. µg^-1) generated using the random hexamer labeling technique (Feinberg and Vogelstein, 1983). The telomere probe was the 400 bp insert of pAT4 (Richards and Ausubel, 1988). The insert of pAT4 consists almost entirely of tandem TTAGG terminal repeats and was provided by Dr Eric Richards. Following hybridization, blots were washed under stringent conditions (0.5 × SSC, 0.5% SDS at 69°C) and visualized by exposure to X-ray film. Quantitation of radioactive bands was performed using a Molecular Dynamics Phosphor Imager.

**rDNA–telomere junction cloning**

Oligodeoxynucleotides were purchased from Gibco BRL. Template DNA was purified from 2-week-old seedlings of the A. thaliana strain Columbia grown on sterile media. PCR reactions were performed using 50 ng of template DNA, 20 pmol of each primer, 200 μM dNTPs, and Vent Polymerase (New England Biolabs), using the buffer supplied with the polymerase. PCR involved 25 cycles of a 1 min denaturing step at 94°C followed by a 2 min annealing/extension step at 72°C. PCR products were gel purified, treated with the Klenow fragment of DNA polymerase I, and ligated into the EcoRV site of pBluescript SK⁺. Positive clones were sequenced by cycle sequencing using the fmol sequencing kit from Promega.

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