Note

Chromatin Immunoprecipitation Reveals That the 180-bp Satellite Repeat Is the Key Functional DNA Element of *Arabidopsis thaliana* Centromeres

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ABSTRACT

The centromeres of *Arabidopsis thaliana* chromosomes contain megabases of complex DNA consisting of numerous types of repetitive DNA elements. We developed a chromatin immunoprecipitation (ChIP) technique using an antibody against the centromeric H3 histone, HTR12, in Arabidopsis. ChIP assays showed that the 180-bp centromeric satellite repeat was precipitated with the antibody, suggesting that this repeat is the key component of the centromere/kinetochore complex in Arabidopsis.

THE centromere is one of the most important domains of eukaryotic chromosomes. The centromere is responsible for sister-chromatid cohesion and serves as the site for spindle-fiber attachment during cell division. Thus, centromeres play a critical role in faithful chromosome segregation and transmission. Although the functions of centromeres are conserved among all eukaryotic species, the DNA sequences in centromeric regions often show little or no homology among related species. In most eukaryotic species, the centromeres are embedded in long tracks of highly repetitive DNA sequences. Satellite repeats are often the major DNA components of centromeres (Csink and Henikoff 1998).

Although the centromeric DNA sequences are significantly diverged among eukaryotic species, several proteins specific to the centromere/kinetochore complex are highly conserved (Dobie *et al.* 1999; Yu *et al.* 2000). The centromere-specific histone H3 variants (CenH3s) are well characterized and their role in centromere function have been demonstrated (see reviews by Henikoff *et al.* 2001; Sullivan *et al.* 2001). The first CenH3, CENP-A, was identified in humans as a histone-H3-related centromere protein (Palmer *et al.* 1987, 1991). Since then CenH3s have been found in all model eukaryotes (reviews by Henikoff *et al.* 2001; Sullivan *et al.* 2001) and recently in plants (Talbert *et al.* 2002; Zhong *et al.* 2002). CenH3s replace the regular H3 histone in centromeric chromatin (Yoda *et al.* 2000; Ahmad and Henikoff 2001; Blower *et al.* 2002). Blocks of CenH3-associated nucleosomes and regular H3-associated nucleosomes are linearly interspersed in functional centromeres (Blower *et al.* 2002). CENP-A is present only in the functional centromeres of dicentric chromosomes in humans (Warburton *et al.* 1997). Thus, identification of DNA sequences that interact with CenH3 is an effective way to recognize specific DNA sequences involved in centromere function.

The centromeres of *Arabidopsis thaliana* have been genetically mapped (Copenhaver *et al.* 1999) and are cytologically located within distinctive centromeric heterochromatin (Fransz *et al.* 1998, 2000). Various types of repetitive DNA elements, including retroelements, transposons, and telomere-like repeats, were identified in the centromeric regions (Richards *et al.* 1991; Thompson *et al.* 1996; Brandes *et al.* 1997). The most abundant DNA element within the genetically mapped *A. thaliana* centromeres is the 180-bp satellite repeat (Martinez-Zapater *et al.* 1986; Maluszynska and Heslop-Harrison 1991; Murata *et al.* 1994; Round *et al.* 1997; Heslop-Harrison *et al.* 1999). Each Arabidopsis centromere contains several megabases of the 180-bp repeat (Kumekawa *et al.* 2000, 2001; Hosouchi *et al.* 2002). The 180-bp repeat is organized into long tandem arrays (Jackson *et al.* 1998) that may be interrupted by the Athila retrotransposon (Fransz *et al.* 2000; Kumekawa *et al.* 2000, 2001). However, the complete sequences of individual Arabidopsis centromeres are impossible to determine using the currently available sequencing technologies (Arabidopsis Genome Initia-

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tive 2000; Henikoff 2002). It remains an open question whether we have identified all the DNA elements located in the centromeres of Arabidopsis chromosomes. It is not known which centromeric repeats, if any, are involved in centromere function.

The CenH3 in Arabidopsis, HTR12, was characterized recently by Talbert *et al.* (2002). The antibody against HTR12 was localized at Arabidopsis centromeres in both mitotic and meiotic cells (Talbert *et al.* 2002). Using the anti-HTR12 antibody we developed a chromatin immunoprecipitation (ChIP) procedure to determine which centromeric repeats, if any, are incorporated into the centromere/kinetochore complex in Arabidopsis.

Approximately 20 g of young leaf tissues from the *A. thaliana* ecotype Columbia were ground to fine powder with liquid nitrogen, resuspended in 10 ml nuclei isolation buffer [60 mm PIPES, 25 mm HEPES, 10 mm EGTA, 0.35 m sorbitol, protease inhibitor (Roche Applied Science, Indianapolis), and 0.1 mm phenylmethylsulfonyl fluoride, pH 6.7] containing 0.1% cellulase and 0.05% pectinase. The suspension was incubated at 37° for 30 min and filtered with cheesecloth of 120-, 45-, and 30-μm meshes. Nuclei were pelleted by centrifuge at 2000 × *g* for 10 min at 4°. The nuclei were washed twice using 10 ml nuclei isolation buffer and suspended in 1.2 ml of micrococcal nuclease digestion buffer (10% sucrose, 50 mm Tris-HCl, pH 7.5, 4 mm $MgCl_2$, and 1 mm $CaCl_2$).

The ChIP procedure was based on protocols developed by Lo *et al.* (2001) with only minor modifications. Nuclei were digested with 10 units of micrococcal nuclease (Sigma, St. Louis) to liberate nucleosomes. The nucleosome samples were first incubated with preimmune rabbit serum (1:100 dilution), then 4% protein A Sepharose (Amersham Biosciences, Piscataway, NJ) for 4 hr, and centrifuged. The supernatant was incubated with the anti-HTR12 antibody (1:400 dilution) overnight and 25% protein A Sepharose for 4 hr. After centrifugation, the samples were separated into *Sup* (unbound) and *Pel* (bound) fractions. The bound fraction was sequentially washed in 1.2 ml washing buffer (20 mm Tris-HCl pH 7.5, 5 mm EDTA) containing 50, 100, and 150 mm NaCl. Bound immune complex was eluted with 1 ml elution buffer (50 mm NaCl, 20 mm Tris-HCl, pH 7.5, 5 mm EDTA, 1% SDS). Nucleic acids were extracted from both the supernatant and wash-dissociated bound fractions and resuspended in 100 μl of TE buffer (pH 8.0).

Equal amounts (10 μl each) of the *Sup* and *Pel* fractions were blotted on membranes. The membranes were sequentially probed with ^{32}P-labeled centromeric DNA probes (Table 1). The amount of hybridization was quantified using a phosphorimager. Mock experiments using preimmunized rabbit serum served as nonspecific binding controls for each ChIP assay. The percentage of immunoprecipitation (IP) [defined as *Pel*/(*Pel*+*Sup*)] of the mock experiments was subtracted in each case from the percentage of IP of the anti-HTR12 treatments. Each experiment was replicated in three independent tubes. We used the 18S·26S ribosomal RNA genes (rDNA) as negative controls. The rDNAs in Arabidopsis are located at the terminal regions of chromosome 2 and 4 and are distant from the centromeres (Fransz *et al.* 1998).

The ChIP experiment was repeated three times and the results are summarized in Figure 1. We used polymerase chain reaction (PCR)-amplified sequences of the 180-bp satellite repeat as a probe in the slot blot hybridization. The PCR-derived probe may include more variants of the 180-bp repeat family than included in the specific plasmid probes. On average, 15.5% [standard error (SE) = ±2.0%, $n = 3$] of the 180-bp repeat was found in the pellet, whereas only 0.7% (SE = ±0.57%, $n = 3$) of the DNA was detected in the pellet when the same blots were reprobed with the *A. thaliana* rDNA sequences. The 180-bp repeat was immunoprecipitated at a significantly higher level compared to the rDNA (*t*-test, $P = 0.002$).

A number of repetitive DNA elements previously identified in the centromeric regions were tested in the ChIP assays. A 620-kb mitochondrial DNA (mtDNA) in chromosome 2 (Stupar *et al.* 2001) was located within the genetically mapped centromere (Copenhaver *et al.* 1999). This mtDNA locus is only ~100 kb away from the 180-bp repeat array (Arabidopsis Genome Initiative 2000). Bacterial artificial chromosome clone T17H1, which contains 76 kb of mtDNA (Stupar *et al.* 2001), was used as a probe in the slot blot hybridization. The IP percentage of mtDNA was 1.2% on average (Figure 2). Similarly, the 5S ribosomal RNA genes were located in the centromeric regions of Arabidopsis chromosomes 2, 3, and 4 (Arabidopsis Genome Initiative 2000). The IP percentage of 5S rDNA is 2.6% on average (Figure 2).

Several medium repetitive DNA sequences, including 106B, 163A, 164A, 278A, and mi167, were reported in the pericentromeric regions of Arabidopsis chromosomes (Thompson *et al.* 1996; Brandes *et al.* 1997). The 106B repeat shows homology with the long-terminal-repeat region of the Athila retrotransposon, but other repeats are not homologous to any known sequences (Thompson *et al.* 1996). The IP percentage of these repeats ranged from 2.0 to 3.9%.

The majority of the transposable elements in the Arabidopsis genome, including Athila, Tat, Tim, Copia, and another Ty3/*gypsy* element with homology to the centromere-specific retrotransposons in cereals (referred to as the CR homolog hereafter; see also Langdon *et al.* 2000), are concentrated in the centromeric regions (Arabidopsis Genome Initiative 2000). Members of all of these transposable elements can be found within the genetically mapped Arabidopsis centromeres (Kumekawa *et al.* 2000, 2001). Athila elements are highly concentrated in pericentromeric regions. Sequencing analysis has revealed insertions of the Athila elements into the 180-bp satellite arrays (Kumekawa *et*

TABLE 1

DNA probes and their represented centromeric repeats used in ChIP assays

Centromeric repeat	Clone	Clone insert size	Reference
18S•26S rDNA	PCR amplified	1 kb	
180-bp repeat	PCR amplified	180 bp	
mtDNA	T17H1	76 kb	Stupar *et al.* (2001)
5S rDNA	pTa794	410 bp	Gerlach and Dyer (1980)
106B	106B	3 kb	Thompson *et al.* (1996)
163A	163A	400 bp	Thompson *et al.* (1996)
164A	164A	450 bp	Thompson *et al.* (1996)
278A	278A	900 bp	Thompson *et al.* (1996)
mi167	Mi167	1.3 kb	Brandes *et al.* (1997)
Athila	F11C12SHLA16E	1 kb	Kumekawa *et al.* (2000)
Tat	F26J23SHLA17N	2.4 kb	Kumekawa *et al.* (2000)
Tma	T03E02SHSA23K	2.3 kb	Kumekawa *et al.* (2000)
Copia	F11C12SHLA16D	3.8 kb	Kumekawa *et al.* (2000)
CR homolog	PCR amplified	4.1 kb	

Plasmid clones 106B, 163A, 164A, 278A, and mi167 were provided by Dr. C. Dean (John Innes Center, Norwich, UK). Probes for detecting transposons Athila, Tat, Tma, and Copia were provided by Dr. N. Kumekawa (Kazusa DNA Research Institute, Chiba, Japan). Part of the 18S•26S rDNA in Arabidopsis was amplified using primers 5′-ctgcccgttgctctgatgattc-3′ and 5′-cctggtaagtttccccgtgttg-3′. The 180-bp repeat was amplified using primers 5′-catattcgactccaaaacactaacc-3′ and 5′-agaagatacaaagccaaagactcat-3′. Sequences representing the CR homolog were amplified using primers 5′-actttgatgttgcgggctgata-3′ and 5′-gtagtcctttcgggttgtcttc-3′.

al. 2000, 2001). Athila had an IP percentage of 4.5%, and the IP percentage of other transposable elements ranged from −0.6 to 3.9% (Figure 2).

In summary, the 180-bp repeat was significantly increased in the precipitated fractions ($P = 0.002$) in ChIP assays. The levels of precipitation of other centromeric repeats were not significantly higher than that of the rDNA control ($P > 0.05$). The ChIP data suggest that the 180-bp repeat is the main DNA element incorporated into the centromere/kinetochore complex. We cannot rule out the possibility that other centromeric repeats are also incorporated into the centromere/kinetochore complex. However, the copy numbers of these repeats in the functional centromeric chromatin do-

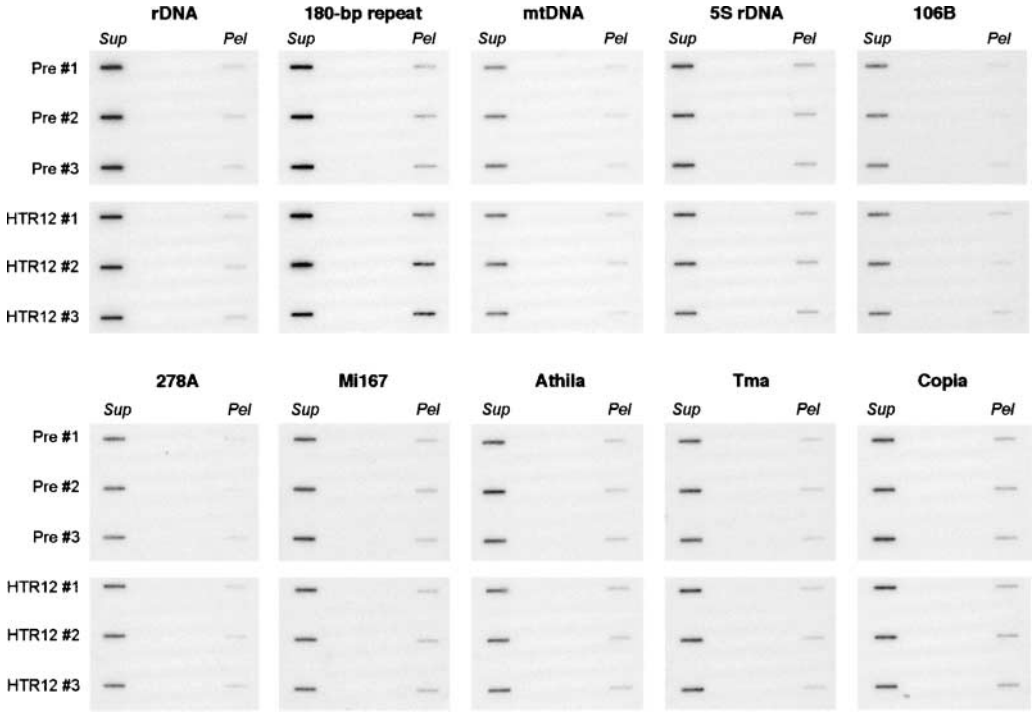

Figure 1.—ChIP analysis of Arabidopsis centromeric repeats. The nucleosome samples were prepared from leaf tissues of *A. thaliana* ecotype Columbia. Unbound (*Sup*) and bound (*Pel*) fractions precipitated with preimmune blood (Pre) and anti-HTR12 antibody (HTR-12) were blotted to membranes and probed with the centromeric repeats. Three samples were blotted in each of the three independent ChIP experiments. Note that the *Pel* fraction hybridization signals derived from the 180-bp repeat are significantly darker in samples precipitated with anti-HTR12 antibody than in samples precipitated with Pre.

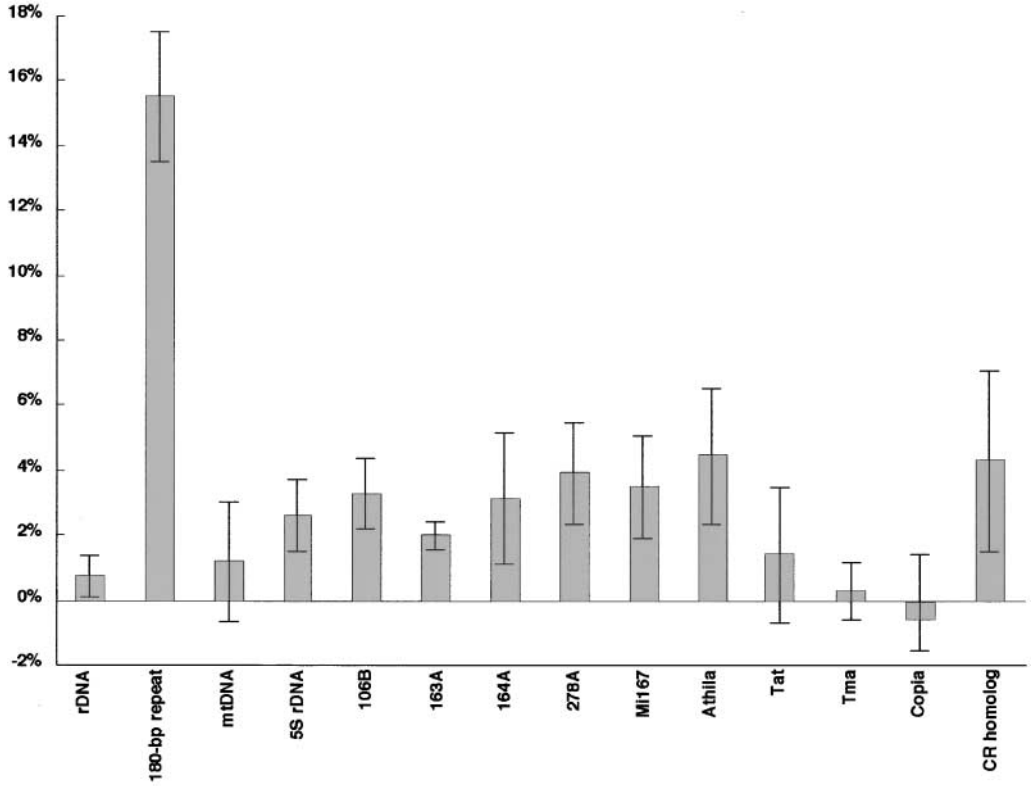

FIGURE 2.—The IP percentage derived from different Arabidopsis centromeric repeats. The columns represent the average IP percentage from three independent experiments. Only the IP percentage from the 180-bp repeat is significantly higher than that of the control (rDNA).

mains would be low and beyond the detection sensitivity of our current ChIP technique.

In a similar ChIP study in maize, ZHONG *et al.* (2002) showed that a centromeric satellite repeat (CentC) and a centromeric retrotransposon (CRM) were precipitated using an antibody against the maize CenH3. CRM belongs to a special retrotransposon family that is highly specific to the centromeric regions of grass chromosomes (MILLER *et al.* 1998; PRESTING *et al.* 1998; LANGDON *et al.* 2000). Frequent insertions of this centromeric retrotransposon into the centromeric satellites were recently demonstrated in rice (CHENG *et al.* 2002) and maize (NAGAKI *et al.* 2003). The ChIP assays in maize suggested that ~33% of the CRM elements are located within the functional domains of maize centromeres (ZHONG *et al.* 2002). In contrast, the Athila elements were not detected in our ChIP assays, suggesting that the Athila elements may rarely insert into the functional domains of Arabidopsis centromeres.

It is not surprising that the 180-bp repeat appears to be a functional component of Arabidopsis centromeres. Satellite repeats are often the main DNA components of eukaryotic centromeres (CSINK and HENIKOFF 1998). The centromeres of maize and rice chromosomes have also been well studied. Both maize and rice centromeres contain satellite repeats (ALFENITO and BIRCHLER 1993; ANANIEV *et al.* 1998; DONG *et al.* 1998). The functional roles of the centromeric satellite repeats in maize and rice have been suggested using ChIP and centromere misdivision studies (KASZAS and BIRCHLER 1996, 1998; CHENG *et al.* 2002; ZHONG *et al.* 2002).

Our ChIP results showed that ~15% of the 180-bp repeat was incorporated into the centromere/kinetochore complex. This ChIP value may be an underestimation because some of the 180-bp repeat bound by HTR12 may not be recovered in ChIP assays. However, the ChIP data suggest that only subsets of the 180-bp satellite arrays are involved in centromere function. Similar phenomena have also been reported in maize and humans. ZHONG *et al.* (2002) showed that ~38% of the centromeric satellite repeat CentC in maize was immunoprecipitated using the anti-CenH3 antibody. Human centromeres contain up to several megabases of the 171-bp α-satellite repeat. However, only subsets of the α-satellite repeats are found in association with CENP-A (WARBURTON *et al.* 1997; BLOWER *et al.* 2002) and show centromeric function when used in artificial chromosome construction (IKENO *et al.* 1998; SCHUELER *et al.* 2001).

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