Differential localization of the centromere-specific proteins in the major centromeric satellite of *Arabidopsis thaliana*

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Accepted 9 February 2004
Journal of Cell Science 117, 2963-2970 Published by The Company of Biologists 2004
doi:10.1242/jcs.01144

Summary

The 180 bp family of tandem repetitive sequences, which constitutes the major centromeric satellite in *Arabidopsis thaliana*, is thought to play important roles in kinetochore assembly. To assess the centromere activities of the 180 bp repeats, we performed indirect fluorescence immunolabeling with antibodies against phosphorylated histone H3 at Ser10, HTR12 (*Arabidopsis* centromeric histone H3 variant) and AtCENP-C (*Arabidopsis* CENP-C homologue) for the *A. thaliana* cell cultures. The immunosignals from all three antibodies appeared on all sites of the 180 bp repeats detected by fluorescence in situ hybridization. However, some of the 180 bp repeat clusters, particularly those that were long or stretched at interphase, were not fully covered with the signals from anti-HTR12 or AtCENP-C. Chromatin fiber immunolabeling clearly revealed that the centromeric proteins examined in this study, localize only at the knobs on the extended chromatin fibers, which form a limited part of the 180 bp clusters. Furthermore, outer HTR12 and inner phosphohistone H3 (Ser10) localization at the kinetochores of metaphase chromosomes suggests that two kinds of histone H3 (a centromere variant and a phosphorylated form) might be linked to different roles in centromere functionality; the former for spindle-fiber attachment, and the latter for chromatid cohesion.

Key words: 180 bp repeat, *Arabidopsis thaliana*, Centromere proteins, Histone H3, Phosphorylation

Introduction

The centromere of eukaryotic chromosomes is an important structure for precise chromatid segregation during mitotic and meiotic cell divisions. Despite its functional importance, the primary DNA structures are quite variable from yeasts to humans. Budding yeast have short and simple centromeres, the minimum required size of which is only ~125 bp, while higher eukaryotes have much larger and/or more complex centromeres (Choo, 1997). In a model plant, *Arabidopsis thaliana*, the centromeric regions of all five chromosome components are occupied by the major satellite, referred to as the 180 bp or pAL1 family (Copenhaver et al., 1999; Maluszynska and Heslop-Harrison, 1991; Murata et al., 1994; The Arabidopsis Genome Initiative, 2000). This repeat family, most of which have a 178 bp repeat unit, is tandemly arrayed in head-to-tail orientation, and form large clusters (Heslop-Harrison et al., 1999; Murata, 2002; Round et al., 1997). The sizes of the clusters have been estimated to range from 1.4 to 2.3 Mb (Haupt et al., 2001) or from 2.7 to 3 Mb (Hosouchi et al., 2002; Kumekawa et al., 2000; Kumekawa et al., 2001). The sizes of functional centromeres, even during meiosis, were investigated in *Drosophila* minichromosomes (Sun et al., 2003) and maize B-chromosomes (Kaszas and Birchler, 1998). Both studies indicated that centromere repeats of 400-500 kb in length are involved in maintaining their functions, although the centromere organization and DNA sequences are very different in the two organisms. However, a wide variation (65 kb to 2 Mb) in the size of CentO satellites that are located within the functional centromere domain of rice chromosomes has been found (Cheng et al., 2002). To date, there are no reports showing the minimum size required for centromere function in *A. thaliana*.

In the region encompassing the centromere, various kinds of proteins assemble to build up a kinetochore complex together with species-specific centromeric DNA. In yeasts and mammals, the interactions among centromere DNA and proteins have been extensively studied. The critical role of human CENP-A and -C proteins for chromosome maintenance has been shown in several studies; e.g. they appear only on active centromeres and not on inactive ones (Choo, 1997). In particular, CENP-A is thought to be a key protein, since it interacts directly with centromere DNA and replaces histone H3 at the kinetochore region of active centromeres (Warburton et al., 1997). Similar centromeric histone H3 variants are widely found in eukaryotes (Smith, 2002). Recently in *Arabidopsis*, the CENP-A homologue has been identified as the HTR12 protein and shown by immunofluorescent labeling to be colocalized with the 180 bp repeats (Talbert et al., 2002). The close association of the protein with the centromeres was also demonstrated by a chromatin immunoprecipitation (ChIP) assay (Nagaki et al., 2003). In addition, histone H3 phosphorylation at Ser10 has been shown to occur preferentially at active centromeric regions in plants (Houben et al., 1999; Kaszas and Cande, 2000). Therefore, antibodies

raised against these proteins, should serve as good markers for identifying the active centromeres as well as for detecting centromeric and phosphorylated histone H3 in *A. thaliana*.

In this study, we performed a cytological investigation of a cultured cell line of *A. thaliana,* and found numerous structural chromosome changes, e.g. the formation of mini-, dicentric and ring chromosomes. The copy numbers of the 180 bp repeats on these aberrant chromosomes and their centromere activities were investigated by fluorescence in situ hybridization (FISH) and indirect immunofluorescence labeling with anti-HTR12, phosphorylated histone H3 (Ser10) and AtCENP-C (*Arabidopsis* CENP-C homologue). Furthermore, we developed a unique chromatin fiber immunofluorescence labeling system for plants, to provide a means to elucidate the centromeric nucleosome structure.

Materials and Methods

Cultured cells

The *Arabidopsis* cell suspension used in this study was derived from the roots of *A. thaliana*, ecotype Columbia, and maintained at 22°C in modified Murashige and Skoog medium supplemented with 2.0 mg/ml 2,4-D (Mathur et al., 1998). Subcultures were made every week, and some cells were treated with 4 μg/ml aphidicoline for 20 hours according to previously described methods (Menges and Murray, 2002).

For fluorescence in situ hybridization (FISH), the cells were treated with 0.05% (w/v) colchicine for 2 hours and then fixed with acetic ethanol (1:3) at 0°C overnight. For immunofluorescent labeling, the cells were treated with enzyme solution [1% (w/v) Cellulase Onozuka RS (Yakult, Japan), 0.15% (w/v) Pectolyase Y-23 (Kyowa Chemical Products, Japan) in 0.6 M sorbitol] for 30 minutes to release protoplasts, and then fixed with 4% (w/v) paraformaldehyde solution in PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) at room temperature for 30 minutes.

Chromosome preparations

Chromosome preparations were made according to the method of Murata et al. (Murata and Motoyoshi, 1995; Murata et al., 1997) with modifications. Two to three days after subculture, the cell suspensions were treated with enzyme solution [(0.3% (w/v) Cellulase Onozuka RS, 0.3% (w/v) Pectolyase Y-23, 0.3% (w/v) Cytohelicase (Sigma) in 10 mM citrate buffer (pH 5.5)] for 1 hour at 37°C and dropped on glass slides with fixative (1:3 acetic methanol) and then dried by heat from a flame. After the cells were fixed with paraformaldehyde solution, they were squashed on the glass slides with coverslips. The coverslips were then removed on dry ice. The specimens were stored in PBS at 4°C.

For chromatin-fiber immunofluorescence labeling, cultured cells were treated with the enzyme solution [(1% (w/v) Cellulase Onozuka RS, 0.15% (w/v) Pectolyase Y-23 in 0.6 M sorbitol, 3 mM MES buffer (pH 5.5)] for 2 hours at room temperature. Released protoplasts were collected by centrifugation and stored in 3 mM MES buffer containing 0.6 M sorbitol. Protoplasts were then treated with 0.1% (w/w) Nonidet P-40 in 0.6 M sorbitol, placed on charged glass slides (Superfrost Plus, Fisher Scientific) and covered with coverslips. After pushing the coverslips down gently to stretch the chromatin fibers, the slides were placed in liquid nitrogen. The coverslips were removed and the slides were put into 4% (w/v) paraformaldehyde in PBS for 30 minutes at room temperature. After fixation, the slides were washed once with PBS and used for immunofluorescence labeling.

FISH and immunofluorescence labeling

Chromosomal DNA was denatured at 76°C for 1 minute in 70% (v/v) formamide in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0). The centromeric 180 bp repetitive sequences were amplified from genomic DNA of ecotype Columbia with a set of primers (ATH180F: GATCAAGTCATATTCGACTC, ATH180R: GTTGTCATGTGTAT-GATTGA), which were designed from 180 bp repeat sequences reported previously (Heslop-Harrison et al., 1999). The amplified 180 bp repetitive sequences were then labeled with Fluorescein-High Prime (Roche). Part of an *Athila* sequence, approximately 4.6 kb in length and including an LTR (long terminal repeat) and ORF1, was amplified with a set of primers (AthilaF: ATGACGCCTTCAG-CAAAAGT, AthilaR: TTCACTCCTCCTTGCGATCT) designed from a database sequence (GenBank/EMBL/DDBJ accession no. X81801) (Pelissier et al., 1995), and cloned into the T-easy vector (Promega). The cloned *Athila* sequence was labeled with biotin using the Biotin Nick Translation Mix (Roche) and detected with Streptavidin-Cy5 (Amersham Pharmacia). The slides were counter-stained with 0.1 μg/ml DAPI (4,6-diamino-2-phenylidole).

Anti-phosphorylated histone H3 at Ser10 (Upstate) and anti-HTR12 (Talbert et al., 2002) were used to detect phosphorylated histone H3 and centromeric histone H3, respectively. Those antibodies were produced in rabbits immunized with synthetic peptides corresponding to residues 7-20 (ARKS^{Ph}TGGKAPRKQL) of human histone H3, and to 3-19 (RTKHRVTRSQPRNQTDA) of *Arabidopsis* HTR12 (cenH3), respectively. In addition, the anti-AtCENP-C antibodies were purified from a rabbit immunized with a synthetic peptide (SKVKSFVSDYKKLVD) corresponding to the C-terminal amino acids of *A. thaliana* CENP-C homologue (accession nos. AB128986 and AB128987) (Ogura et al., 2004). The antibodies were diluted 1:200, 1:2000 and 1:200, respectively for application. Signals were detected with Alexa Fluor 546-labeled anti-rabbit antibody (Molecular Probes). In some experiments, anti-HTR12 and anti-AtCENP-C were directly labeled with Alexa Fluor 555 and Alexa Fluor 488, respectively using the Zenone antibody labeling system (Molecular Probes). After immunofluorescence detection, FISH experiments were performed by the same protocols described above.

The hybridization signals were visualized and recorded using a chilled CCD camera (Axiocam, Zeiss) and the images were pseudo-colored and processed using Axiovision (Zeiss) and Photoshop 6.0 (Adobe). The sizes of the 180 bp repeats were estimated on the basis of the FISH signal strengths on metaphase chromosomes by Image Gauge ver.3.4 (Fuji Photo Film).

Results

Chromosome changes and occurrence of centromere repeats

Since the *Arabidopsis* cell cultures used in this study have been maintained for more than 5 years, numerous chromosomal aberrations have occurred. Chromosome numbers among 30 cultured cells varied from 2n=15 to 67 with a mean of 33.5, which is much higher than that of normal plants (2n=10). Structural changes were also frequently observed; some chromosomes were smaller and others larger than those of normal plants (1-2 μm). Among them, dot-like minichromosomes (Fig. 1A), and ring-shaped chromosomes were noted (Fig. 1B).

In normal *Arabidopsis* plants, the centromeric regions of all ten chromosomes are occupied by members of the 180 bp repeat family, although the copy numbers and cluster sizes are variable among chromosomes (Haupt et al., 2001). The size of chromosomes in cultured cells varied considerably, but the 180 bp repetitive sequences were found on all chromosomes investigated by FISH. Even on the dot-like chromosomes, weak signals from the 180 bp repetitive sequences could be

detected (Fig. 1A). Although the ends of SAT-chromosomes, which carry a nucleolar secondary constriction, were sometimes extended and looked like minichromosomes without 180 bp repeats (Fig. 1A), their origins were confirmed by FISH using 18S rDNA as a probe (data not shown). Dicentric-like chromosomes carrying two distinct 180 bp repeat sites were occasionally found (Fig. 1C). The most interesting chromosomes found were also dicentric, but a number of weak signals appeared between two distinct terminal signals (Fig. 1D). In the direct labeling FISH technique employed here, the fluorochrome signal strengths on the centromeric regions were roughly correlated with the estimated size of the 180 bp cluster in *Arabidopsis* plants (data not shown). Therefore, the wide signal variation detected in cultured cells was thought to be closely related to increase and decrease in the copy numbers of the 180 bp repeats. Although there was no clear correlation between the copy number and chromosome size, very weak signals on some small chromosomes were possibly indicative of the minimum copy numbers of the 180 bp repeats allowable for chromosome maintenance during mitosis (Fig. 1E,F).

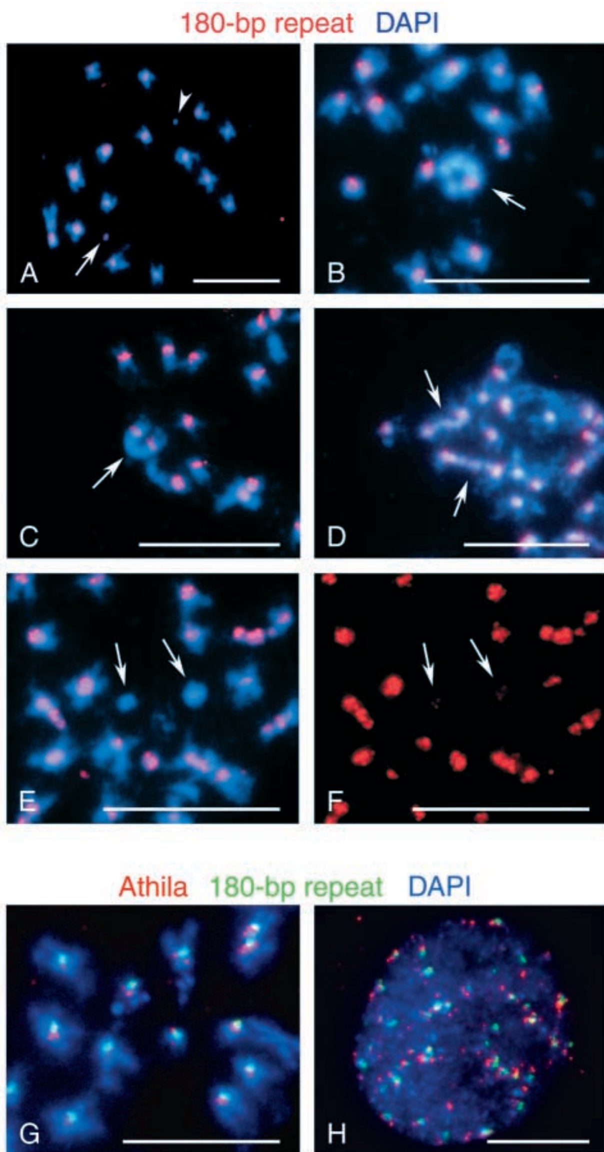


Fig. 1. FISH images of *A. thaliana* cell cultures probed with 180 bp repetitive sequence (A-H) and with *Athila* (G,H). Chromosomes and nuclei were counterstained with DAPI (blue). The 180 bp repetitive sequences are shown in red (A-F) or green (G,H) and *Athila* as red (G,H). A-F show the following features: (A) the smallest chromosome found (arrow) and the stretched end of SAT-chromosome (arrowhead); (B) a ring-shaped chromosome (arrow); (C) a putative dicentric chromosome with two distinct 180 bp signals (arrow); (D) two chromosomes with two distinct and minor multiple 180 bp signals (arrows); (E) chromosomes carrying very small amounts of 180 bp repeats (arrows); (F) the same metaphase cell as in E showing only the 180 bp signals (enhanced to be visualized clearly). (G,H) A prometaphase (G) and an interphase (H) cell, probed with 180 bp repeats (green) and with *Athila* (red). Scale bars: 5 μm.

In the *A. thaliana* genome, retroelements, particularly *Athila*, are known to disperse over the centromeric and pericentromeric regions (The Arabidopsis Genome Initiative, 2000). Our FISH experiments probed with *Athila* DNA [LTR + ORF1; 212-4837 in accession no. X81801 (Pelissier et al., 1995)] also revealed the centromeric and pericentromeric localization in cultured cells (Fig. 1G). Green 180 bp signals were found to flank rather than overlap with red *Athila* signals in most prometaphase chromosomes, and in interphase nuclei, *Athila* appeared preferentially at the peripheral regions of the 180 bp repeats (Fig. 1H).

Detection of centromeric proteins and their relationship to the 180 bp repeats

In *Arabidopsis*, the 180 bp repetitive sequences are clustered at the centromeric regions, and are believed to be related to kinetochore assembly. As described above, however, the cluster sizes varied considerably in the cultured cells. To ascertain the centromere activity of the 180 bp clusters detected by FISH, we applied three kinds of antibodies; one against *Arabidopsis* centromeric histone H3 or HTR12 (Talbert et al., 2002), one against AtCENP-C and one against human phosphorylated histone H3 at Ser10. In this study, we have developed an efficient technique that makes it possible to detect sequentially centromeric proteins and DNA and to reveal their precise spatial relationship.

Immunosignals from anti-HTR12 appeared on all chromosomes observed, but were limited to the outer layers of the centromeres (Fig. 2). Subsequent FISH analyses showed that HTR12 proteins colocalized with the 180 bp repetitive sequences, but did not completely overlap with them (Fig. 2). The shape and signal strength of HTR12 proteins on the chromosomes were quite uniform, whereas the number of the 180 bp repetitive sequences was variable. For the chromosomes with low copy numbers of 180 bp sequences, the signals from the 180 bp sequences and from anti-HTR12 overlapped completely at the kinetochore regions or outer layers of the centromeres (Fig. 2G). In contrast, for the chromosomes with a large copy number of 180 bp repeats, FISH signals covered only part of the outer (kinetochore) regions (Fig. 2D-F). On the dicentric chromosomes with two 180 bp repetitive sites, HTR12 signals appeared at both 180 bp sites (Fig. 3D). In anaphase bridges, HTR12 signals appeared at both ends of the structures (Fig. 3E). The *Athila* DNA sequences were

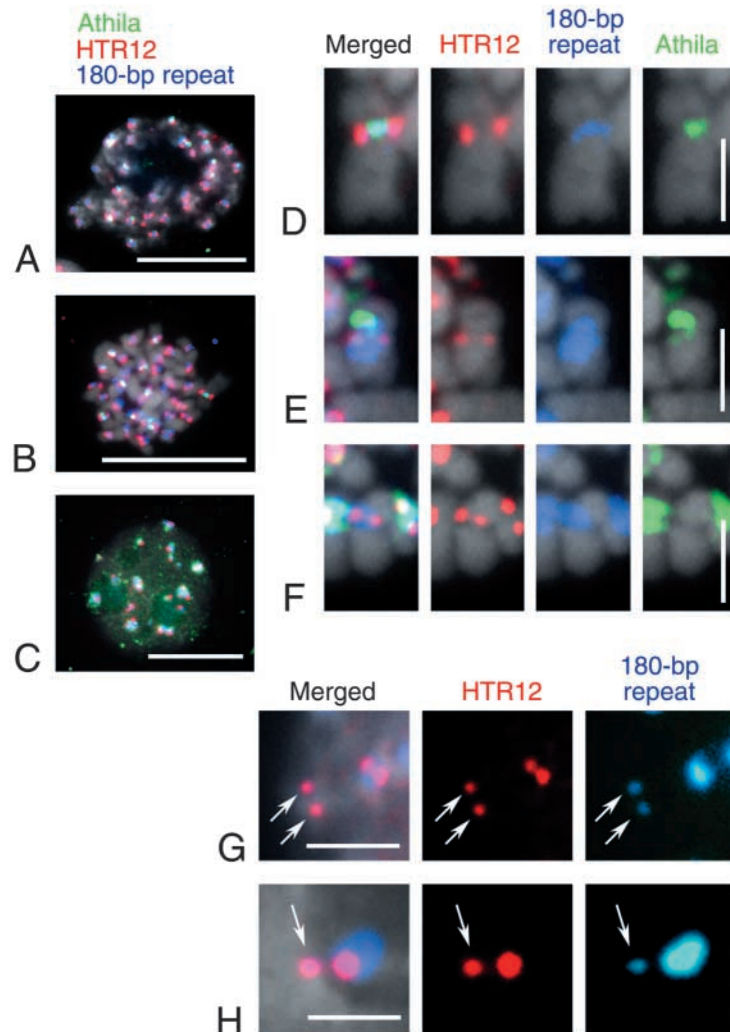


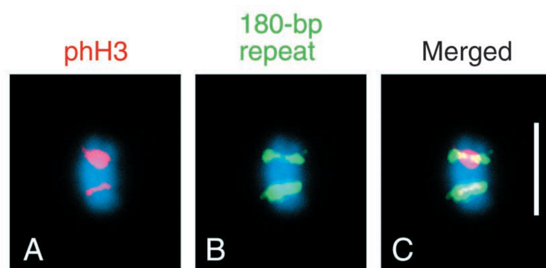
Fig. 2. Immunofluorescence labeling of HTR12 and FISH detection of 180 bp repeats and *Athila.* (A-C) Immunosignals of HTR12 (red), FISH signals of 180 bp repeats (blue) and *Athila* (green) on prometaphase chromosomes (A), metaphase chromosomes (B) and in an interphase nucleus (C). Scale bars: 5 µm. (D-F) HTR12, 180 bp and *Athila* signals on the three (as in A-C) chromosomes, and the superimposed images. Scale bars: 1 µm. (G,H) HTR12 (red) and 180 bp repetitive sequence (blue) on two different sized prometaphase chromosomes (G) and in an interphase nucleus (H). Arrows indicate completely overlapping signals. Scale bars: 1 µm.

detected near the 180 bp sites, but they did not overlap with the anti-HTR12 signals (Fig. 2D-F).

At interphase, anti-HTR12 signals almost always colocalized with the 180 bp repetitive sequences, but did not encompass all of the area covered by the 180 bp signals (Fig. 2G,H), as observed in metaphase chromosomes. The *Athila* signals at interphase were found to be located near the 180 bp sites, but in most cases they did not overlap with HTR12 (Fig. 2C), as was observed in prophase to metaphase cells.

The observation that the 180 bp regions were not fully covered with HTR12 suggested that the CENP-A homologue, i.e. HTR12 protein, binds only to part of the 180 bp repeat clusters. To ascertain the localization of HTR12, we developed a chromatin-fiber immunolabeling and FISH technique. This made it possible to detect the HTR12 protein with its antibody and subsequently the 180 bp repeat and *Athila* DNA on the same chromatin fibers (Fig. 4). As shown, chromatin fibers were well extended on the slides. However, the immunosignals from HTR12 appeared preferentially on the chromatin tangles. Up to 30 of the dot-shaped signals could be counted in each case, and the numbers observed were variable among nuclei, but generally seemed to correspond to the number of chromosomes or centromeres present. In contrast, almost all the FISH signals from the 180 bp repeats were observed as threads, although some parts were tangled to form knobs (Fig. 4B,G). Interestingly, these large knobs corresponded to the HTR12 immunosignals (Fig. 4C,G). The *Athila* retroelements were shown to be close to or overlap with the 180 bp repeats on metaphase chromosomes (Fig. 2D-F); however, in chromatin fibers, the FISH signals were discontinuous and scattered over the chromatin fibers (Fig. 4D). The HTR12 immunosignals did not colocalize with *Athila* signals, but appeared on the borders of the 180 bp clusters (Fig. 4E).

The antibody against part of AtCENP-C was also



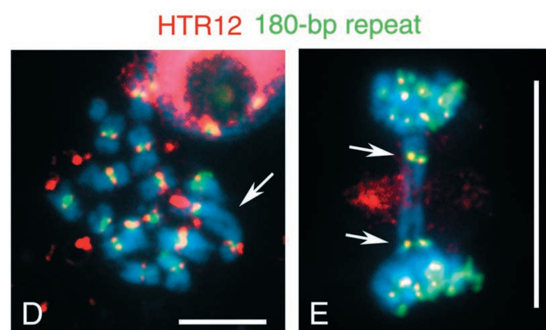


Fig. 3. Immunofluorescence labeling of phosphorylated histone H3 and HTR12 on putative dicentric chromosomes. Chromosomes were counterstained with DAPI (blue). (A) Immunosignals from phosphorylated histone H3 (red), (B) FISH signals from 180 bp repetitive sequence (green) on a dicentric chromosome, (C) superimposition of A and B. Scale bar: 2 µm. (D) A metaphase cell containing a putative dicentric chromosome (arrow). HTR12 and two 180 bp repeat sites appear as red and green, respectively. Scale bar: 2 µm. (E) An anaphase cell with chromatin bridges. Arrows indicate HTR12 signals (red) and FISH signals of 180 bp repeats (green). Scale bar: 5 µm.

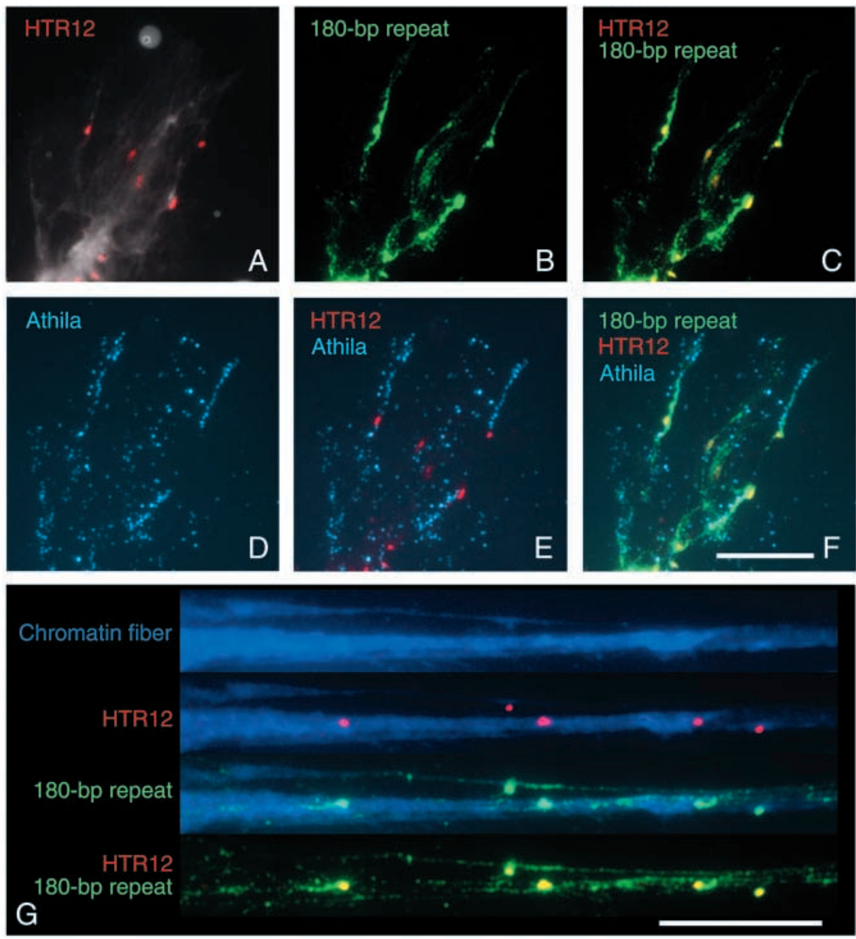


Fig. 4. Immunofluorescence labeling of HTR12, and FISH detection of 180 bp repetitive and *Athila* sequences on extended chromatin fibers. (A) Immunosignals from HTR12 (red) on DAPI-staining extended chromatin fibers (white). (B) FISH signals from 180 bp repeats (green). (C) Superimposition of the HTR12 signals in A and the image in B, with overlapping regions shown in yellow. (D) FISH signals from *Athila* (blue). (E) Superimposition of HTR12 signals in A and the image in D. (F) Superimposition of 180 bp repeat signals in B and the image in E. Scale bar: 5 μm. (G) Immunosignals from HTR12 and FISH signals from 180 bp repeats on fully extended chromatin fibers. The knobs of 180 bp repeats are colocalized (yellow) with immunosignals of HTR12. Scale bar: 5 μm.

applied to the cultured cells. Immunosignals of anti-AtCENP-C appeared on all chromosomes observed, and their size and shape were similar to those of anti-HTR12 that appeared in the same region (Fig. 5). AtCENP-C was found to be located at the kinetochore regions or the outer layers of the centromeres and completely colocalized with HTR12, but did not fully overlap with the 180 bp repeats.

Phosphorylation at Ser10 of histone H3 is known to occur in both animal and plant chromosomes; however, its centromeric localization has only been found in plants chromosomes such as rye, barley, broad bean (Houben et al., 1999; Manzanero et al., 2000), corn (Kaszas and Cande, 2000) and *A. thaliana* (F.S. and M.M., unpublished data). Fluorescence immunolabeling with anti-phosphorylated histone H3 revealed that Ser10 of histone H3 at the centromeric regions of all chromosomes was phosphorylated (Fig. 6B,F). All these regions were shown by subsequent FISH analyses to carry the 180 bp repetitive sequences (Fig. 6A,B,E,F). The signal distribution of the phosphorylated histone H3 was limited to the centromeric regions of metaphase chromosomes, which largely overlapped with the 180 bp repeat sequences. The signals from phosphorylated histone H3 appeared on metaphase chromosomes, but not in prophase or anaphase cells (data not shown). In all dicentric chromosomes observed, phosphorylation signals always appeared on both 180 bp sites (Fig. 3A-C). However, the signal strengths were variable and were not necessarily proportional to the signal strength of 180 bp repetitive sequences. Phosphorylated histone H3 did not colocalize with HTR12 proteins on centromeric regions, and always appeared in the internal parts of the centromeres, which was in contrast to the outer localization observed for HTR12 (Fig. 6C,G).

Discussion

Requirement of the 180 bp repeats for chromosome maintenance

The 180 bp family is the major centromeric satellite, and occupies the centromeric regions of all chromosomes in *A. thaliana* (Copenhaver et al., 1999; The Arabidopsis Genome Initiative, 2000). Therefore, it is thought that the clusters of the 180 bp family assemble the kinetochore proteins, although this assertion has not been substantiated to date. In the cultured cells used in this study, both numerical and structural changes of chromosomes have occurred. The copy numbers of the 180 bp repeats on chromosomes were quite variable, but there were no chromosomes lacking the 180 bp repeat site. This suggests that the 180 bp repeats are essential for maintaining the function of the centromere and for transmitting chromosomes to daughter cells.

Since the signal strengths detected by direct FISH with fluorochrome probes are known to be correlated with the copy numbers of the target DNA sequences, the weak signals on small chromosomes were thought to reflect the minimum

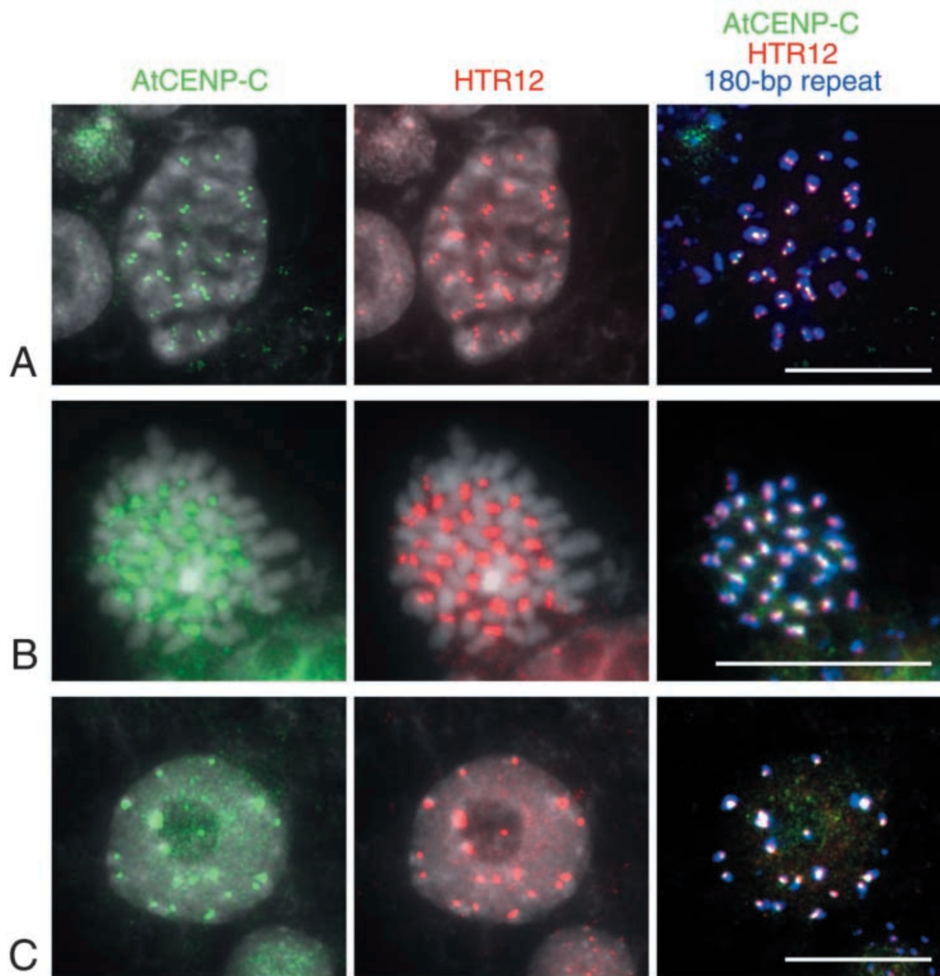


Fig. 5. Immunolabeling of anti-AtCENP-C and anti-HTR12, and FISH with 180 bp repeats as a probe. Immunosignals of anti-AtCENP-C (green) and anti-HTR12 (red) and FISH signals of 180 bp repeat (blue) detected on a prometaphase cell (A), a metaphase cell (B) and an interphase cell (C). Counterstaining of chromosomes or nucleus with DAPI appears gray. Scale bars: 5 μm.

viable copy numbers of the 180 bp repeats. The digital imaging quantification of the signals indicated that the approximate size of the smallest 180 bp site is 80-110 kb, assuming that the 180 bp cluster of chromosome 1 is 2.26 Mb (Haupt et al., 2001) or 3.0 Mb in length (Hosouchi et al., 2002). This estimate is comparable to the size of CentO repeats in chromosome 8 of rice (Cheng et al., 2002).

For chromosomes having two distinct 180 bp sites, we concluded that both sites had centromere activities, since anaphase bridges were frequently observed and the centromere-specific proteins and modifications were detected as discussed below. However, the centromere activities of the multiple minor 180 bp sites observed between two distinct terminal sites on specific chromosomes (Fig. 1D) were not ascertained.

The 180 bp repeats and centromere activity

The sizes of 180 bp repeats on chromosomes were variable in the cultured cells used here, and therefore it was of interest to determine their activities in the centromere. In mammalian chromosomes, centromere activities are known to be closely associated with the localization of specific centromeric proteins such as CENP-A and -C (Choo, 1997). This sort of close relationship between the localization of proteins and centromere activities has hitherto not been clearly demonstrated in plants. However, since the centromeric proteins homologous to human CENP-A and -C have been found or predicted in maize (Dawe et al., 1999; Zhong et al., 2002) and *A. thaliana* (Yu et al., 2000; Talbert et al., 2002; Ogura et al., 2004), it should be also possible to detect centromere activity in plants by localization of these homologues. Our sequential detection of HTR12 (a centromeric histone H3 variant of *A. thaliana*), AtCENP-C (*Arabidopsis* CENP-C homologue) and the 180 bp repeats revealed that these two centromeric proteins colocalize with the 180 bp repeats. This indicates that all 180 bp repetitive sites detected have centromere activity, except in the case of the multiple minor sites observed between two distinct sites (Fig. 1D). In plants, phosphorylation of histone H3 at Ser10 appears at centromeric or pericentromeric regions of chromosomes at prophase to anaphase (Houben et al., 1999; Kaszas and Cande, 2000). This protein modification is thought to be necessary for maintaining sister chromatid cohesion at centromeric or pericentromeric regions rather than for condensation. It was also reported that this phosphorylation is not observed in inactive centromeres in barley (Houben et al., 1999). In the *A. thaliana* cell cultures examined here, immunosignals of anti-phosphorylated histone H3 appeared on all distinct 180 bp repetitive sites detected by FISH. The phosphorylation was also detected at both sites of the 180 bp repetitive sequences on putative dicentric chromosomes (Fig. 3A-C). The

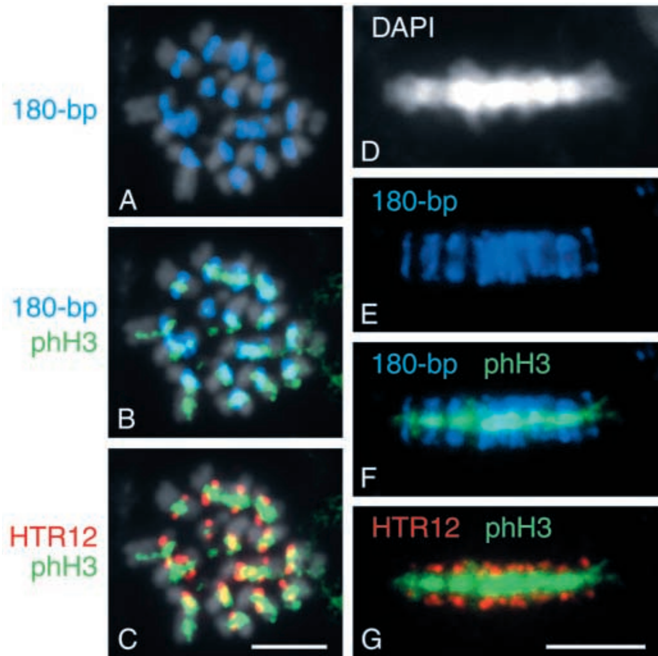


Fig. 6. Immunofluorescence labeling of phosphorylated histone H3 and HTR12, and FISH detection of 180 bp repetitive sequences on mitotic metaphase chromosomes. (A-C) Polar view of chromosomes spread on the equatorial plate. (D-G) Equatorial view of chromosomes arranged on the equatorial plate. (A,E) FISH signals from 180 bp repeats (blue), (B,F) FISH signals from 180 bp repeats (blue) and immunosignals from phosphorylated histone H3 (green), (C,G) immunosignals from phosphorylated histone H3 (green) and from HTR12 (red). Chromosomes counterstained with DAPI appear gray (A-C) and pale gray (D). Scale bars: 2 μm.

centromere activity on these 180 bp sequence sites was also supported by observation of anaphase bridges, since the bridges were thought to bring together chromosomes with two or more active centromeres. These findings and previous reports indicate a close correlation between histone H3 phosphorylation and centromere activity.

In human cell cultures, both histone H3 and CENP-A were shown to be phosphorylated at Ser10 (analogous Ser7 in CENP-A) (Zeitlin et al., 2001). However, the human anti-phosphohistone H3 (Ser10) used in this study seemed to recognize phosphorylation of the ordinary *Arabidopsis* histone H3 rather than that of the centromeric histone H3 variant (HTR12). This was thought to be a result of the considerable differences in the N-terminal amino acid sequence of HTR12 compared to those of human and *Arabidopsis* histone H3, despite the fact that Ser10 is conserved amongst all of them. This observation was also supported by the differential localization of HTR12 and Ser10 phosphorylation of histone H3 in metaphase cells (Fig. 6C,G). Therefore, this finding suggests that nucleosome structures containing ordinary, though phosphorylated, histone H3 are also formed around the centromeric regions in *A. thaliana*. The lack of overlap between centromeric H3 and phospho-H3 was also found in mammals (van Hooser et al., 2001) and *Drosophila* (Blower et al., 2002). These findings indicate that the similar structural features of centromeric chromatin are conserved in plants and animals.

Distribution of centromere histone H3 over the 180 bp clusters

The HTR12 protein is a centromere-specific histone H3 variant in *A. thaliana,* and was shown to colocalize with the 180 bp repetitive sequences of all centromeres (Talbert et al., 2002). The *Zea mays* centromeric histone H3, CENH3 was also detected at the kinetochore regions of the centromere and colocalized with centromere-specific tandem repeat CentC and with centromeric retroelement CRM (Zhong et al., 2002). These results indicate wide conservation of CENP-A-like proteins and their close relationship to the centromeric satellites. In our study, the spatial relationship between HTR12 protein and 180 bp repetitive sequences was investigated by sequential combination of immunolabeling and FISH. In the cell cultures studied here, drastic changes in the copy numbers of 180 bp repetitive sequences had occurred, however, all chromosomes carried the 180 bp repetitive sequences despite their variation in size (Fig. 1E,F). For chromosomes with low numbers of 180 bp repetitive sequences, immunofluorescence from anti-HTR12 and FISH 180 bp repetitive sequences was found to completely overlap with the kinetochore regions. This result supports the previous report (Talbert et al., 2002), and suggests that HTR12 protein and 180 bp repetitive sequences form a high-order structure at the kinetochore region.

Binding of HTR12 proteins to the 180 bp repeats was also demonstrated by chromatin immunoprecipitation (ChIP) assays (Nagaki et al., 2003) (H. Sato, F.S and M.M., unpublished data). However, both results indicated that only a portion (12-15%) of the 180 bp repeats precipitate with anti-HTR12. This agrees with our present findings that the immunosignals from anti-HTR12 could not entirely cover the 180 bp FISH signals in interphase nuclei (Fig. 2C) and in metaphase chromosomes carrying large 180 bp clusters (Fig. 2H). This suggests that HTR12 proteins localize only on a limited number of copies of 180 bp repeats. Our chromatin-fiber immunolabeling and FISH technique demonstrated clearly that the immunosignals from HTR12 appear preferentially on the condensed knobs of the 180 bp repeats, but not on the extended fibers (Fig. 4). This new finding revealed that only a limited proportion of the 180 bp tandem repeat cluster on each chromosome binds to HTR12 proteins for the assembly of the kinetochore structure. From the observations made in this study, it is not clear whether or not the knobs are in fact kinetochores.

In *Drosophila* and humans, both centromeric histone H3 variant (Cid and CENP-A) and ordinary histone H3 appeared to be interspersed as alternating blocks at the centromeric domain (Ahmad and Henikoff, 2002; Blower et al., 2002). However, this sort of organization could not be detected in *Arabidopsis* centromeres. It might be because of differences in the centromeric chromatin structure between plants and animals, or the different experimental procedures and specimen preparations employed. More detailed studies are needed.

In *Z. mays*, the CENP-A homologue CENH3 protein was suggested to form a complex with a centromere-specific repetitive sequence CentC and a centromeric retroelement *CRM* (Zhong et al., 2002). Potential roles of centromeric retrotransposons in centromere formation have been discussed (Cheng and Murata, 2003; Gindullis et al., 2001; Hudakova et al., 2001), but in *Oryza sativa,* the contribution of centromere-specific retrotransposon *CRR* was questioned (Cheng et al.,

2002). In *A. thaliana,* centromeric retrotransposon *Athila* families were shown to be distributed on pericentromeric regions and flank the core of the 180 bp satellites (Hosouchi et al., 2002; Kumekawa et al., 2000; Kumekawa et al., 2001; The Arabidopsis Genome Initiative, 2000). In our FISH experiment, small and large *Athila* signals appeared at centromeric and/or pericentromeric regions on almost all chromosomes of cultured *Arabidopsis* cells. However, the major immunosignals for HTR12 protein did not colocalize with FISH signals of *Athila*. This suggests that *Athila* retroelements do not play important roles in assembling centromeric proteins and in functioning centromeres, although the *Athila* elements are fairly divergent (Wright and Voytas, 2002) and the whole elements in the genome might not be covered with the *Athila* probe used in this study.

We thank C. Koncz and M. Umeda for supplying the *A. thaliana* cell culture and Paul B. Talbert for supplying the antibody against HTR12. We are also grateful to J. S. (Pat) Heslop-Harrison and Trude Schwarzacher for their technical suggestions. This work was supported by the Core Research for Evolutionary Science and Technology, Japan Science and Technology, Japan.

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