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Comparison of Surface Structures between Extended and Condensed Stages of Barley Chromosomes Revealed with Atomic Force Microscopy

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Summary Chromosomes are super-molecules consisting of DNA, histone and chromatin proteins, which specifically appear within a cell at the cell division. We analyzed barley chromosomes by atomic force microscopy (AFM) to elucidate its structural basis. Mitotic chromosomes were taken from root tips of barley (*Hordeum vulgare* L., cv. Minorimugi) using the EMA (enzymatic maceration and air-drying) method after synchronization of cell cycle. Both the air-dried or critical point dried specimens were observed in air by a dynamic force mode. This observation technique enables to obtain three-dimensional image data on the surface structure of barley chromosomes at high resolution without any metal coating. The details of the higher order chromosomal structure such as chromatin fibers were clarified with the biological significance. Acidic treatment (*e.g.*, acetic acid treatment) for removing proteins was useful to obtain clear images of basic chromosomal structure. Thus, it is concluded that the AFM has a great potential for investigation of molecular structures of chromosomes.

Key words Atomic force microscopy (AFM), Chromosomal structure, Barley, Chromatin.

The genetic information of the eukaryotic cell is stored in chromosomes. These structures are generated by condensation of chromatin fibers, which consist mainly of DNA and histone proteins. Numbers of papers have reported that modes of chemical modifications in DNA and histone proteins are closely related to the regulation of gene expression (reviewed by Rice and Allis 2001). They have revealed that regulation of the chromatin superstructure is closely related to the basic biological function such as transcription, replication, repair and DNA packaging through the cell cycle (Demeret *et al.* 2001). Primary control occurs through interactions between specific regulatory DNA sequences and large variety of transcription factors. Transcriptions are also regulated by which post-translational modification of histone proteins produces the structural change of chromatin (Struhl 1998); the phosphorylation of histones, for example, plays a role in inducing chromatin condensation (Dmitry *et al.* 2001). Acetylation of histone H4 is widely discussed as a factor, which causes the structural change of chromatin and serves for transcriptional regulation (Turner 2000, Wako *et al.* 2002, 2003).

Viewed from the ultra-structural aspect, the basic structure of chromatin is the nucleosomes, which consist of histone octamer (H2A, H2B, H3, H4) and double-stranded DNA; the DNA is wound twice around the histone octamer to produce the "beads-on-a-string" form (reviewed by

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Kornberg and Lorch 1999). Nucleosomes further form the higher structure of 30 nm fiber (chromatin fiber) in diameter (*e.g.*, Leuba and Zlatanova 2002). It is still not fully understood how the chromatin fibers are organized into 30 nm fiber and then chromosomes at cell division. Further information, preferably three-dimensional information on the higher-order structure of the chromosomes is required.

Thus, the present study has applied the atomic force microscope (AFM) to observations of chromosomes. The AFM belongs a family of scanning probe microscopes (SPM) and can obtain topographic images of the sample surface by monitoring the interaction force between the probing tip and sample surface. The AFM has a potential for visualizing non-conductive materials in both air and liquid environment with high resolution. In the present study, we show AFM images of barley chromosomes and discuss the plant chromosomal higher structure based on the AFM results.

Materials and methods

Preparation of barley chromosomes

Root tips of *Hordeum vulgare* L. (barley), cultivar "Minorimugi" ($2n=14$) were used in the present study. The seeds were germinated at 25°C in dark and roots of 1–2 cm in length were excised for chromosomal preparations. Two methods were applied for synchronization of cell cycles. First, the roots were pretreated with distilled water at 0°C for 24 h, fixed with ethanol/acetic acid (3 : 1) at room temperature for several hours and stored at −20°C until use. The roots were washed in tap water for 20 min and the meristematic portions (*ca.* 2 mm long) without the root caps were dissected using a small knife. They were macerated in an enzyme solution containing 4% Cellulase Onozuka RS (Yaklut, Tokyo), 1% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo), 10 mM EDTA, pH 4.2 at 37°C for 60–90 min. After washing with distilled water, each root tip was placed on a glass slide (Matsunami, Osaka) and squashed either in ethanol/acetic acid (3 : 1) or 45% acetic acid by pushing a cover slip or by tapping with the tip of fine forceps.

Acetic acid treatment and drying method

Some of specimens squashed in ethanol/acetic acid were directly air-dried, and the others were dehydrated in a graded series of ethanol (70, 80, 90, 100%) at room temperature, transfered to isoamyl acetate, and dried in a critical point dryer using liquid CO_2.

Specimens squashed in 45% acetic acid were, on the other hand, treated as follows: The squashed root tip sandwiched between the glass slide and coverslip was warmed in 45% acetic acid by heating the slide with an alcohol lamp for several seconds. This treatment was repeated until cytoplasmic proteins around chromosomes appeared to be removed under a phase-contrast microscope. Some of them were further treated in a humid chamber with 45% acetic acid for 10–36 h. These specimens were dipped in 70% ethanol, rinsed with water, treated in 1% osmium for 5 min, dehydrated in a graded ethanol series, transfered to isoamyl acetate, and dried in the critical point dryer using liquid CO_2. The specimens prepared by the enzymatic maceration method were subjected to either air-drying or 45% acetic acid treatment for 24 h in a humid chamber before air-drying.

AFM imaging

AFM studies were carried out using a SPA-300 scanning probe microscope controlled by a SPI 3700 probe station (Seiko Instruments Inc. Chiba, Japan). This microscope was equipped with a piezo translator with a maximum x–y scan range of 100 μm width and a z range of 1.2 μm. Cantilevers used were rectangular with a force constant of 40 N/m and a resonance frequency of 350 kHz (SI-DF 40, Seiko Instruments Inc.). The specimens were observed in air with a dynamic force mode. All images were displayed simultaneously as the topographic (height) mode and vari-

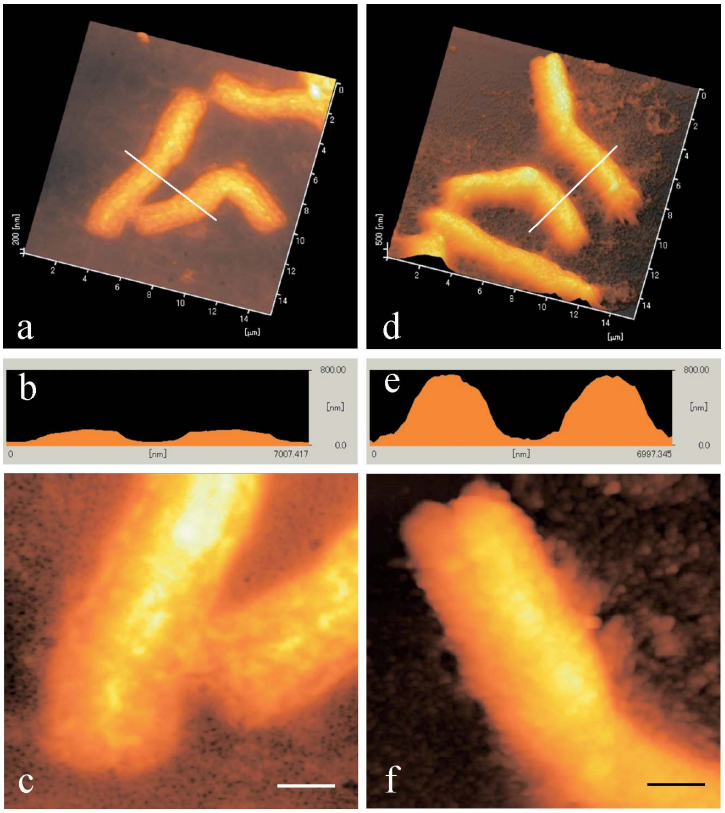

Fig. 1. AFM images of barley chromosomes at metaphase stage. a. Barley metaphase chromosomes which are simply spread in ethanol/acetic acid and air-dried after the enzymatic maceration. The AFM data are displayed as the height image. b. Vertical sectioned image under the line in Fig. 1a. The air-dried chromosomes are about 180 nm in height. c. Enlarged AFM image of Fig. 1a. Bar shows 1 μm. d. The critical point-dried chromosomes. e. The critical point-dried chromosomes are about 800 nm in height. f. The enlarged AFM image of Fig. 1d. Granular substances present around the critical point-died chromosome; the substances are probably due to nuclear and/or cytoplasmic protein complexes attaching to chromosomal surface structure and transferring to the daughter cells. Bar shows 1 μm.

able deflection mode.

Results

AFM in a dynamic force mode provided us with the three-dimensional surface images of barley chromosomes in air. The images contained quantitative information on the sample height as well as the width. We firstly observed the metaphase chromosomes (Fig. 1a), which were spread in ethanol/acetic acid (3 : 1) and simply air-dried after the enzymatic maceration. Chromosomes in the air-dried specimens were very flat (150–200 nm) due to the effect of the surface tension of water (Fig. 1a, b). The cylindrical profile with characteristic constriction of the centromeric region could be roughly determined under a low magnification as indicated by arrows (Fig. 1a). The surface of the chromosomes and their vicinity appeared continuously covered with a thin layer of unknown substances (Fig. 1c). Thus, it was difficult to analyze the surface details of the chromosomes under the preparing conditions currently applied. We then obtained AFM images of critical point-dried metaphase chromosomes prepared with the same method as above. The chromosomes showed a

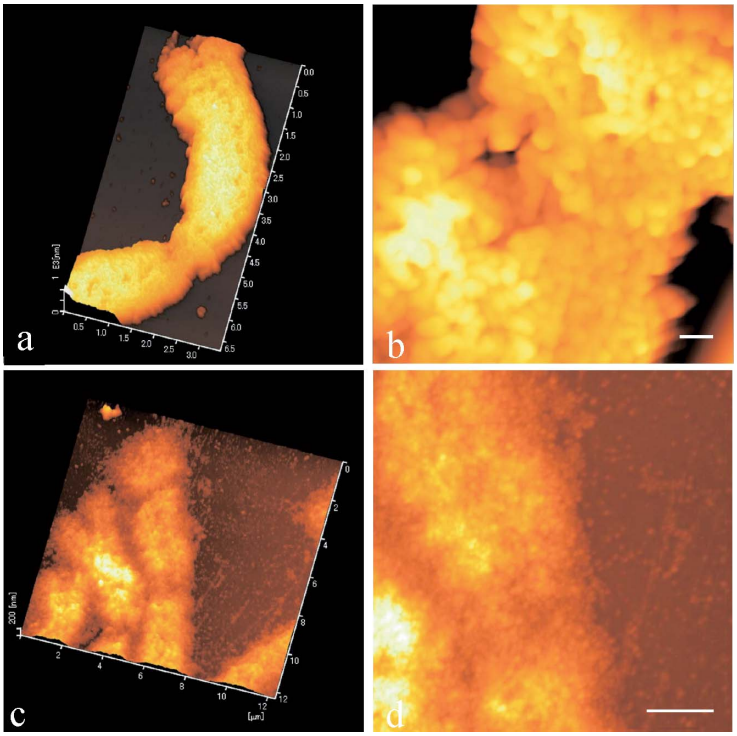

Fig. 2. The effect of acetic acid treatment for metaphase chromosomes. a. Chromosome treated in warm 45% acetic acid before critical-point drying. Since cytoplasmic proteins were removed in this method, the surface structure of the chromosome can be observed clearly and precisely. b. Enlarged AFM image at the centromeric region. The chromosomes are covered with globular protuberances on their surface, in which are sharply displayed in. Several fibrous structures (*ca.* 60–80 nm) were present in this region and connected the two arms. Bar shows 100 nm. c. Chromosomes treated in 45% acetic acid at 30°C for 30 h. The surface structure of the chromosome is shown as the height mode. d. Closer view of the centromeric region. Thin fibrous structures are present around chromosomes as shown by an arrow, probably because this treatment remove surrounding proteins to appear the higher-order structure of the chromosomes. Bar shows 1 μm.

well-defined cylindrical shape about 650–800 nm in height (Fig. 1d, e). The surface of chromosomal arms as well as the centromere exhibited granular structures that were also observed on the surface of glass slide around the chromosomes (Fig. 1f). These granular substances were, thus, considered as nuclear and/or cytoplasmic protein complexes, which were carried into new daughter cells and were not removed under the conditions.

We then observed samples that were prepared by warming in 45% acetic acid followed by critical point-drying. In this method, cytoplasmic proteins surrounding the chromosomes were effectively removed. The surface of the metaphase chromosomal arms showed a globular (or knobby) appearance (Fig. 2a, b). The NOR (Nucleolar organizing region) chromosome appeared in Fig. 2c was identified as barley chromosome 7 (5H) (Kakeda *et al.* 1991). The size of the knobby substances was rather uniform and the images reminded us of the dense package of knobby substances or fibers in the chromosomes. The centromeric region was, on the other hand, characterized by the presence of several fibrous structures (60–80 nm wide). These fibers connected the two arms of the chromosome (Fig. 2b). When specimens were prepared by the squashing method in 45% acetic acid and treated in a humid chamber with 45% acetic acid at 30°C for 30 h, metaphase chromosomes flattened and appeared considerably unraveled (Fig. 2c, d). These chromosomes had fine (60–80 nm wide, Fig. 2d) granules on their surface. Long and thin (60–80 nm thick, Fig. 2d) fibers often

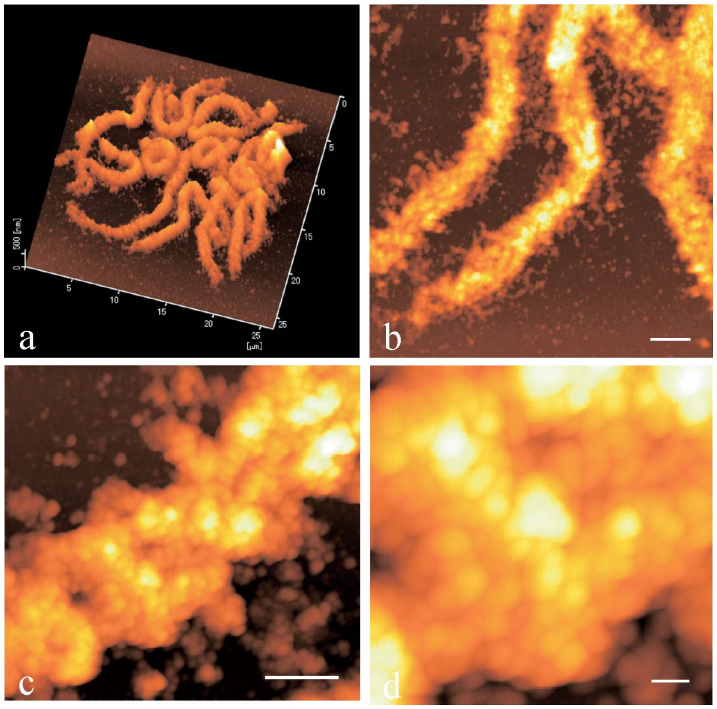

Fig. 3. Prometaphase chromosomes treated with the same method as in Fig. 2. a. Entire view of whole prometaphase chromosomal spread ($2n=14$). The chromosomes at prometaphase stage are looser in structure than those of metaphase. b–d. Closer views of the prometaphase chromosomes. The chromosome itself consists of globular structures, which are similar in size and structure to those observed in the metaphase chromosomes. Bars in Fig. 3b–d show 1 μm, 500 nm, and 100 nm, respectively.

extended from the chromosomes to the surroundings as indicated by arrows. Granules of the same size were also scattered around the chromosomes.

In order to obtain more detailed information on the chromosome structure, we also observed prometaphase chromosomes, which were treated with warmed 45% acetic acid and prepared by critical point-drying (Fig. 3a). At a low magnification, the prometaphase chromosomes were characterized by the presence of a series of transverse between chromatids or oblique grooves within the chromatid, which, as a whole, appeared to ran spirally or in zigzag way along the long axis of the single chromatids (Fig. 3b). The pitch of the grooves ranged from 500 to 600 nm (Fig. 3b, c). At a high magnification, the prometaphase chromosomes consisted of globular structures, which was similar in size and structure to those observed in the metaphase chromosomes (Fig. 3d).

Discussion

The atomic force microscope (AFM) (Binning *et al.* 1986), is an instrument which provides three-dimensional surface images of samples by scanning a sharp probing tip over the sample surface. Several unique features of AFM are very useful for biological studies (Ushiki *et al.* 1996, 2002, Hansma *et al.* 2004). Firstly, the AFM can operate both in air and in liquid (Hoshi *et al.* 2004). Imaging in aqueous solution permits the observation of biomolecules under physiological conditions (Drake *et al.* 1989). Secondly, the AFM provides a high signal/noise ratio. Thirdly, conformational changes of biomolecules can be directly visualized (reviewed by Engel and Muller 2000).

Although, some reports of AFM topography of plant chromosome structure in eukaryotic cells, have been reported (Winfield *et al.* 1995, McMaster *et al.* 1996, Ohtani *et al.* 2002), the three-dimensional fine structure of chromosomes has mainly been studied by scanning electron microscopy (SEM) (Iwano *et al.* 1997), yet. Our findings have shown that AFM provides quality three-dimensional images of the chromosome, which are comparable with SEM images. As shown in the present study, AFM has the following advantages over SEM: 1) AFM can directly observe samples without metal coating at high resolution; and 2) AFM images can contain quantitative information on the sample height as well as the width. Thus, we consider that AFM has great potential for contributing to the chromosomal research together with other imaging techniques, such as SEM.

The present study has also shown a suitable preparation method for AFM imaging of barley chromosomes. Our findings indicate that critical-point drying of samples after treatment with warm acetic acid preserves well the fine structure of the chromosome; the simple ethanol/acetic acid treatment removes cytoplasmic protein complexes on and around the chromosome, while the long treatment of acetic acid tends to unravel the original structure of the chromosome. Previous investigators demonstrated that fixation with acetic acid/alcohol selectively removes histone H1 from chromosomes, while non histone protein are retained (Sumner *et al.* 1973, Brody 1974). Histone H1 proteins are thought to be responsible to link adjacent nucleosomes or tie the DNA fibers to and from a nucleosome. Thus, the long treatment of acetic acid may remove histone H1, which decays the higher-order arrangement of the chromosome. The similar results were obtained in human chromosomes after acidic treatment (Sone *et al.* 2002).

There are some hypotheses for the nuclear and chromosome structure such as solenoid structure model (Finch and Klug 1976), solid solenoid model (Bulter 1984), double helical crossed linker structure model (Williams *et al.* 1986), zigzag model (*e.g.*, Leuba and Zlatanova 2002), *etc.* Concerning the models for the metaphase chromosome structure, there are two major candidates: the radial loop model and radial coil model (Pienta 1991). In the former model, the loops of 30 nm fibers are arranged in the chromosomes in radial fashion, in such way that they form the central axis of the chromosome. In the latter model, the 30 nm (solenoid) fibers are wound in a helical fashion into a 200 nm fiber, which is coiled in turn to form the chromatid. In the present study, we observed the globular structures with a diameter of 60–80 nm densely packed in the metaphase chromosomes, which were treated with acetic acid. These globular structures may represent part of folded 30 nm fiber loop faced on chromosome. However, further studies will be needed to settle these arguments. It may be also necessary to observe chromosomes prepared by other methods such as treatment with a buffer containing NaCl and detergent treatment with different concentrations.

Our AFM images also indicate that the centromere is unique in structure; multiple fiber-like structures connected two arms in this portion. This finding is consistent with the previous SEM studies in the plant (Wanner *et al.* 1991, Iwano *et al.* 1997) and mammalian (Rizzi *et al.* 1995) chromosomes. Another remarkable finding is the presence of spiral grooves in the prometaphase chromosome that were not observed in the metaphase chromosome. Previous studies by light microscopy have already shown the presence of the spiral or zigzag structure in both animal and plant chromosomes treated with a buffer solution (Ohnuki 1968). This structure may also correspond to the helical structure revealed by SEM in the metaphase chromosome from pollen mother cell of *Trillium kamtschaticum* ($2n=10$) (Nakanishi *et al.* 1969).

In conclusion, the present study has applied the AFM to the studies on the higher order structure of plant chromosomes. In this study, we succeeded in observing the fundamental structure of barley chromosomes after a 45% acetic acid treatment. Thus, AFM has great potential for visualizing three-dimensionally the chromosomal structure. It may be also attractive to study by AFM the difference of the chromosomal structure between small type (S-type) plant chromosomes (*e.g.*, rice) and large type (L-type) plant chromosomes (*e.g.*, barley) because they have different condensation

dynamics (Fukui 1996).

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