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Surface structures consisting of chromatin fibers in isolated barley (*Hordeum vulgare*) chromosomes revealed by helium ion microscopy

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Abstract

The chromosome compaction of chromatin fibers results in the formation of the nucleosome, which consists of a DNA unit coiled around a core of histone molecules associated with linker histone. The compaction of chromatin fibers has been a topic of controversy since the discovery of chromosomes in the 19th century. Although chromatin fibers were first identified using electron microscopy, the chromatin fibers on the surface of chromosome structures in plants remain unclear due to shrinking and breaking caused by prior chromosome isolation or preparation with alcohol and acid fixation, and critical point drying occurred into dehydration and denatured chromosomal proteins. This study aimed to develop a high quality procedure for the isolation and preparation of plant chromosomes, maintaining the native chromosome structure, to elucidate the organization of chromatin fibers on the surface of plant chromosomes by electron microscopy. A simple technique to isolate intact barley (*Hordeum vulgare*) chromosomes with a high yield was developed, allowing chromosomes to be observed with a high resolution scanning ion microscope and helium ion microscope (HIM) imaging technology, based on a scanning helium ion beam. HIM images from the surface chromatin fibers were analyzed to determine the size and alignment of the chromatin fibers. The unit size of the chromatin fibers was 11.6 ± 3.5 nm and was closely aligned to the chromatin network model. Our findings indicate that compacting the surface structure of barley via a chromatin network and observation via HIM are powerful tools for investigating the structure of chromatin.

Keywords: chromatin fiber; helium ion microscope (HIM); *Hordeum vulgare*; plant chromosome

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Competing interests

All authors listed had contributed sufficiently to the project to be included as authors, and all those who are qualified to be authors are listed in the author byline. We declare no competing interests among all authors and project sources.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data analyzed during this study are included in this published article.

Code availability

Not applicable.

Author Contributions

NO and KF conceived and designed research. CS and RP conducted experiments. GR contributed the development of image analysis tools. CS analyzed data. CS and NO wrote the manuscript. All authors read and approved the manuscript.

Abbreviations

APM: amiprofos methyl

CPD: critical point drying

EM: electron microscopy

FIB/SEM: focused ion beam-SEM

HIM: helium ion microscope

HU: hydroxyurea

IL: ionic liquid

PMSF: phenylmethylsulfonyl fluoride

PI: propidium iodide

SEM: scanning electron microscopy

SHIM: scanning helium ion microscope

SEs: secondary electrons (SEs)

TEM: transmission microscopy (TEM)

DTT: 1,4-dithiothreitol (DTT)

DAPI: 4',6-diamidino-2-phenylindole (DAPI)

Introduction

Chromosomes contain DNA fiber, histone proteins, and non-histone proteins (Pooley et al 1974; Skinner and Ockey 1971; Heyden and Zachau 1971; Shih and Bonner 1969; Huberman and Attardi 1966). The compaction of chromosomes begins with the packaging of DNA into chromatin fibers during interphase. During mitosis, chromatin fibers are compacted into high-order structures, called chromosome, before being separated into two daughter cells. The basic unit of chromatin fibers is the nucleosome, which consists of approximately 147 bp of DNA units coiled around a core of eight histone molecules (two subunits of H2A, H2B, H3, and H4) (Segal et al. 2006) associated with linker histone H1. The size of the nucleosome without the linker is 10 nm (Maeshima et al. 2014). Nucleosome units connect with other units to form 10-nm fibers, known as “beads on a string” (Baldi et al. 2020). Then, higher levels of chromatin fibers appear as shallow supercoiled 30 nm structures, known as solenoids (McGhee et al. 1983). The process of chromatin fiber compaction for the formation of chromosomes has been disputed since the chromosome was discovered in the 19th century. Several models of folding, including the hierarchical helical folding model (Sedat and Manuelidis 1978), dynamic matrix model (Wanner and Formanek 2000), radial loop model (Paulson and Laemmli 1977), chromatin network model (Poirier and Marko 2002), consecutive/nested loop model (Gibcus et al. 2018), and stacked layer loop model (Daban 2015), have been proposed to explain mitotic chromosome folding. The chromatin network model has been regularly referenced in chromosome structure studies (Swedlow and Hirano 2003; Woodcock and Ghosh 2010; Baxter and Aragón 2012). This model states that the chromatin fibers crosslink every ~15 kb of DNA, giving rise to a network structure that gives chromosomes an elastic property, whereby chromosomes can relax and remain the same length after stretching many times (Poirier and Marko 2002).

Light microscopy cannot be used to visualize the chromosome structure due to the resolution of light vision wavelengths at 0.22 μm (Brock 1984). To overcome the limiting resolution of light microscopes, researchers attempted to elucidate the inner and outer structure of chromosome using advanced electron microscopy (EM), including atomic force microscopy (Ohmido et al. 2005), super-resolution microscopes (Morse and Simpson 1988), scanning electron microscopy (SEM) (Iwano et al. 1997), transmission microscopy (TEM) (Manzanero et al. 2002), and focused ion beam-SEM (FIB/SEM) (Uchiyama et al. 2005; Poonperm et

al. 2015; Wako et al. 2020a). As a result, chromosome fibers were visualized and chromosomal proteins, such as TopoII α and KIF4, were located. However, the process of chromosome compaction remains unclear. The main problem of using EM to visualize biomaterials is low contrast image caused by non or semi conductive materials (Clode 2006).

Helium ion microscopy (HIM), or scanning helium ion microscope (SHIM), is a state-of-the-art nanofabrication imaging technique, which harnesses the positive charge of helium ions interacting on the surface of samples (Joens et al. 2013). This technique allows helium ions to be deposited deep into biological samples using high beam energy due to mass of ion higher than electron, producing a small beam spot and high secondary electrons (SEs), demonstrating the brightness of SE emissions (Joens et al. 2013). Therefore, images obtained using HIM show high contrast, a high depth of field, and a high resolution, compared to images obtained using SEM. HIM is expected to produce images with a maximum resolution at 0.5 nm (Wanner and Formanek 2000; Bell 2009; Joens et al. 2013) and has low beam damage compared with conventional EM, because helium ions can penetrate deeper in biological samples. Therefore, the backscatter electrons on the surface are a lower propensity than EM. Recently, HIM was used to study the effects of magnesium ions on the structure of the human chromosome, composed of chromatin fibers with a diameter of 11 nm without the need for a metal coating cause of sufficiency SEs and low beam damage (Dwiranti et al. 2014). Thus, HIM is a powerful tool for the nano-level imaging and analysis of the surfaces of biological samples. However, HIM has yet to be used for the study of the structure of plant chromosomes.

Although both plant and vertebrate cells are eukaryotic, plant chromosomes are more difficult to extract than vertebrate chromosomes, as plant cells have cell walls and polysaccharides in the cytoplasm. These components are difficult to remove from chromosome solutions, and can obscure chromosomes during the observation. A standard procedure for plant chromosome isolation includes fixation with ethanol-acetic acid (Martin et al. 1994; Sugiyama et al. 2004). However, this can change the structure of the chromosome by dehydration, as well as destroy chromosome proteins. Moreover, a low mitotic index is also difficult to collect in a large number of chromosomes. In the past, the flow sorting technique was introduced in plants for the high-yield of chromosomes (Lysák et al. 1999). Using this procedure, plant cells were treated with hydroxyurea (HU) to accumulate meristematic cells at the G₁/S interphase for synchrony (Adams and Lindsay 1967), while L-

Amiprophos methyl (APM) was used to depolymerize microtubules for accumulation in metaphase (Morejohn and Fosket 1984). This method has been used to increase the mitotic index above 50% and avoid dehydrating the chromosomes in barley (Doležel et al. 1992). However, chromosomes had to be stained with fluorescence dyes, namely 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI), to make them distinguishable from the nuclei and cell debris. Pre-staining chromosomes with DAPI is incompatible with other techniques that use the different chemicals for counterstaining.

For observation with ordinary EM, chromosomes are typically prepared under critical point drying (CPD) conditions to decrease the distortion of the three-dimensional structure due to the adverse effect of surface tension forces, or with metal coating to increase the secondary electron signal (Squarzoni et al. 1994; Dwiranti et al. 2014). However, these treatments create artifact structures on chromosomes, thus altering the native structures. Recently, the ionic liquid method was introduced for the chromosomes preparation for observation with EM, as an alternative to the critical point drying methods in order to prevent dehydration and to maintain the native chromosome structure (Dwiranti et al. 2012). The coating method for HIM is another important procedure that allows for the observation of chromosomes with their native structure. This study aimed to elucidate the compaction and organization of chromatin fibers in plant chromosomes with their native outer structure intact. Our developed technique was simple to isolate high yields of native and intact barley (*Hordeum vulgare*) chromosomes without the need for alcohol-acid fixation or pre-staining. Chromosomes were prepared using an ionic liquid method, with the procedure adjusted to observe chromosomes at a nano-level resolution using HIM. The unit length of the chromatin fibers and the compaction models in the barley chromosome are discussed in detail.

Materials and methods

Barley root germination and synchronization

Barley seeds (*Hordeum vulgare*, $2n=14$) cultivar 'Minorimugi' were used. The barley seeds were germinated on a moist filter paper for 40-48 h in the dark at $25\pm0.5^{\circ}\text{C}$ until the length of the root tips was 1-1.5 cm. To collect mitotic stage cells, the seeds were transferred to Hoagland's solution (Hoagland and Arnon, 1950)

containing 2 mM hydroxyurea (HU) (TCI, Japan), and incubated for 18 h to synchronize the cells in G₁/S phases. Then, the roots were washed briefly with distilled water and transferred to HU-free Hoagland's solution for 6.5 h. The roots were incubated in 2.5 μ M Amiprofos methyl (APM) (Merck & Co., Inc., NJ., USA) in Hoagland's solution for 2.5 h to arrest the cells at the metaphase stage (Lysák et al. 1999).

Isolation of the barley chromosome

Eighty roots with a length of 1-1.5 cm were cut from the seeds, washed briefly with distilled water, and fixed with 2% formaldehyde (Fujifilm-Wako, Osaka, Japan) in Tris buffer (10 mM Tris, 10 mM Na₂EDTA, 100 mM NaCl, 0.1% Triton X-100, pH 7.5) at 4°C for 20 min. The roots were washed with Tris buffer for 5 min and cut into 0.5-1.5 mm meristem regions before transferring to modified LB01 buffer (15 mM Tris, 2 mM Na₂EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM mercaptoethanol, 1 mM 1,4-dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% Triton X-100, pH 7.5) in a 1.5-ml centrifuge tube (Doležel et al. 1989). The chromosomes were released into the modified LB01 buffer by homogenizing 30-60 times using plastic pestles (Merck, USA). The resulting suspension was filtered through a 50- μ m pore size nylon mesh to remove large tissues and cellular fragments. The flow-through was layered over 40% (w/v) sucrose in Tris buffer and centrifuged at 50 xg (RCF) for 15 min. Nuclei and chromosome clumps (Doležel et al. 1992) were accumulated in the upper sucrose layer, while isolated chromosomes remained in the supernatant layer. To collect isolated chromosomes, the supernatant was transferred and layered over 70% glycerol storage buffer (15 mM Tris-HCl pH 7.2, 2 mM EDTA, 80 mM KCl, 0.2 mM spermine, 0.5 mM spermidine, 1 mM DTT, 0.0175% Empigen BB, and 70% glycerol) (Uchiyama et al. 2005) and centrifuged at 920 xg (RCF) for 10 min.

Chromosome preparation for helium ion microscopy (HIM)

Chromosomes solution was dropped on silicon wafers (TED PELLA Inc., CA, USA) coated with 0.01% poly-L-lysine (Merck & Co., Inc., NJ., USA) for 15 min on ice and fixed with 2.5% (v/v) glutaraldehyde in PBS at 4°C overnight. After fixation, the chromosomes were washed three times with PBS for 5 min each. For HIM observation, the chromosomes were treated with 0.5% ionic liquid (IL) butyl-methyl-imidazolium tetrafluoroborate (BMI-BF₄) (TCI, Japan) for 1 min and dried in -0.1 MPa vacuum pumping system for 2 h (Dwiranti et al. 2012). Before observing with HIM, the silicon wafers with chromosome were coated with a

thin layer of tungsten (SVC-700 LRF; PR Sputtering system) (5-nm thickness). The HIM (Zeiss Orion NanoFab/Helium Ion Microscope) was operated using an acceleration voltage of 30 keV, a resolution at 0.5 nm (He), and a beam current 0.1-100 pA (He).

Imaging processing and chromatin fiber length calculation

ImageJ (version 1.51) (Schneider et al. 2012) was used to analyze the chromatin fiber images, while chromosome images obtained by HIM were subjected to several imaging processes, according to Wako et al. (2020). The analysis of the images obtained by HIM was performed in a series of steps. At the 1st step to 3rd step, the chromatin fiber area was defined from HIM image. At the 4th step to 5th step, mid lib lines were drawn on the chromatin area. At the 6th step to 8th step, the chromatin fiber width lines were drawn perpendicular to the mid lib lines. At the 9th step, the size of the width lines from the chromatin area was measured. The width lines were denoted by various colors.

Results

Isolation of barley mitotic chromosomes

To analyze the outer chromosome structure at the native nano level resolution using an advanced scanning ion microscopy, namely helium ion microscope (HIM), we developed an effective barley chromosome isolation procedure. HIM required a high amount of barley chromosome for observation. To this end, barley roots germinated for 48 h were treated with two chemicals for cell cycle synchronization, namely hydroxyurea (HU), to block the cells in the G₁/S interphase, and Amiprophos-methyl (APM), to accumulate cells metaphase by depolymerizing the microtubules (Adams and Lindsay 1967; Morejohn and Fosket 1984). These two synchronization steps were based on the procedure reported by Lysák et al. (1999). The barley cells recovered from HU blocking within 1 h, and the mitotic index was highest at 7 h after release from 2 mM HU. The meristematic region of the observed and counted cells at mitosis was stained by DAPI and observed under a fluorescence microscope (Fig. 1a). The meristematic regions of the barley roots were 0.5-1.5 mm in length from the root to the tips. The procedures of root synchronization are treated with 2 mM HU for 18 h, then released for 6.5 hr and 2.5 mM APM for 2.5 h to accumulate cells in metaphase. During HU treatment, the meristematic

cells were arrested at the cell stage and remained at G₁/S interphase. In this study, after removing from HU blocking, the cells restarted the cell cycle and showed the highest metaphase cells at 6.5 h. Anaphase cells are also increased at 7 hr. A total of 1,000 cells were counted from three roots, raising the ratio of mitotic cell to 58% after synchronization. Ultimately, 91% (530 metaphase in 580 mitotic cells) of the root mitotic cells were synchronized in metaphase; anaphase and telophase were not observed. Thus, this isolation procedure allows for the collection of large amounts of high quality barley chromosome for HIM imaging.

The quality of the purified chromosome solution was examined by staining with DAPI and observation using fluorescence microscopy. As a result, the chromosomes appeared to maintain their chromosome structure, with no protein degradation, PMSF was used to inhibit protease and dehydration during isolation (Fig. 1b). Up to 31 chromosomes per μL of 70% glycerol buffer were concentrated. Although the chromosome solution contained a small amount of cell debris and interphase nuclei, they did not obscure the chromosome during observation by HIM.

Observation of the surface fibrous structure of barley chromosome by HIM

The images of the chromosomes observed by HIM were expected to be clear with a high contrast, depth of field, and resolution. To this end, the isolated barley chromosome was prepared for the observation of their surface structure using HIM. Briefly, 5 μL of the chromosome suspension (~155 chromosomes) was dropped on the silicon wafer and fixed with 2.5% (v/v) glutaraldehyde to preserve the chromosome structure for the crosslinking of the amine reactive group (Migneault et al. 2004). Then, the chromosomes samples were treated with 0.5% ionic liquid (IL). The excess ionic liquid was removed, followed by drying in a desiccator with a -0.1MPa vacuum pump for 2 h. The silicon slide was coated with 5 nm of tungsten to increase the conductivity on the surface. Without the tungsten coating, the chromosomes were charged up by the low helium ion induced secondary electron generated on the surface of the chromosome, resulting in insufficient secondary electrons for detection by HIM. The tungsten coating gives a high secondary electron yield at the surface, while maintaining a particle size below 1 nm (Heu et al. 2019). Thus, it preserves the chromosome surface while adding a thin layer to improve image contrast, and is less disruptive of the chromosome surface compared with other metal coatings. Thereafter, the barley chromosome was visualized by HIM with an acceleration voltage of 30 keV. The length of the barley chromosome was 4.6 μm with a width of 630 nm (Fig. 2a). The chromatin

fibers were clearly observed at a 25,000-fold magnification (Fig. 2b and 2c). At this high magnification, the fibrous structures were able to be observed on the chromosomes throughout the longitudinal axis of the chromosome. The entire chromosome appeared to be composed of these fibrous structures, suggesting that these structures were the chromatin fibers. The chromatin fibers were folded and connected in no particular directions (Wanner et al. 1991). The majority of the chromatin fibers in the chromosome arms were loosely packed and had a low density, allowing the individual fibers to be visualized (Fig. 2a). However, in some functional specific regions of the chromosomes, for example, the terminal and pericentromeric regions, the chromatin fibers appeared to be tightly packed, making the fibers look more like globular structures (Fig. 2b and 2c, arrows). In addition, we observed a unique figure of chromatin fibers in the centromere region. The chromatin fibers there appeared as straight lines, stretching toward both arms of the chromosomes, parallel in arrangement, and loosely compacted (Fig. 2c, arrowheads).

Image analysis of chromatin fibers

ImageJ was used to analyze the chromatin fibers at the surface of the barley chromosome. Image analysis of the HIM images was performed following a series of steps. At the 1st step, the chromosome image was converted from RGB to 8-bit grayscale and normalized by setting the gray value from minimum 0 to maximum 255 (Fig. 3a). At the 2nd step, the corresponding methods and parameters for the defining chromosome area were searched using a macro script. The results indicated the proper parameters for the chromosome as the mean (radius =10, parameter 1=2). At the 3rd step, the chromatin fiber area was defined using the auto local threshold function with the mean shown in white (Fig. 3b). The mean method was used to select the threshold as the mean of the local greyscale distribution. At the 4th step, the distance map was drawn (white regions) using Chamfer Distance Map of MorphoLibJ plugin (Legland et al. 2016). This plugin computes an approximate distance between the foreground to the nearest background pixel (Fig. 3c). At the 5th step, the mid lib lines were calculated from the chromatin area (white lines) using the Ridge Detection plugin (Steger 1998). This plugin detects a distance map and draws lines (Fig. 3d). At the 6th step, the junction point on the mid lib lines was searched using the macro script. At the 7th step, the junction point of the mid lib lines was removed by the macro script (Fig. 3e). At the 8th step, the chromatin fiber width lines were drawn perpendicular to the mid lib lines. At the 9th step, the size of the width lines was measured from the chromatin

area. The width lines were denoted by various colors based on their lengths (Fig. 3f). As a result of this image analysis, a total of 29,675 width lines and 7,879 mid lib lines of fibers were generated from one chromosome image. Among these, 2056 width lines and 40 mid lib lines were generated from the centromere region.

Calculation of the size of chromatin fibers in barley

Calculating the chromatin fiber size from the whole chromosome surface yields a reliable result that is close to the real size of chromatin. Throughout the barley chromosome, chromatin fibers with a size of 11 nm were detected (Fig. 4a and 4b), while a size of over 28 nm was detected at the connecting point of the chromatin fibers (Fig. 4c and 4d). In addition, the size of the chromatin fibers of the centromeric region was 11.1 ± 3.2 nm. The average size of width lines was calculated from chromatin fibers, as presented in the histogram (Fig. 5). The result shows that the size of the chromatin fibers varied from 1.1 nm to 31.0 nm, with 66.2% of the diameters of the chromatin fibers ranging from 8-14 nm ($n = 19,644$) of the total size of the chromatin fibers and the average chromatin fibers size was 11.6 ± 3.5 nm ($n = 29,675$). These results suggest that the surface structure of the chromosome consists of a chromatic fiber network (Poirier and Marko 2002), where fibers have a length of approximately 11 nm and 28 nm when at the connecting point.

Discussion

Barley has been used as a monocot plant model to study the structure of chromosomes due to its large chromosome using high resolution microscopes. The structures of the barley chromosome have been studied extensively using various types of electron microscopes, including SEM (Iwano et al. 1997; Wanner and Formanek 2000; Houben et al. 2007; Scheoder-Reither et al. 2012; Wanner et al. 2015), TEM (Manzanero et al. 2002), and FIB/SEM (Schroeder-Reiter et al. 2009; Hamano et al. 2014). Despite the barley chromosomes outer structure was visualized, it is difficult to represent the appearance of chromatin fibers based on their native structure. The main methods of chromosome isolation at fixation step, mainly with alcohol and acetic acid (3:1), and chromosome preparation, mainly by critical point drying (CPD), were unable to preserve chromosomal proteins (Kaneyoshi et al. 2015). In fact, both methods were known to dehydrate native chromosomal proteins. To solve this problem, we developed a procedure by which to isolate the barley chromosome for observation

by ultrahigh resolution microscope with a scanning helium ion beam. The two experimental improvements presented in this study were the isolation procedure and the synchronization procedure.

Since the chromosomes of different species of plants have distinct characteristics, different chromosome isolation methods need to be developed specifically for plant species in order to obtain metaphase chromosomes. The plant metaphase chromosomes of several plant species have been successfully isolated (Szabados et al. 1981; Hadlaczky et al. 1983; De Laat and Blaas 1984; Mii et al. 1987). However, the isolation procedures used frequently include alcohol-acetic acid treatment, which can alter the ultrastructure of the chromosomes. In order to avoid the fixation of alcohol-acetic acid treatment and preserve the native chromosome structures, Griesbach et al. (1982) isolated *Nicotiana tabacum* chromosomes using exploding protoplasts in a buffer with a neutral pH. Williams et al. (1993) used 2% formaldehyde to fix and manipulate chromosomes to isolate *Vicia faba* chromosomes. Thus, similar to previous studies, we developed a chromosome isolation method specifically for barley, which allows for the native structure of the barley chromosome to be preserved for its observation at the nano scale.

In this study, we improved HIM sample preparation consisting of these three steps: effective synchronization for barley chromosomes, alcohol-free fixation and dehydration-free preparation. This synchronization procedure can increase mitotic index reaching to 58% in roots meristem, and eventually 91% of mitotic cell at metaphase considering a high number of chromosomes in plant root's meristem easy to collect metaphase chromosomes for the HIM sample preparation. For the synchronization procedure, we combined HU and APM treatment. For cell synchronization, we modified the procedure of APM treatment, performed for 6.5 h after the release from HU treatment, slightly modified to a previous report (Lysák et al. 1999). Because after 2 h of APM treatment, most of the mitotic cells were found at prophase, and, as a result, the chromosomes were over 12 μm long and significantly entangled. The chromatin fibers in prophase chromosomes are flattened out and loosely arranged in the barley chromosomes, similar to a previous report (Wanner et al. 1991). Therefore, APM treatment was prolonged to 2.5 h to allow for the sufficient accumulation of cells in metaphase. APM is analogous to colchicine blockage by depolymerizing the mitotic spindles (Morejohn and Fosket 1984). Thus, it is an effective method for metaphase cell synchronization in barley. In the presence of colchicine, some cells occasionally rescued from the metaphase arrest and go through to anaphase, because the spindle assembly

checkpoint was not fully activated after the treatment with the microtubular depolymerization for several hours. This induces chromosome separation in the absence of spindle fibers. This phenomenon is called colchicine-induced anaphase, or C-anaphase (Chamla 1988; Yih and Lee 2003). C-anaphase chromosomes show separated centromeres and appear as single chromatids (Yih and Lee 2003). Similar to colchicine, APM treatment could induce C-anaphase, and this phenomenon is enhanced when cells were treated with APM for longer than 2 h (Lysák et al. 1999). The majority of the isolated barley chromosomes appeared as single chromatids after this procedure. We hypothesized that these might be C-anaphase chromosomes, as APM treatment was prolonged to 2.5 h. However, when comparing the width of our chromosome (630 nm) (Fig. 2a) with that of the normal barley metaphase chromosome (approximately 1.32-1.81 μm) (Schroeder-Reiter 2004; Wanner et al. 2005; Schneider et al. 2012), our chromosome was half as wide and conform with C-anaphase chromosomes. Replacement of alcohol and acetic acid fixation with 2% formaldehyde can prevent the chromosome protein dehydration and maintain native chromosome structure. Then, the barley cells were lysed and the chromosomes were extracted into the LB01 lysis buffer (Doležel et al. 1989) supplemented with equal amounts of PMSF and DTT as used for the isolation of human chromosomes in a previous study (Uchiyama et al. 2005). After the chromosomes were purified, they were stored in 70% glycerol buffer, according to Uchiyama et al. (2005), to maintain the quality of the chromosomes and increase their concentration.

In this study, HIM was used to visualize the outer structure of the barley chromosome (Fig. 2). As an advanced microscope, HIM provides images with a resolution down to 5 nm (Joens et al. 2013), which is higher than that of the conventional scanning electron microscope. HIM produces images with an increased depth of field and a high surface contrast without the metal coating of biological samples, while minimizing sample damage. HIM has been used to compare the effects of treatment at the sub nanometer level (Sawafta et al. 2014) across biological surface studies, including the imaging of human colon cancer cells (Bazou et al. 2011) and the interaction between *E.coli* bacteria and dragonfly wing (Bandara et al. 2020). In the research field of chromosomal structures, HIM has been used to compare the effects of magnesium ions on the structure of the human chromosome with Osmium coating and non-coating (Dwiranti et al. 2014), proving that HIM is an excellent tool for the study of the outer structure of chromosomes at the sub-nanometer level in biological samples.

We hypothesized that the native structure of chromatin fiber can visualize by the ionic liquid preparation and tungsten coating. Dwiranti et al. (2014) observed dehydrated isolated chromosomes prepared with CPD. However, using this method in the present study, the chromosome areas were dark without metal coating, and we were unable to observe the surface of the barley chromosomes. In our study, the traditional ethanol dehydration and CPD step were replaced with ionic liquid (IL) treatment to ensure the chromosomes remained hydrated. The ion inducing secondary electron generation ratio of hydrated proteins is lower than that of dehydrated proteins. IL-treated chromosomes may have been unable to produce sufficient secondary electrons compared to the CPD treated chromosomes, resulting in dark chromosome areas with depleted ion-induced secondary electron emission (Hill and Rahman 2011; Baggott et al. 2018). The depleted secondary electron emission area occurred with the use of a highly insulating material. Incident He^+ accumulates in the chromosome surface under He^+ ion irradiation. To resolve this problem, the chromosomes were coated with 5 nm of tungsten to enhance the conductivity of the chromosome surface. Tungsten has a high secondary electron yield property and a grain size of <1 nm (Heu et al. 2019). Tungsten coating produces a very thin coating layer and disrupts the surface of the sample less than with platinum (2-3 nm) or gold (10-12 nm) (Heu et al. 2019), whose particle sizes are bigger than that of tungsten. However, the coating with tungsten 5 nm can increase the emission of the secondary electron at the chromosome surface and also increase the thickness of the chromatin fiber in the native structure. The top of the chromatin fiber is thicker than the uncoating region, as seen by Juarez et al. (2018) demonstrated that the same sputtering equipment using aluminum coating showed an increase of a few nanometers. The size of the chromatin fibers at the side might be the same or a few nanometers bigger than the native structure. In our study, we measured the side of the chromatin fiber, and found that the size of native chromatin fiber might be 11.6 nm or a few nanometers smaller.

Using HIM, the average size of the chromatin fibers was 11.6 ± 3.5 nm, with the majority of the chromatin fibers showing a diameter in the range 8-14 nm along the outer structure of the barley chromosome (Fig. 4, 5). For our calculations, 29,675 width lines from throughout the chromosome were used to calculate a value close to that of the real size. The size was close to the beads-on-string basic structure of chromatin fiber (Zhou et al. 2019; Boopathi et al. 2020), similar to previous studies on barley chromatin fiber using SEM (Wanner et al. 1991). Without metal coating, the size of human chromatin fibers is 11.17 ± 2.9 nm (Dwiranti et al. 2014). Although the size of the chromatin fibers of barley chromosomes observed by HIM is comparable

with the SEM images obtained by Wanner et al. (1991), the detailed surface structures of the barley chromosome were different. While Wanner et al. (1991) found chromatin fibers with a knot-like swelling structure on their surfaces, we observed chromatin fibers with a smooth surface. This difference may have arisen due to differences in the preparation of the chromosome. In our study, we avoided dehydrating or degrading the chromosomal proteins by using ionic liquids, which preserve the native chromosomal structure. In contrast, Wanner et al. (1991) prepared chromosomes using acetic acid and ethanol for cell fixation, which dehydrate and degrade chromosomal proteins (Kaneyoshi et al. 2015), resulting in chromatin fiber shrinkage and the appearance of knot like swelling at condensed areas. The chromatin fibers in both arms were tightly compact (Fig. 2b), similar to the findings reported by Wanner and Formanek (2000). The formation of a globular structure at the chromosome terminal (Fig. 2b) and the pericentromeric region (Fig. 2c) made individual chromatin difficult to discriminate, implying that chromatin fibers in these regions are highly compact. This was similar to the study of the human chromosome by Dwiranti et al. (2014), in which globular structures were found throughout the surface of the human chromosomes. However, the human chromosomes were more condensed than the other barley chromosome regions. Interestingly, the size of the globular structure on the human chromosomes (31.7 ± 3.9 nm) in Dwiranti et al. (2014)'s study is comparable with the size of the chromatin fiber connecting points (29.10 nm) in the present study (Fig. 4c, 4d). This may be the same structure in high and low compaction, or represent the formation of a 30-nm solenoid structure. However, at the centromeric region of the barley chromosome, the chromatin fibers exhibited narrow and parallel arrangement stretching to both chromosome arms with loosely compacted. The formation of narrow and parallel might cause by alignment of the kinetochore complex associates with CENP-A nucleosome at centromere for attachment with spindle fiber (Cleveland et al. 2003; Kixmoeller et al. 2020). The complex of kinetochore could connect with an opened conformation of chromatin. After the kinetochore associate with the centromere, the complex makes chromatin fibers around the kinetochore region stretched to accept the binding of spindle fiber, resulting as the narrow and parallel formation.

Using the chromosomal protein procedure and chromatin fiber connecting form represent network model (Poirier and Marko 2002) on the surface of the chromosome, the chromatin fibers of the barley chromosome were found to measure 11.6 ± 3.5 nm. The network model indicates that the crosslink of chromatin fibers occurs every ~15 kb DNA, similar to our connecting points, with the elastic property of the network

structure shown in the centromeric region, at which the chromatin fibers were stretched to both sides with a loose compaction. These results suggest that certain chromosomal proteins exist to help the chromatin fiber crosslink and maintain the chromosome length during metaphase. Ultimately, HIM was found to be suitable for the visualization of the outer structure of chromosomes with a high contrast and at a high magnification on the nanoscale.

Conclusion

In this study, we developed a procedure to isolate a large amount of high quality barley chromosomes for observation and imaging with HIM in order to analyze the outer structure of the barley chromosome in metaphase for the first time. The isolation procedures developed in this study allowed for the preparation of highly concentrated chromosomes with their surface proteins preserved. The clear chromatin fibers of the barley chromosome were found to measure 11.6 ± 3.5 nm in size and form a network model on the chromosome surface. The chromatin fibers were found to exhibit a higher compaction at the terminal and pericentromeric regions of the barley chromosome, observed in the form of globular structures. The chromosome fibers at the centromere were parallel in arrangement and loosely compacted, although both chromosome arms contained tightly packed fibers.

The procedures developed in the present study can be applied to other biomaterials, such as the inner structure of the chromatin axis with DNA and protein interactions. Thus, in future studies, we will investigate both the outer and inner structures of chromatin with the aim of fully elucidating the process of chromatin compaction in the plant chromosome. The flood gun in HIM could be a useful procedure to neutralize the charge, while observing the outer structures of the biomaterials. In the future study, we will use the flood gun to neutralize the uncoated chromosome surface to investigate the outer structure and use FIB/SEM with slice and view functions to investigate the internal chromosome structure. ChromEMT technique (Ou et al. 2017) is also useful to visualize the internal DNA axis of a chromosome.

Author Contribution Statement

NO and KF conceived and designed research. CS and RP conducted experiments. GR contributed the development of image analysis tools. CS analyzed data. CS and NO wrote the manuscript. All authors read and approved the manuscript.

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Figures

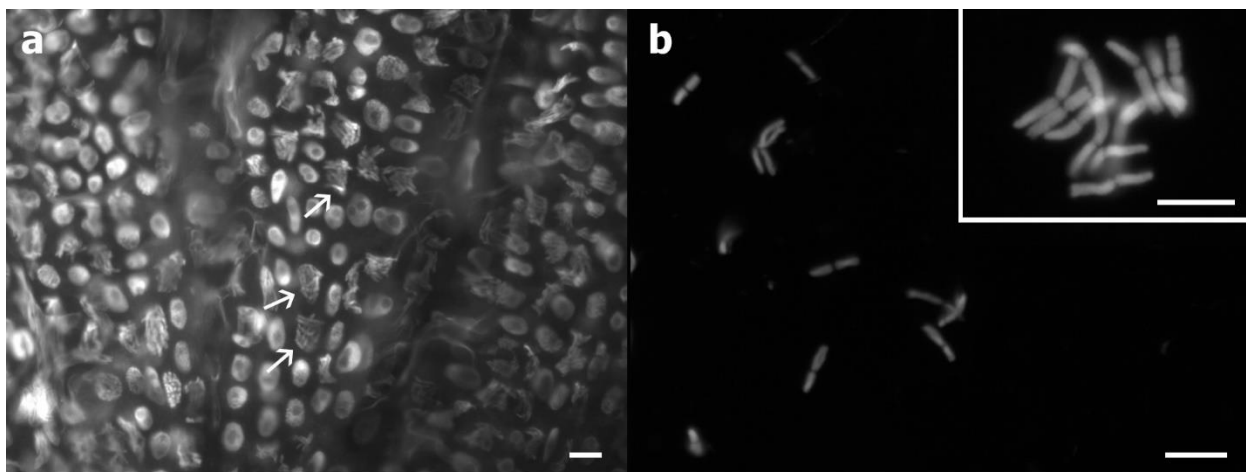


Fig. 1 Barley root tips and isolated chromosomes. Chromosomes were stained with DAPI. **(a)** Cells in the root tip region after synchronized with 2.5 mM HU for 18 h and 2.5 μ M APM for 2.5 h. Arrows show metaphase cells. **(b)** Isolated chromosomes from chromosome purification method. Scale bar = 10 μ m

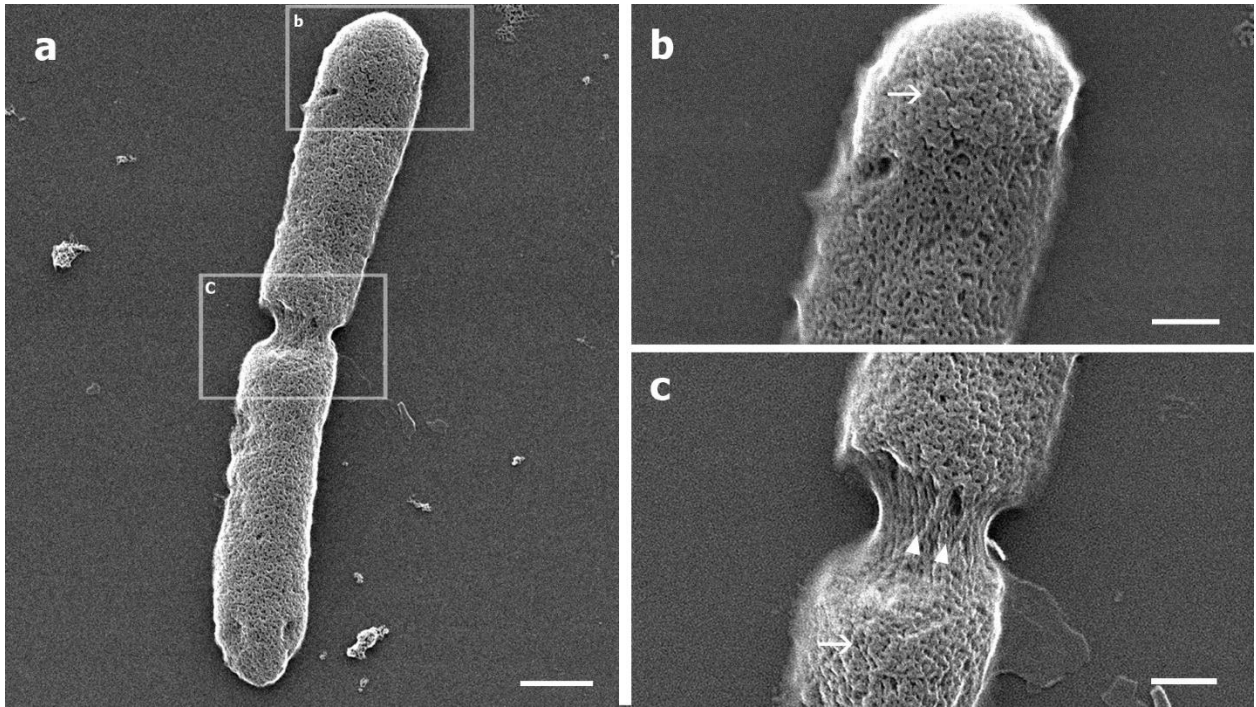


Fig. 2 HIM image of barley chromosome. (a) Overall metaphase barley chromosome. (b) Magnified image of the terminal region of the barley chromosome. (c) Magnified image of the centromeric region of the chromosome. Arrows present the high compacted globular structure. Arrowheads show the parallel arrangement and loose compaction of the chromatin fibers. Scale bar: (a) 500 nm and (b, c) 200 nm.

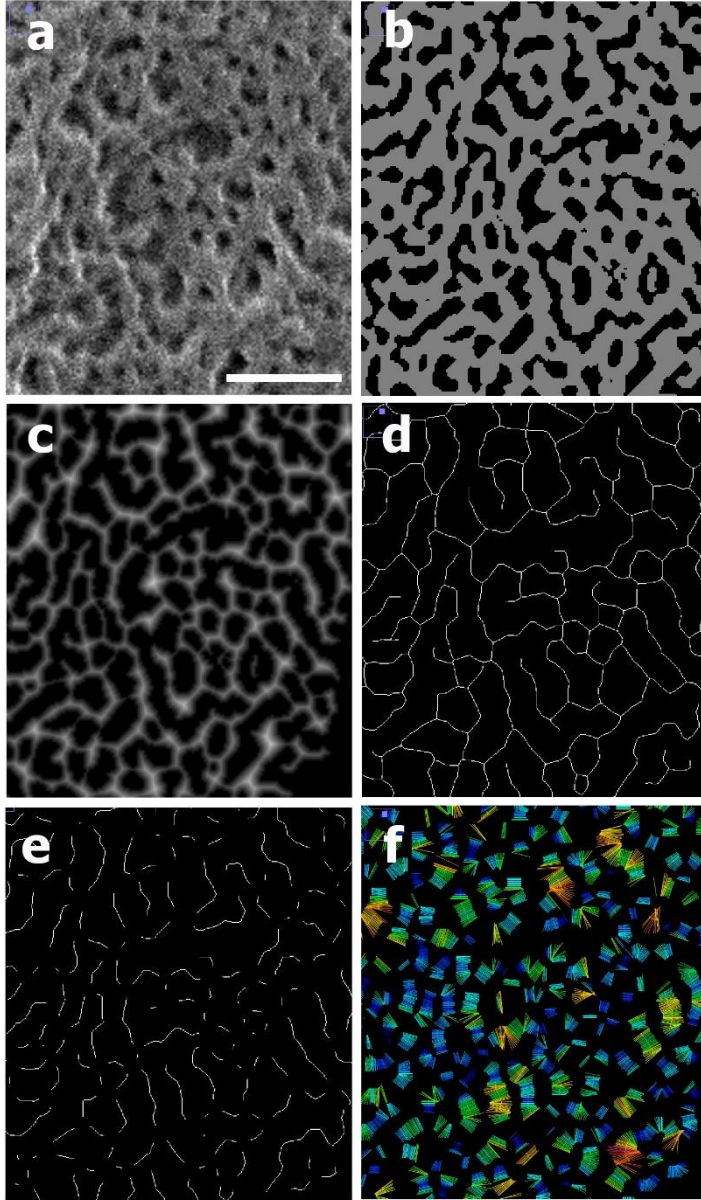


Fig. 3 Image analysis based on the procedure of measuring the diameter of the chromatin fibers. **(a)** The barley chromosome image was normalized by setting the gray value from minimum 0 to maximum 255. **(b)** The chromatin fiber area was defined by auto local threshold function. **(c)** The distance map was drawn from white regions by Chamfer Distance Map of MorphoLibJ plugin. Gray values of chromatin fiber area indicate the distance to the black background area. **(d)** The mid lib lines were calculated from the chromatin area showing white lines using the Ridge Detection plugins. **(e)** The junction points were removed from the mid lib lines. **(f)** The width lines are shown in various colors based on their lengths. Scale bar = 200 nm

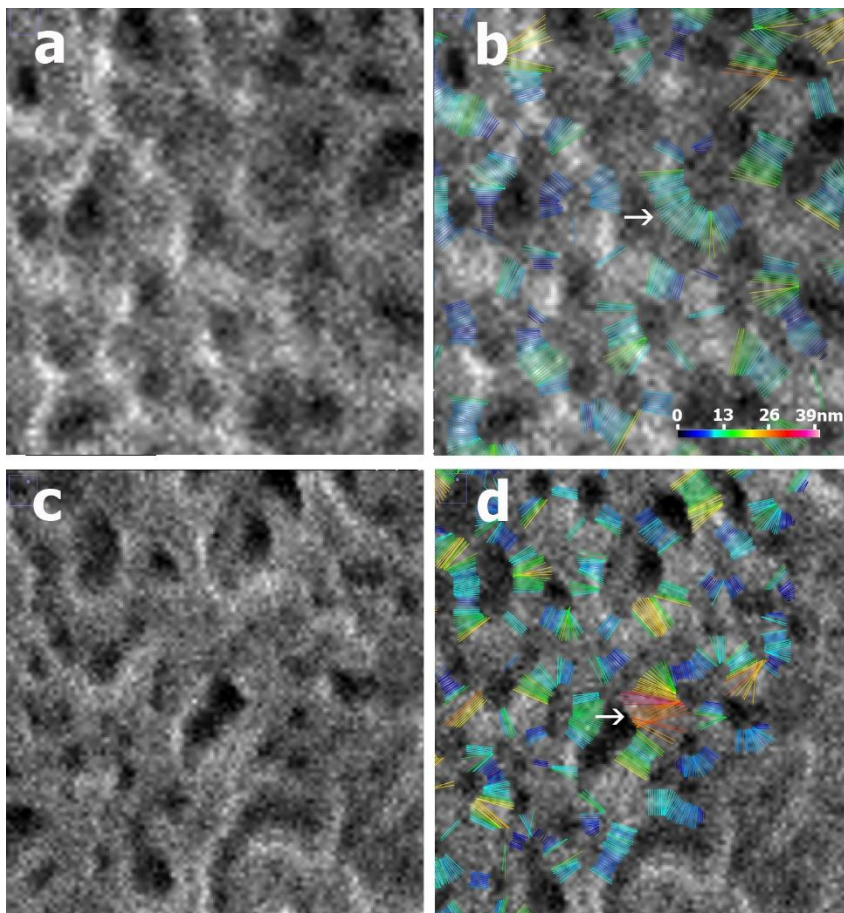


Fig. 4 Chromatin fiber areas and width lines. **(a, c)** Chromatin fibers were defined with gray area. **(b, d)** Merging image of chromatin fiber areas with colored width lines. **(b)** Arrow shows an 11.3 nm width line. **(d)** Arrow shows a 28.5 nm width line.

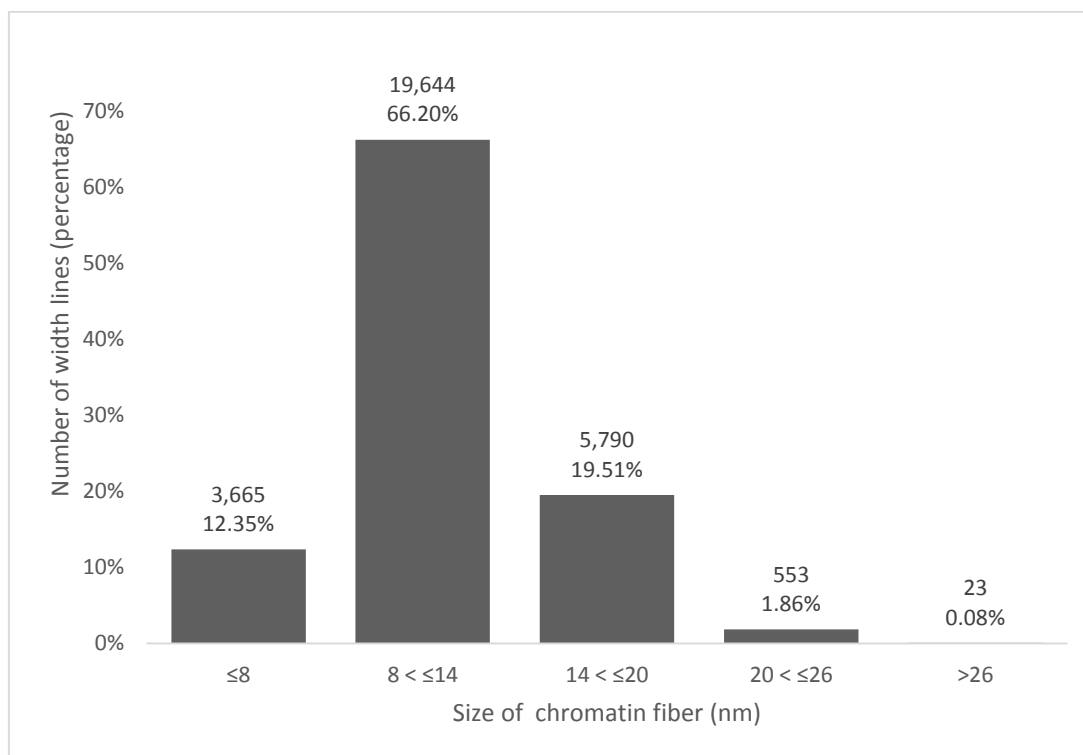


Fig. 5 The diameters of chromatin fibers in the barley chromosome. Histogram showing the distribution of the diameters of chromatin fibers ($n = 29,675$). The average size of chromatin fibers was 11.6 ± 3.5 nm, and 66.2% of the chromatin fibers were 8-14 nm.