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Karyotype Analysis of Buckwheat Using Atomic Force Microscopy

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Abstract: Karyotype analysis and classification of buckwheat chromosomes were performed without chemical banding or staining using atomic force microscopy (AFM). *Fagopyrum esculentum* (common buckwheat) and *Fagopyrum tartaricum* (Tartarian buckwheat) chromosomes were isolated from root tissues using an enzymatic maceration technique and spread over a glass substrate. Air-dried chromosomes had a surface with ridges, and the height of common and tartary buckwheat were approximately 350 and 150 nm. Volumes of metaphase sets of buckwheat chromosomes were calculated using three-dimensional AFM measurements. Chromosomes were morphologically characterized by the size, volume, arm lengths, and ratios. The calculated volumes of the *F. esculentum* and *F. tartaricum* chromosomes were in the ranges of 1.08–2.09 μm^3 and 0.49–0.78 μm^3, respectively. The parameters such as the relative arm length, centromere position, and the chromosome volumes measured using AFM provide accurate karyomorphological classification by avoiding the subjective inconsistencies in banding patterns of conventional methods. The karyotype evolutionary trend indicates that *F. esculentum* is an ancient species compared to *F. tartaricum*. This is the first report of a cytological karyotype of buckwheat using AFM.

Key words: chromosomal volumes, buckwheat chromosomes, atomic force microscopy, karyotype

Introduction

Buckwheat is nature's best source of rutin, a beneficial vitamin C complex flavonoid. Buckwheat's protein is superior to that of many cereal grains, as it includes eight essential amino acids that cannot be synthesized by the human body (Pomeranz et al., 1975). Because of the nutritious advantages of buckwheat, this crop has been considered as a functional food and a medicinal plant. Minor crops are usually less investigated, and hence the genomic resources of buckwheat are limited. Bitter tartary (*Fagopyrum tartaricum*) and sweet common (*Fagopyrum esculentum*) buckwheat have eight basic (Morris, 1951) chromosomes ($2n = 2x = 16$), but the chromosome sizes are small, which makes the cytogenetical analyses complicated. Karyotype analysis complements the marker-assisted breeding through identification of genetic linkage maps. Characteristics of karyotype and variation of the species are an essential part of biosystematics and can provide evidence for further study of molecular phyletic evolution.

Conventional methods of karyotyping are dependent on the visual analysis of banded metaphase chromosomes and on the staining characteristics, which is subjective. Atomic force microscopy (AFM) is a multifunctional molecular toolbox in nanobiotechnology (Muller & Dufrêne, 2008) with the advantage of being able to image single biomolecules under physiological conditions. The key advantage of AFM over scanning electron microscopy (SEM) and other conventional microscopy techniques is that AFM can produce three-dimensional (3D) surfaces of the biological specimen up to atomic resolution in the case of flat and periodic structures.

Only a few AFM studies on the structural analysis of plant chromosomes have been reported: barley (Schaper et al., 2000; Yoshino et al., 2002; Sugiyama et al., 2003), corn and wheat (McMaster et al., 1996), and common soapwort (Di Bucchianico et al., 2008). In our study, the main objective was to observe by AFM buckwheat chromosomes without staining or banding and to obtain topographic features specific to each chromosome. The specific objective is to quantitatively karyotype the chromosomes of *F. tartaricum* and *F. esculentum* buckwheat based on volume parameter.

Materials and Methods

Seeds of *F. esculentum* and *F. tartaricum* were germinated on moist filter papers in petri dishes at room temperature in the dark. For synchronization of the cells, the roots were incubated for 18 h at 25°C in 1.25 M hydroxyurea. Primary root tips were pretreated in an ice water mixture for 24 h. Root tips were collected at a length of 1–2 cm, pretreated in 0.05% colchicine solution for 4 h at 25°C, washed 3× with water (Hadlaczky et al., 1983), fixed in 99% ethanol–acetic acid (3:1) solution, and stored at −20°C. After washing in distilled water for 10 min, the root tips were incubated in an enzyme solution of 2% cellulase Onozuka RS (Yaklut, To-

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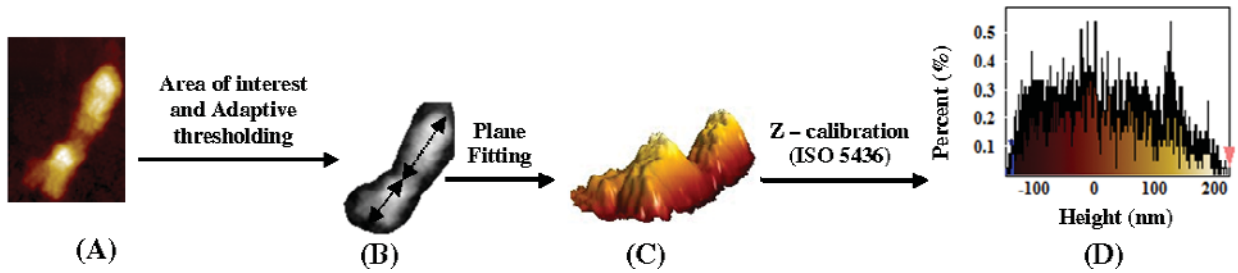

Figure 1. Schematic of the volume calculation process for buckwheat chromosomes (**A**) two-dimensional AFM image of chromosome number 1 from common buckwheat. (**B**) Arm length measurement of the chromosome from the centromere, ◄► short arm length ◄·····► long arm length. (**C**) 3D rendering of the chromosome after plane correction. (**D**) Volume calculation after Z-calibration.

kyo), 2% pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo), 1.5% Macerozyme R200 (Yakult), 7.5 mM EDTA, and 7.5 mM KCl, pH 4.2 at 37°C. The enzyme reaction mixture incubation time was 50 and 60 min for *F. tartaricum* and *F. esculentum*, respectively. The macerated tissues were transferred to alcohol cleaned glass slides and squashed in 30% acetic acid by tapping with the tip of forceps. The squashed specimens were further treated by warming in 30% acetic acid by heating the glass slide for a few seconds. This treatment helped to remove the cytoplasmic contamination and cellular debris surrounding the chromosomes as observed under the phase-contrast microscope (Model BX50, Olympus Corporation, Tokyo, Japan). The samples were then rinsed with 1 × SSC and DW for 3 and 1 min, respectively. Seven well-scattered metaphase sets of chromosomes from each species were selected for karyotype analysis.

The samples were observed with a phase contrast microscope and photographed to determine the location of the chromosomes to be studied by AFM. The glass slides were marked underneath as a possible region of interest for AFM imaging. AFM (Nanowizard, JPK Instruments, Berlin) was used for scanning the samples. Standard silicon cantilevers (Olympus Corporation) with a spring constant of $k = 42\ \mathrm{Nm}^{-1}$ was used. All AFM measurements were carried out in atmospheric air at room temperature (25°C) using the intermittent contact mode with resonant frequency around 300 kHz. The scan speeds were in the range of 0.2 to 0.3 Hz. Both topographic and error signal images were acquired simultaneously during AFM imaging. The same cantilever tip was used for imaging each set of chromosomes to avoid difference in tip profiles. The analysis and measurement of the images were made using SPIP software (Image Metrology, Copenhagen).

Tartary (*F. tartaricum*) and common buckwheat (*F. esculentum*) were chosen because these are the two most important species of buckwheat. Karyotype analyses of both species of buckwheat were conducted by calculating the volume and arm ratio (AR) of individual chromosomes. The AR is defined by the length of the long arm of the chromosome divided by the length of the short arm of the chromosome. Nomenclature for the centromeric positions of chromosome follows Levan et al. (1964), and the karyotype classification follows Stebbins (1971) and Paszko (2006).

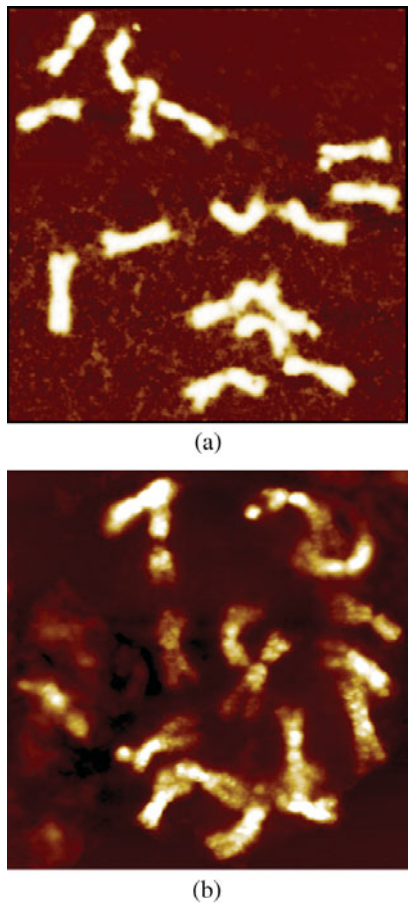

Figure 2. Complete metaphase spread of a typical (**A**) *F. esculentum* and (**B**) *F. tartaricum* buckwheat chromosomes.

In a 3D visual field of chromosome spreads, the area of interest was selected at the base of each chromosome. For the corresponding AFM image $z(x, y)$ data, the plane $z_p(x, y)$ was fitted by the polynomial function $z_p(x, y) = z_0 + \sum_{i=1}^{I} a_i x^i + \sum_{i=1}^{I} b_i y^i + \sum_{i=1}^{I} c_i xy$, where the coefficient a_i and b_i were found by minimizing the square sum error. The step height of the region of interest was calculated in accordance (ISO, 2000) to the definition in ISO 5436-1. The volume of the chromosomes was then calculated (Fig. 1) by multiplying the average of chromosome heights with the selected area at the base of each chromosome.

Table 1. Arm Ratio Pairings and Volumes of Common Buckwheat Chromosomes.

Pair	Arm Ratio (Long/Short)	Pair	Volume (μm^3)
15	1.74	4	2.095 ± 0.044
10	1.63	1	1.968 ± 0.052
14	1.57	3	1.884 ± 0.038
6	1.48	6	1.841 ± 0.037
4	1.36	8	1.813 ± 0.011
9	1.27	9	1.752 ± 0.014
8	1.26	2	1.745 ± 0.105
7	1.25	7	1.734 ± 0.024
2	1.22	15	1.722 ± 0.010
5	1.21	12	1.714 ± 0.016
16	1.20	10	1.573 ± 0.020
13	1.16	5	1.552 ± 0.042
11	1.13	14	1.273 ± 0.004
3	1.13	13	1.201 ± 0.057
1	1.11	16	1.165 ± 0.033
12	1.02	11	1.089 ± 0.078

Table 2. Arm Ratio Pairings and Volumes of Tartary Buckwheat Chromosomes.

Pair	Arm Ratio (Long/Short)	Pair	Volume (μm^3)
11	2.23	4	0.777 ± 0.054
1	2.21	6	0.759 ± 0.043
7	1.78	11	0.746 ± 0.054
9	1.70	2	0.732 ± 0.052
16	1.68	1	0.715 ± 0.063
15	1.58	5	0.698 ± 0.057
3	1.55	9	0.688 ± 0.064
8	1.53	10	0.669 ± 0.080
2	1.52	7	0.656 ± 0.077
13	1.50	15	0.632 ± 0.095
6	1.49	3	0.605 ± 0.012
10	1.48	13	0.587 ± 0.096
4	1.22	16	0.562 ± 0.104
14	1.17	12	0.518 ± 0.077
5	1.15	8	0.503 ± 0.083
12	1.15	14	0.492 ± 0.074

Results and Discussion

Images of metaphase chromosomes of common (*F. esculentum*) and tartary (*F. tartaricum*) buckwheat chromosomes are shown in Figure 2, where the chromosome number $2n = 2x = 16$ (diploid) is observed for both species. The chromosomes from the cell nucleus are localized within an area of $\sim$25 μm^2. Tartary chromosomes appeared to be more closely arranged compared to the dispersed nature of the distribution of the common buckwheat chromosomes. Distinct arms and the centromeres were clearly visible in both the common and tartary buckwheat species, allowing the symmetry to be characterized. The metaphase chromosomes allowed accurate volume measurement, as the chromosomes were well separated without overlapping and with minimal cellular debris.

The chromosome volumes and their AR measured from the 3D AFM image data for seven sets each of common and tartary buckwheat are summarized in Tables 1 and 2, respectively. The chromosomes of both species are numbered based not on biological origin but on the order of imaging. Individual identification and numbering of chromosomes would need a high number of samples considering the overlapping of parameters in different classes of buckwheat.

The pairings on the basis of volume for common and tartary buckwheat chromosomes are shown in Figures 3 and 4, respectively. The corresponding ideograms are shown in Figure 5. The chromosomes are displayed with the shorter arm at the top and positioned so that the centromeres are on the same horizontal line. All 16 chromosomes of common buckwheat are metacentric, while 14 of the tartary buckwheat chromosomes are meta and 2 (number 2 and 11) are sub-metacentric. Sub-metacentrics displayed a shift from the parity, while the meta-centrics were clearly symmetrical (Levan et al., 1964). The proportion of chromosomes with AR >2:1 was 0 and 2 for common and tartary buckwheat, respectively. The karyotypes of common and tartary buckwheat are of Stebbins 1A and Stebbins 2A type.

Buckwheat chromosomes were imaged in both liquid and air-dried mode. In-liquid imaging swells the chromosomes and changes the morphology greatly. The heights of the chromosomes were increased by a factor of 6 when imaged in-liquid under PBS buffer solution. In addition, the hydrated chromosomes become softened and thus do not reflect the actual morphology of the chromosomes. A further challenge associated with in-liquid imaging is that the substrate surface should be functionalized for rigid adherence of the chromosomes. Imaging of the chromosomes on days 0, 2, 4, and 8 after isolation shows that there is no significant difference in the morphology of chromosomes. The dehydration of chromosomes is negligible in air, which indicates that the chromosomes were preserved and were stable in air.

There is no agreement between the chromosome pairings based on AR compared with the volume measurements. This could be explained by the random nature of the chromosome deposition on the glass slide and the sample preparation method. Irrespective of the deposition of chromosomes, the volume remains the same. Hence, the volume presents itself as a more accurate parameter in karyotyping and in the classification of chromosomes.

The volume of tartary buckwheat chromosomes is smaller compared to that of the common buckwheat chromosomes. The difference in volumes of chromosomes between tartary and common buckwheat are in agreement with their genome sizes with a C-value of 0.56 for *F. tartaricum* and 1.39 for *F. esculentum* (Nagano et al., 2000). The nuclear DNA content of common buckwheat is 2.48 times (Nagano et al., 2000) more than that of tartary buck-

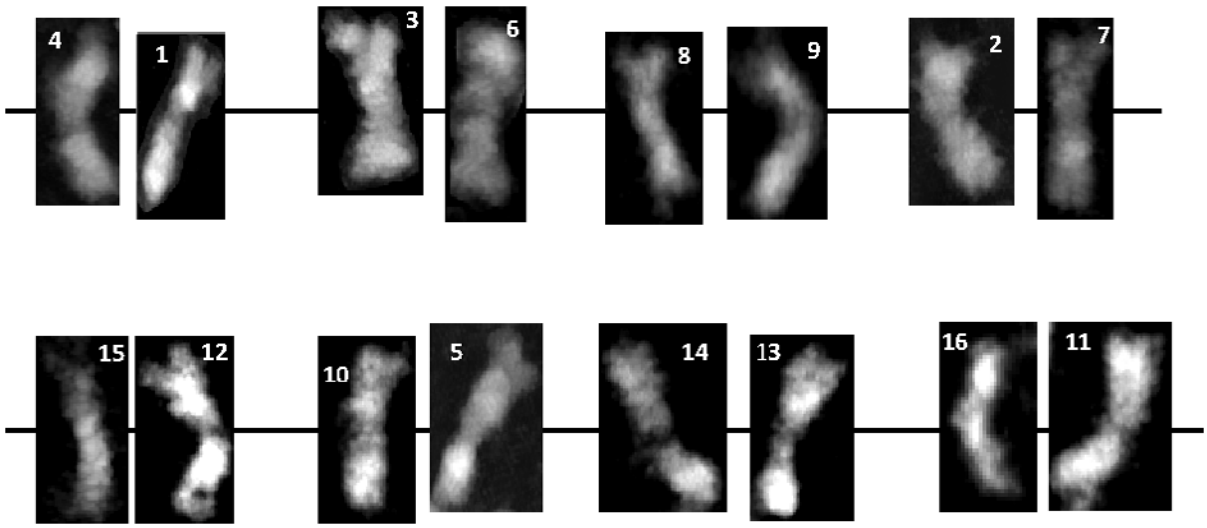

Figure 3. The karyotyped set of *F. esculentum* chromosomes, arranged in order of descending volumes.

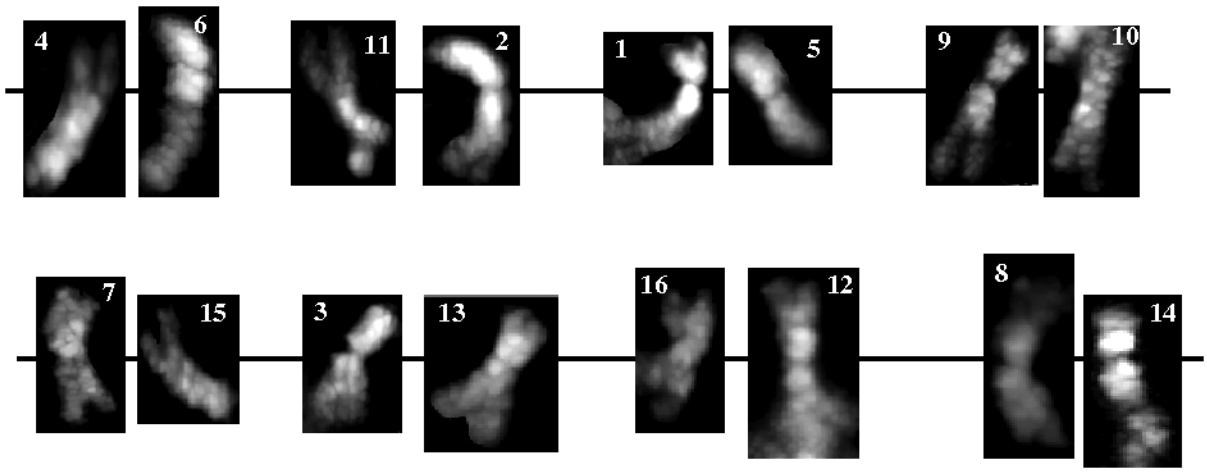

Figure 4. The karyotyped set of *F. tartaricum* chromosomes, arranged in order of descending volumes.

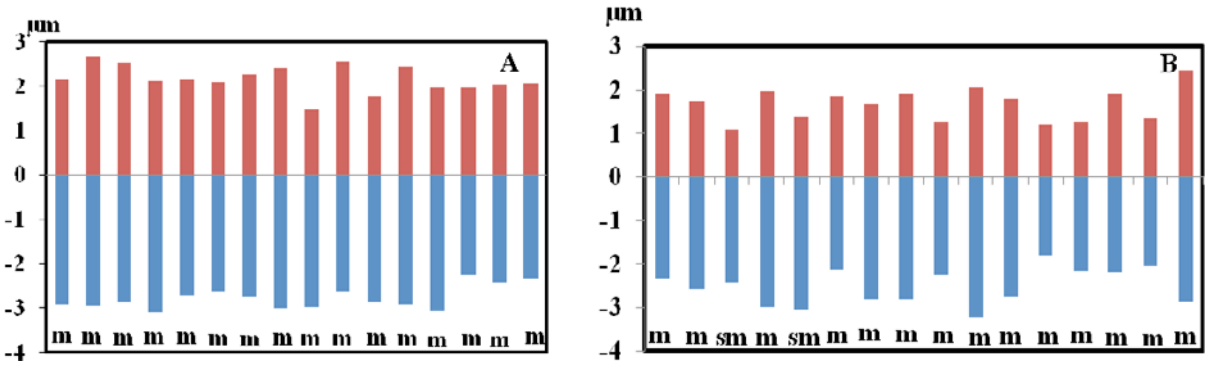

Figure 5. Idiogrammatic representation of the diploid karyotype of (**A**) *F. esculentum* and (**B**) *F. tartaricum*, arranged in order of descending volumes.

wheat while the volume of common buckwheat chromosomes is approximately 2.5 times larger than tartaricum. The relative chromosome volume and mean relative DNA content per chromosome for barley are highly correlated with a coefficient of 0.99 (Bennett et al., 1982). The results of our study confirm that the chromosomal volumes are proportional to the genome size and indicate that the DNA differences lie within the chromosomes.

The heights of the chromosomes for common buckwheat were in the range of 300 to 350 nm, while for tartary

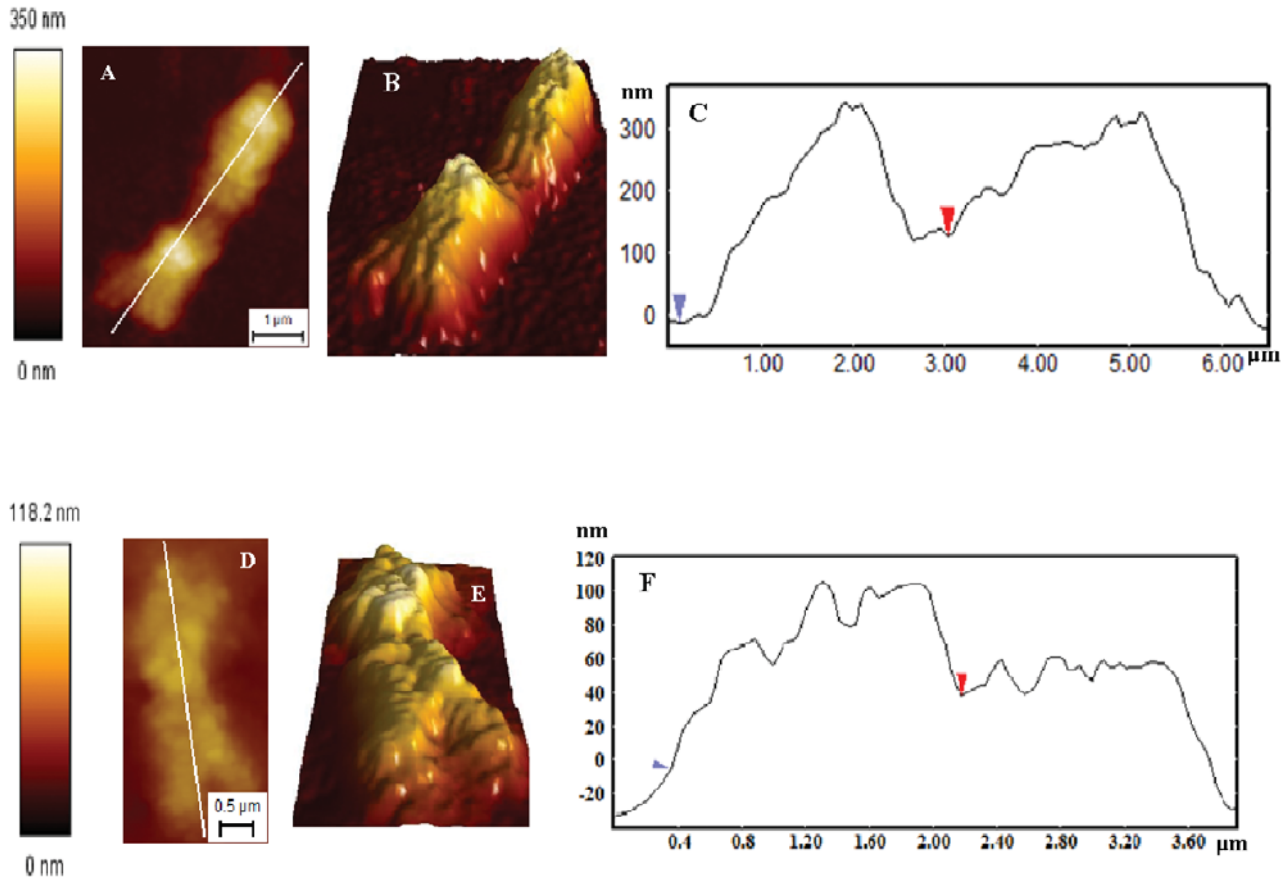

Figure 6. Surface morphology of the (**A**, **B**) common and (**D**, **E**) tartary buckwheat chromosomes. (**A**, **D**) Topographic images. (**B**, **E**) 3D view of the topographic images. (**C**, **F**) Section profile of the chromatid axes indicated by the lines in topographic images. Red arrows in **C** and **F** indicate the position of centromere. Blue arrows in **C** and **F** indicate the surface position of the substrates. The *Z* scales represent vertical image ranges of the topographic images. The scale bar in **A** and **D** indicates 1 and 0.5 μm, respectively.

buckwheat the range was between 100 to 150 nm. Typical section profiles of the chromatid axes for common and tartary buckwheat are shown in Figure 6.

The alternating ridges and grooves observed on the surface of chromosomes (Fig. 2) might correspond to the G-positive and G-negative banding pattern. Closer observation of parts of the surface of chromosomes (data not shown) reveals that the paired chromatids are composed of coiled chromatin fibers, which are looser in the ridges (G-negative) than in the grooves (G-positive) location on the chromosomes. Our observations comply with the previous study on human metaphase chromosomes by Hoshi and Ushiki (2001).

The relationship between species of plants can possibly be compared and analyzed based on the asymmetry index of chromosomes (Stebbins, 1971). To evaluate the evolution and compare the karyotype asymmetry between common and tartary buckwheat, we adopted the two coefficients proposed by Paszko (2006), namely CV_{CI} (Coefficient of Variation of Centromeric Index) and CV_{CL} (Coefficient of Variation of Chromosome Length) in calculating the asymmetry index. The calculated asymmetry index (AI) for common and tartary buckwheat are 2.12 and 2.28, respectively. Interpretation of the AI value is that the heterogeneity of tartaricum chromosome is higher and the karyotype is more asymmetrical than the common buckwheat. The higher symmetrical nature of common buckwheat (lower asymmetrical index) indicates that the *F. esculentum* is an original species and *F. tartaricum* belongs to secondary evolutionary units. Our results confirm previous reports that used SEM and polymerase chaine reaction techniques for the analysis of buckwheat chromosome structure (Yang et al., 2010).

It is recognized that tip convolution could cause an impact in the accuracy of the measurement of the edges of chromosomes and those that are closer to each other. Since the possible error caused by tip convolution effect will be the same for all chromosomes, the effect of error is neglected in classifying the chromosomes.

The results of our study offer significant potential for accurately locating the precise position of a gene on the chromosome. Knowing the molecular location will allow cytogeneticists to determine how far the gene is from other genes on the same chromosome and will help in sequencing information. Separation of chromosomes from cytoplasmic proteins and cellular debris at the earliest stage of the

isolation procedure is critical, considering the small size of buckwheat chromosomes. This is the first report of the karyotype analysis of *F. esculentum* and *F. tartaricum* by means of a cytogenetical approach using AFM. We provide an objective and quantitative method for classifying chromosomes using volume parameter.

Summary

Karyotype analysis and classification of chromosomes can be performed using AFM. The relative arm length, centromere position, and the chromosome volumes measured using AFM are accurate compared to the conventional methods that involve staining and banding. The chromosomal volumes are proportional to the genome size. *F. esculentum* is an ancient species compared to *F. tartaricum* buckwheat.

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