

Short Communication

## Live Cell Imaging Reveals Plant Aurora Kinase Has Dual Roles During Mitosis

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**The proper segregation of chromosomes during mitosis is required for accurate distribution of genetic information by two daughter cells. Here, we used live cell imaging of microtubules and kinetochores after treatment with an Aurora kinase inhibitor, hesperadin, in tobacco BY-2 cells to analyze the function of plant Aurora kinase during mitosis. Hesperadin treatment induced the delay of CenH3 alignment on the spindle equator. Furthermore, two types of dynamics of lagging CenH3s were observed during chromosome segregation. The findings indicate that the plant Aurora kinase has dual roles; correction of aberrant kinetochore–microtubule attachment and dissociation of cohesin during chromosome alignment and segregation.**

**Keywords:** Chromosome segregation — Cohesin dissociation — Kinetochore–microtubule attachment — Lagging chromosome — Plant Aurora kinase.

Abbreviations: ANOVA, analysis of variance; BY-2, Bright Yellow-2; BY-GTRC, BY-2 cells stably expressing GFP- $\alpha$ -tubulin–RFP–CenH3; CCA, complete CenH3 alignment; NEBD, nuclear envelope breakdown; RFP, red fluorescent protein.

Chromosome segregation errors during mitosis result in aneuploidy. Certain cellular processes are sensitive to change in gene dosage. Aneuploidy involves the loss or gain of one or more individual chromosome, resulting in gene dosage imbalances on the affected chromosome (Weaver and Cleveland 2007). Plants generally have more tolerance to extra chromosomes than animals. Even in plants, however, aneuploid genomes are not entirely stable (Matzke et al. 2003). Aneuploidy can occur as a result of defects in duplication, maturation or segregation of centrosomes, chromosome cohesion defects, spindle checkpoint defects and improper attachment of chromosomes to spindle microtubules (Kops et al. 2005). Aurora B is involved in proper attachment of chromosomes to spindle microtubules. Aurora B inactivation by different methods produces multiple chromosome segregation defects in mammalian cells,

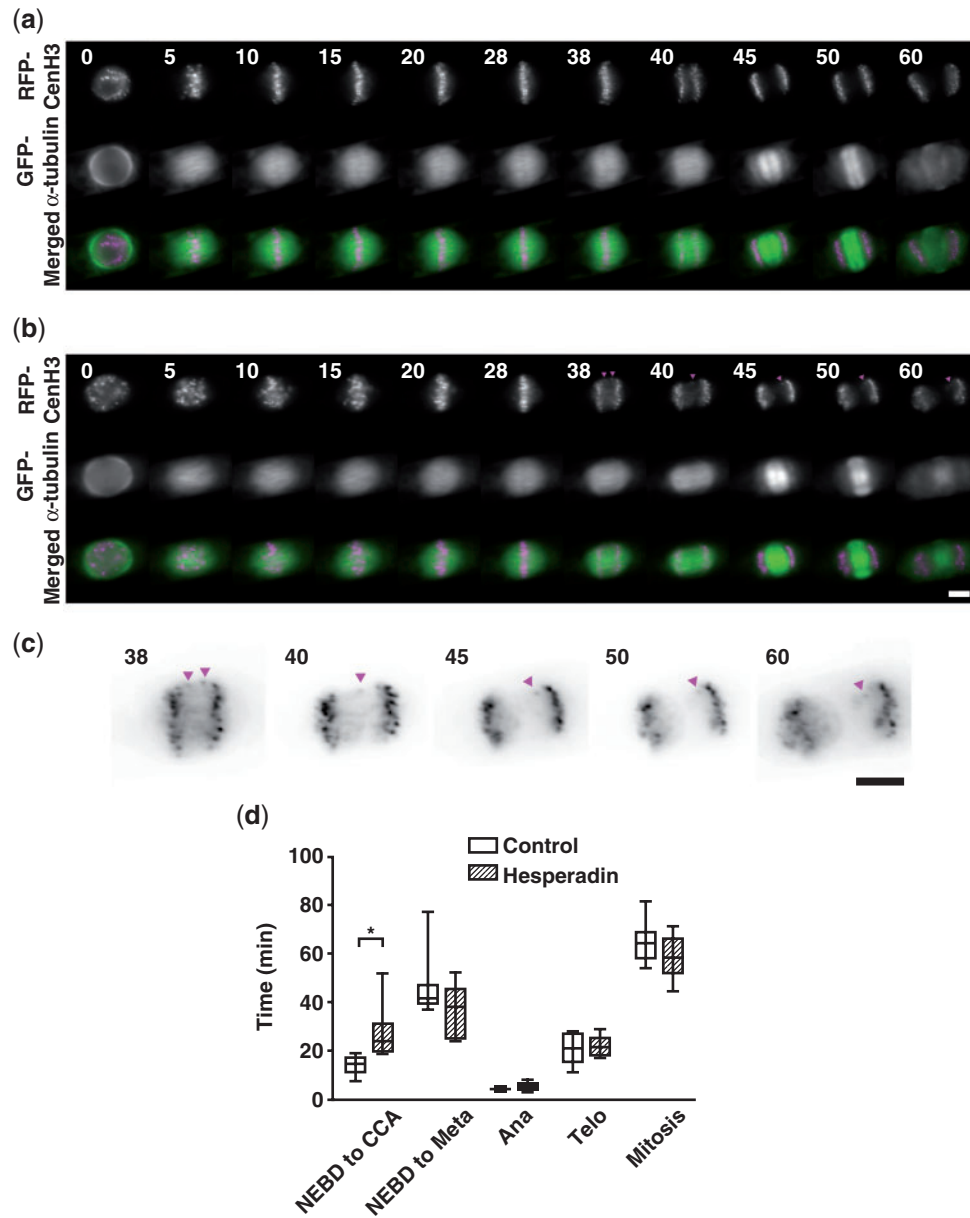
including lagging chromosomes (Hauf et al. 2003, Cimini et al. 2006).

In plants, three Aurora kinase genes (*AtAUR1*, 2 and 3) have been identified in *Arabidopsis thaliana*, and all three AtAURs phosphorylate histone H3 at Ser10 in vitro (Demidov et al. 2005, Kawabe et al. 2005). In addition, AtAUR2 may regulate cell division by competing with the AtAUR2 splicing variant (Kurihara et al. 2007). In tobacco BY-2 cells (*Nicotiana tabacum* cv. Bright Yellow-2), an Aurora kinase inhibitor, hesperadin, suppressed phosphorylation of histone H3 at Ser10 and Ser28, indicating inhibition of plant Aurora kinase. Moreover, hesperadin induced aberrant chromosome segregation, such as frequent formation of lagging chromosomes and micronuclei (Kurihara et al. 2006). However, the mechanism of formation of lagging chromosome during chromosome segregation in plants remains unclear.

To analyze the dynamics of kinetochores and microtubules in living hesperadin-treated BY-2 cells, we initially generated a transgenic tobacco BY-2 cell line expressing green fluorescent protein (GFP)-fused  $\alpha$ -tubulin and tdTomato [a tandem dimer variant of red fluorescent protein (RFP)]-fused CenH3 (centromeric histone H3: *Arabidopsis* HTR12). RFP–CenH3 was used to visualize kinetochores. We observed the transgenic BY-2 cells, designated as BY-GTRC (BY-2 cells stably expressing GFP- $\alpha$ -tubulin–RFP–CenH3), using a wide-field inverted microscope system. After nuclear envelope breakdown (NEBD), kinetochore microtubules were organized at prometaphase. As the cells reached metaphase, CenH3s were observed to oscillate and align rapidly on the spindle equator. After complete CenH3 alignment (CCA), CenH3s were equally separated and phragmoplasts appeared at anaphase and telophase (Fig. 1a, Supplementary Movie S1). Thus, the dynamics of kinetochores and microtubules could be observed in BY-GTRC cells.

To investigate the effect of Aurora kinase inhibition on the dynamics of kinetochores and microtubules, we performed live cell analysis in hesperadin-treated BY-GTRC cells. After >1 h elapsed from hesperadin treatment in BY-GTRC cells, the dynamics of GFP- $\alpha$ -tubulin and RFP–CenH3 were observed from NEBD. During prometaphase,

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**Fig. 1** Delay of CenH3 alignment on the spindle equator in hesperadin-treated BY-GTRC cells. Live cell time-lapse microscopy was performed in BY-GTRC cells after >1 h treatment with control (a) or 5  $\mu$ M hesperadin (b). Merged images of RFP-CenH3 (magenta) and GFP- $\alpha$ -tubulin (green) are shown in color. (c) Enlargement and inverted images of RFP-CenH3 shown in (b). Arrowheads indicate lagging CenH3s. Numbers indicate time (minutes) after NEBD. Scale bars: 10  $\mu$ m. (d) Box-and-whisker plots showing time spent at each stage during mitosis in control (open box) or hesperadin-treated (shaded box) BY-GTRC cells. In the box-and-whiskers graphs, the base and the top of the box indicate the 25th and 75th percentile, the line within the box the 50th percentile, and the whiskers extend to the highest and lowest measurements. The only statistically significant differences are in NEBD to CCA ( $n=10$ ;  $P<0.001$ , two-way ANOVA with Bonferroni post hoc test).

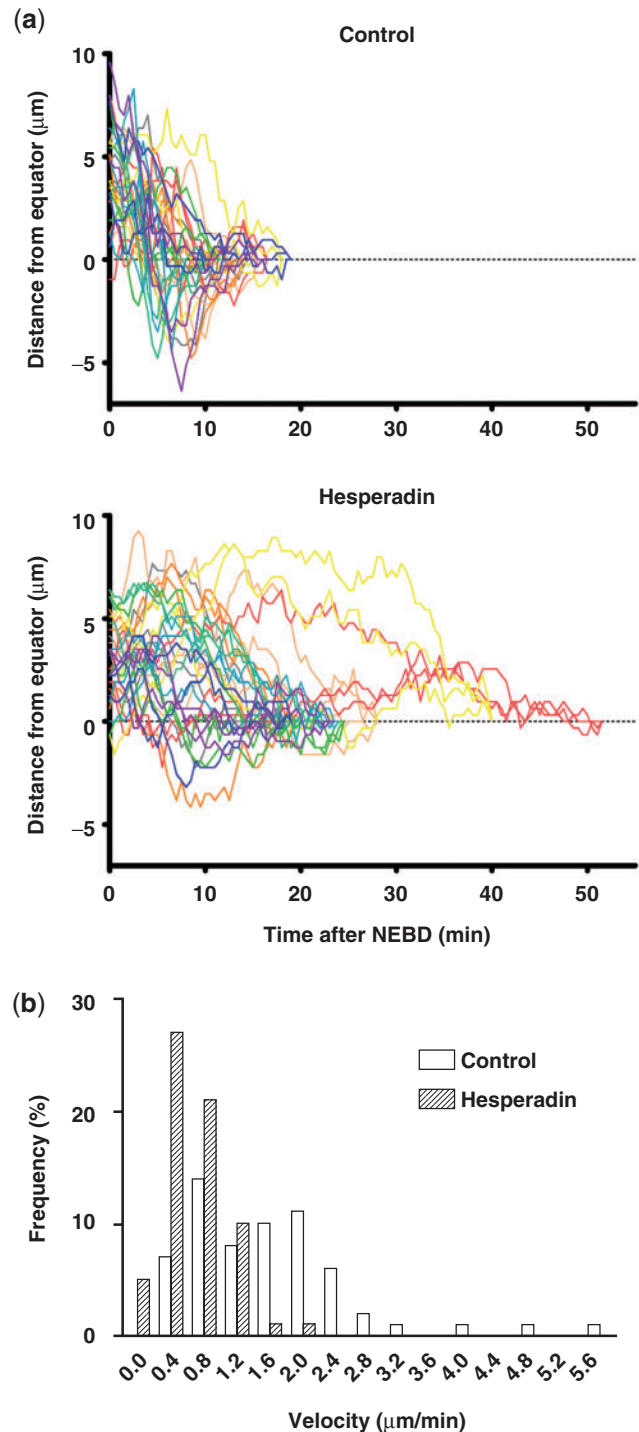
CenH3s were aligned on the spindle equator 28 min after NEBD in the hesperadin-treated cell (Fig. 1b), whereas alignment occurred at 14 min in the control cell (Fig. 1a). The time from NEBD to CCA was 27.4 min (mean) with a range of 19.0–52.0 min in the hesperadin-treated cells, longer than in the control that had a mean time of 14.3 min with a

range of 8.0–19.0 min (Fig. 1c,  $P<0.001$ ,  $n=10$ ). This result indicates that CenH3 alignment on the spindle equator was delayed in hesperadin-treated cells. Moreover, lagging CenH3s were observed during anaphase in the hesperadin-treated BY-GTRC cells (Fig. 1b, c). However, CenH3s were eventually aligned on the spindle equator and segregated

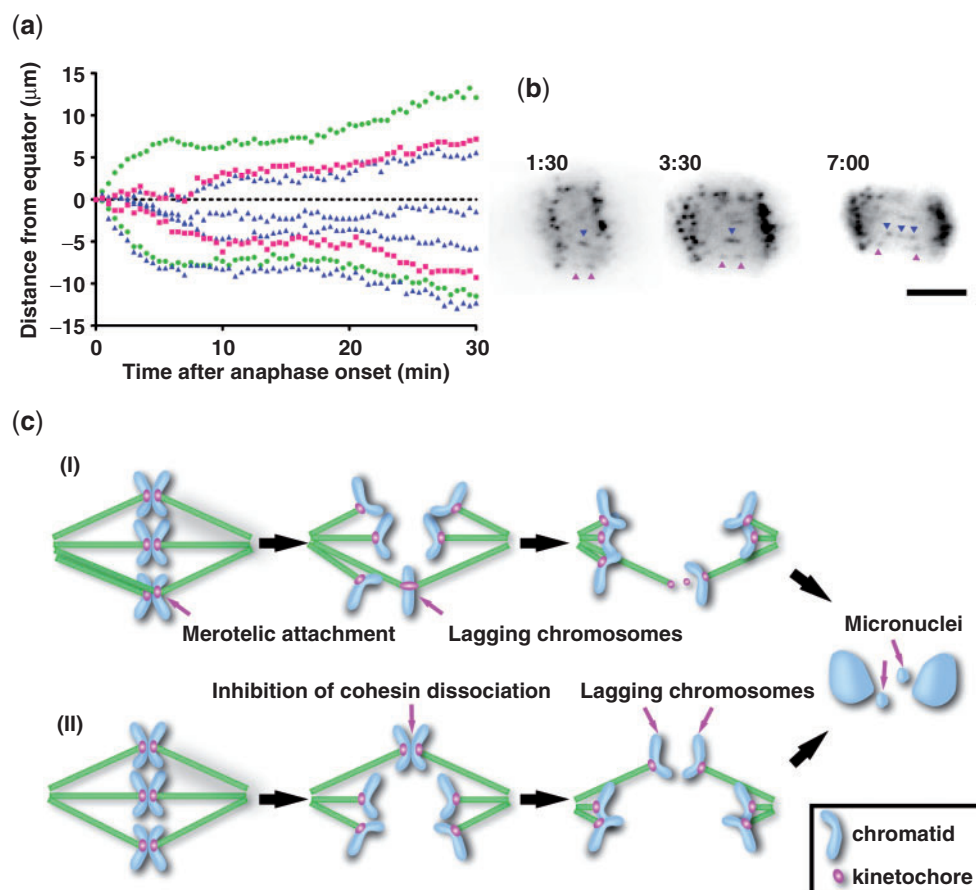
around 40 min after NEBD in the control and 38 min in the hesperadin-treated cells (Fig. 1a, b). The time from NEBD to metaphase was 36.8 min with a range of 23.5–52.0 min in the hesperadin-treated cells, which was not significantly different from the control at 45.4 min with a range of 37.0–77.5 min (Fig. 1c,  $P > 0.05$ ,  $n = 10$ ). The dynamics of microtubules in the control and hesperadin-treated cells did not differ significantly in these observations (Fig. 1a, b, Supplementary Movies S1, S2). The time spent in mitosis also did not differ significantly between the control and hesperadin-treated cells [Fig. 1c: control, 64.3 min (54.0–81.5 min); hesperadin, 58.6 min (44.5–71.5 min);  $P > 0.05$ ,  $n = 10$ ]. These results indicate that hesperadin induced the delay of CenH3 alignment on the spindle equator and lagging CenH3s during chromosome segregation, while other mitotic events were normal.

Manual tracking of RFP–CenH3 was performed to analyze the dynamics of CenH3 in the hesperadin-treated BY-GTRC cells in more detail. After manual tracking, the distances of CenH3 from the spindle equator were measured. Although CenH3s were oscillating to align on the spindle equator, the time from NEBD to CCA was increased in the hesperadin-treated cells, compared with the control cells. Moreover, the slopes of these graphs in the hesperadin-treated cells were less than that of the control cells (Fig. 2a). This result indicated that the oscillation velocity of CenH3 in the hesperadin-treated cells was slower than that in the control cells. The distribution of CenH3 oscillation velocity in the hesperadin-treated cells became slower (Fig. 2b). The average velocity was  $1.6 \pm 1.0 \mu\text{m min}^{-1}$  (mean  $\pm$  SD) in the control cells, and  $0.7 \pm 0.4 \mu\text{m min}^{-1}$  in the hesperadin-treated cells. Thus, hesperadin induced the decrease of CenH3 velocity to the spindle equator during chromosome alignment in BY-GTRC cells.

Bi-oriented kinetochore–microtubule attachment is essential for proper chromosome segregation. However, two types of aberrant attachment can occur during chromosome alignment. In syntelic attachment, both sister kinetochores are attached to microtubules from the same spindle pole. In a merotelic attachment, one sister kinetochore is attached to microtubules from both poles (Pinsky and Biggins 2005). Although the syntelic chromosome is positioned close to the pole (Lampson et al. 2004), the merotelic chromosome can align on the spindle equator because the merotelic chromosome is bi-oriented (Pinsky and Biggins 2005). However, the merotelic chromosome moves to the spindle equator with slower velocity than the chromosomes with proper kinetochore–microtubule attachment. These results suggest that aberrant kinetochore–microtubule attachment, merotelic attachment, occurred during chromosome alignment in hesperadin-treated BY-GTRC cells.



**Fig. 2** The oscillation velocity of CenH3 was slower in hesperadin-treated BY-GTRC cells. (a) Distance of CenH3 from the spindle equator in control (upper) or hesperadin-treated (lower) BY-GTRC cells. Thirty-one CenH3s in 10 independent control or hesperadin-treated cells were analyzed. Each color indicates the CenH3s in the same cell. (b) Velocity distribution of CenH3 movements shown in (a). Velocity distributions of RFP–CenH3 were analyzed in control (open box) or hesperadin-treated (shaded box) BY-GTRC cells.



**Fig. 3** Merotelic attachments occurred in Aurora kinase inhibition. (a) Distance of CenH3 from the spindle equator vs. time during chromosome segregation in hesperadin-treated BY-GTRC cells. Three types of the dynamics of CenH3 were observed. The green graph indicates normal dynamics. The blue graph indicates where one CenH3 was lagged, while the other was normal. Lagging CenH3 was separated into three parts. The magenta graph indicates where both CenH3s were lagged. (b) Lagging CenH3s were observed during chromosome segregation in hesperadin-treated BY-GTRC cells. Blue and magenta arrowheads indicate lagging CenH3s, which are calculated as shown in (a, blue and magenta graphs). Numbers indicate time after onset of anaphase. Scale bar: 10  $\mu\text{m}$ . (c) Schematic diagram of the two distinct pathways for lagging chromosome formation in plant Aurora kinase inhibition.

In mammalian cells, Aurora kinase inhibition induced syntelic attachment followed by misalignment (Hauf et al. 2003, Lampson et al. 2004). However, we did not observe the chromosome remaining near the pole with syntelic attachments in the hesperadin-treated BY-GTRC cells. Partial Aurora kinase inhibition induced lagging chromosomes at anaphase by increasing the number of merotelic kinetochores (Cimini et al. 2006). Then, to investigate the effect of the higher concentration of hesperadin, the dynamics of CenH3 were observed in 50  $\mu\text{M}$  hesperadin-treated BY-GTRC cells. Although the time from NEBD to CCA was longer than that of the control cells, CenH3 were observed to align on the spindle equator even in this high concentration of hesperadin (Supplementary Movie S3).

Lagging CenH3s were observed in almost all the hesperadin-treated BY-GTRC cells (Supplementary Table S1). The frequency of lagging chromosomes observed

in hesperadin-treated BY-2 cells (62% in Kurihara et al. 2006) was higher than that in the micronuclei (36% in Kurihara et al. 2006). The observation that some lagging CenH3s moved to daughter nuclei suggested that lagging chromosomes can be reincorporated in either of the daughter nuclei (Supplementary Movie S2, S3).

To analyze the formation of lagging chromosomes, manual tracking of CenH3 during chromosome segregation was performed. The trajectory of CenH3 to the spindle equator from anaphase to telophase in a 5  $\mu\text{M}$  hesperadin-treated BY-GTRC cell showed two types of lagging CenH3 dynamics during chromosome segregation (Fig. 3a, Supplementary Movie S4).

The first type showed that the dynamics of one CenH3 of a chromosome was the same as the normal type (Fig. 3a, green), but the other CenH3 was lagged during anaphase (Fig. 3a, blue). Furthermore, a stretched CenH3



was observed (Fig. 3b, blue arrowheads). This CenH3 was lagged, and stretched on the spindle equator. Finally, the stretched CenH3 was separated into three parts. This observation suggests that Aurora kinase inhibition induced merotelic attachment in the BY-GTRC cells. Considering these results, if syntelic attachment had occurred in the hesperadin-treated BY-GTRC cells, the microtubule capture from the opposite pole to the syntelic kinetochore would result in merotelic attachment. Thus, the CCA was delayed compared with the control cells. During anaphase, the merotelic kinetochore was stretched by pulling from both poles.

In the second type, both CenH3s of a chromosome remained on the spindle equator at the onset of anaphase and separated later (Fig. 3a, magenta; Fig. 3b, magenta arrowheads). This observation suggests that hesperadin induced inhibition of cohesin dissociation on this chromosome. Sister chromatid separation is required for loss of cohesin, and coincides with the dissociation of cohesin (Nasmyth and Haering 2005). In vertebrate cells, two distinct pathways are involved in cohesin removal (Waizenegger et al. 2000). In prophase, most of the cohesin is removed from chromosome arms, except from centromeric regions. The so-called 'prophase pathway' is required for the Polo-like kinase and Aurora B (Gimenez-Abian et al. 2004). When all the chromosomes are bi-oriented, centromeric cohesin is removed by separase (Hauf et al. 2001). In plants, *Arabidopsis* separase AESP is required for the removal of cohesin during meiosis (Liu and Makaroff 2006). Thus, the plant Aurora kinase would play a role in the removal of cohesin from chromosomes during mitosis.

In summary, live cell imaging of kinetochores and microtubules in BY-GTRC cells revealed that the plant Aurora kinase has dual roles in chromosome alignment and segregation. The Aurora kinase inhibitor, hesperadin, induced the delay of CenH3 alignment by slowing oscillation velocity. Furthermore, stretched lagging CenH3s were observed at anaphase in the hesperadin-treated cells (Fig. 3b, blue arrowheads). Therefore, these results suggest that inhibition of the plant Aurora kinase with hesperadin induced aberrant kinetochore-microtubule attachment, such as merotelic attachments (Fig. 3c [I]). Thus, we propose that the plant Aurora kinase regulates kinetochore-microtubule attachments during chromosome alignment. In addition, the plant Aurora kinase is also involved in cohesin dissociation during chromosome segregation, because both CenH3s were lagged on the spindle equator in the hesperadin-treated BY-GTRC cells (Fig. 3b, magenta arrowheads; Fig. 3c [II]). A previous study using fixed cells reported only a function of plant Aurora kinase for chromosome segregation (Kurihara et al. 2006). However, live cell imaging with visualized kinetochores and

microtubules enables observations of distinct roles for the plant Aurora kinase in chromosome alignment and segregation.

## Materials and Methods

Tobacco BY-2 cells were maintained as previously described by Nagata et al. (1992). BY-2 cells were cultured in modified Linsmaier and Skoog medium in a rotary shaker at 26°C in the dark.

The RFP expression vector was constructed by replacement of GFP from the spUC-GFP vector (Fujimoto et al. 2004) by TA-cloned tdTomato (Shaner et al. 2004) of the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) and renamed spUC-tdTomato. The RFP-CenH3 expression vector was constructed by insertion of the TA-cloned *Arabidopsis thaliana* CenH3 gene (HTR12: At1g01370) of pCR2.1 into spUC-tdTomato. The *Sse*8387I-digested spUC-tdTomato-CenH3 was ligated with *Pst*I-digested binary vector pMDC99 (Curtis and Grossniklaus 2003).

The binary vector 35S::RFP-CenH3 was introduced into *Agrobacterium tumefaciens* strain EHA101. Transformation of BY-GT16 cells stably expressing GFP-fused  $\alpha$ -tubulin (Kumagai et al. 2001) with RFP-CenH3 was conducted as previously described by Fujimoto et al. (2004) except for selection of transformants with 500 mg l<sup>-1</sup> claforan, 50 mg l<sup>-1</sup> kanamycin and 30 mg l<sup>-1</sup> hygromycin, and the cell line obtained was designated BY-GTRC.

Hesperadin (Boehringer Ingelheim Austria, Vienna, Austria) at 5 or 50  $\mu$ M, or dimethylsulfoxide (control) was added to 3-day-old BY-GTRC cells and cultured for a further 1 h. BY-GTRC cells were transferred into Petri dishes with poly-L-lysine-coated coverslips at the bottom (Matsunami Glass Ind., Ltd., Osaka, Japan). The dishes were placed on the inverted platform of a fluorescence microscope (IX-81; Olympus) equipped with a cooled charged-coupled device (CCD) camera (CoolSNAP HQ2; Roper Scientific, Houston, TX, USA). Images were acquired every 30 s with a 40 $\times$  objective lens (UApo/340, NA 1.35, oil immersion). Image processing was performed with the following software: MetaMorph (Universal Imaging Corporation, Downingtown, PA, USA), WCIF ImageJ software ([www.uhnresearch.ca/facilities/wcif/imagej/](http://www.uhnresearch.ca/facilities/wcif/imagej/)) and Adobe Photoshop CS3 Extended (Adobe Systems, San Jose, CA, USA).

Two-way analysis of variance (ANOVA) with a Bonferroni post hoc test was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

Individual RFP-CenH3 movement in BY-GTRC cells was tracked using the Manual Tracking plug-in of the WCIF ImageJ. Manual tracking was carried out by mouse-clicking on the time-lapse images. After manual tracking, the distances of CenH3 from the spindle equator were calculated with Microsoft Excel. The spindle equator was assigned as the position where CenH3s were aligned at the time of complete CenH3 alignment. CenH3 movement was judged when unidirectional movement was  $\geq 2 \mu$ m.

## Supplementary data

Supplementary data mentioned in the article is available at *Plant and Cell Physiology* online.

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