I.4 Chromosome Dynamics in Tobacco BY-2 Cultured Cells

S. Matsunaga[1], N. Ohmido[2], and K. Fukui[1]

1 Introduction

During mitosis in eukaryotes, the nuclear genome is organized into highly condensed structures referred to as chromosomes. The dynamic processes of chromosome condensation and segregation play crucial roles in the equal separation of genetic information to both daughter cells. Since disruption of these processes is harmful to cells, causing, for example, chromosome aneuploidy, appropriate regulation is indispensable. Chromosome dynamics involves modification of the molecules that regulate these processes during mitosis.

The usefulness of tobacco BY-2 (*Nicotiana tabacum* cv. Bright Yellow-2) cultured cells in analyses of chromosome dynamics has been recently demonstrated. Tobacco BY-2 cells possess the following advantages. Their mitotic chromosomes are not small and monocentric, which is advantageous because analyses of chromosome dynamics are generally performed under a microscope. The haploid genome size of the amphidiploid species *Nicotiana tabacum* ($2n = 4x = 48$) is about 4,500 Mb (Arumuganathan and Earle 1991) and the total nuclear genome is divided into 48 mitotic chromosomes. The length of these mitotic chromosomes varies from 2 to 6 μm (Kenton et al. 1993; Moscone et al. 1996), which is larger than that in *Arabidopsis thaliana* (c.a. 2 μm). Such chromosome size makes it possible to analyze the detailed morphology and structure of chromosomes. Although BY-2 cultured cells are derived from *N. tabacum*, it is probable that a certain level of ploidy change and chromosome abnormality occurs during cell culture. However, any problem encountered in studies of chromosome dynamics has not been reported to date.

Another advantage of tobacco BY-2 cells is their ability for high synchronization. Because mitotic chromosomes only emerge in the short mitotic phase, synchronous cell systems are very useful for studying chromosome dynamics. A highly synchronous method has been established in tobacco BY-2 cells (Nagata et al. 1992) and the usefulness of synchronized BY-2 cells has been widely demonstrated in plant cell cycle studies (Ito 2000). Synchronized BY-2 cells provide the most efficient mitotic chromosomes because a mitotic index of more

[1] Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita 565-0871, Osaka, Japan, e-mail: sachi@bio.eng.osaka-u.ac.jp
[2] Faculty of Human Development, Kobe University, 3-11 Tsurukabuto, Nada, Kobe 657-8501, Hyogo, Japan

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than 90% is attained through sequential synchronization with aphidicolin and propyzamide (Nagata and Kumagai 1999).

Recently, the green fluorescent protein (GFP) has become the most powerful and popular tool in plant cell biological analyses. Dynamic analyses of proteins fused with GFP allow direct in vivo observations of proteins or organelles in living plant cells. This application has also been used for imaging chromosomes and nuclei in living cells. We recently conducted analyses of GFP-fused proteins involved in chromosome dynamics using tobacco BY-2 cells.

2 Dynamic Analysis of Condensin Complexes

Condensin complexes are highly conserved among eukaryotes (Hirano 2002). Condensin I is composed of five subunits: two core subunits (CAP-E/SMC2 and CAP-C/SMC4) (where CAP is chromosome associated protein and SMC is structural maintenance of chromosome) which belong to the structural maintenance of chromosomes (SMC) family, and three non-SMC subunits (CAP-D2, CAP-G, and CAP-H) (Losada and Hirano 2005; Table 1). SMC family proteins participate in a number of processes involved in chromosome dynamics (Hirano 2002; Jessberger 2002; Hagstrom and Meyer 2003). CAP-D2 and CAP-G share a highly degenerate, repeating motif known as the HEAT repeat (Neuwald and Hirano 2000) and CAP-H belongs to the kleisin superfamily (Schleiffer et al. 2003). Several studies have revealed that condensin subunits are essential for in vitro and in vivo chromosome condensation and segregation (Hirano and Mitchison 1994; Hirano et al. 1997; Hagstrom et al. 2002; Wignall et al. 2003).

Another condensin complex (condensin II) has been identified in HeLa cells (Ono et al. 2003). Condensin II shares the same pair of SMC subunits with condensin I; however, it contains a different set of non-SMC subunits (CAP-D3, CAP-G2, and CAP-H2). Moreover, although condensin II is also localized on

Table 1. Subunits of condensin complexes

	S. cerevisiae	*H. sapiens*	*A. thaliana*
SMC2 (I and II)	Smc4	hCAP-C	AtCAP-C
SMC4 (I and II)	Smc2	hCAP-E	AtCAP-E1, E2
HEAT (IA)	Ycs4	hCAP-D2	CAB72176
HEAT (IB)	Ycs5/Ycg1	hCAP-G	BAB08309
Kleisin (IC)	Brn1	hCAP-H	AtCAP-H
HEAT (IIA)	–	hCAP-D3	At4g15890
HEAT (IIB)	–	hCAP-G2	At1g64960
Kleisin (IIC)	–	hCAP-H2	AtCAP-H2

mitotic chromosomes, condensin I and II show distinct distributions along the axis of a single chromosome (Ono et al. 2004). Condensin II and I associate with chromosomes sequentially in this order and have different functions with respect to mitotic chromosome assembly (Hirota et al. 2004).

A few plant-related studies have revealed that SMC2 subunits are involved in development in *A. thaliana* (Liu et al. 2002; Siddiqui et al. 2003). *A. thaliana* contains two SMC genes, *AtCAP-E1* and *AtCAP-E2*, with *AtCAP-E1* comprising more than 85% of the total SMC2 transcripts. Embryo lethality caused by double mutation of SMC genes suggests that AtCAP-E1 and AtCAP-E2 are involved in development in *A. thaliana*.

Recently, dynamic analyses of the non-SMC subunits of *Arabidopsis* condensins, AtCAP-H and AtCAP-H2A, were performed using tobacco BY-2 cells (Fujimoto et al. 2005). Figure 1 shows the subcellular localization of GFP-AtCAP-H in tobacco BY-2 cells at different stages. In interphase, GFP signals were predominantly localized in the cytoplasm of cells transformed with *GFP-AtCAP-H*. GFP-AtCAP-H was found in the cytoplasm until the end of prophase. In prometaphase, some signals were detected on the chromosomes, while in metaphase, almost all signals moved to the chromosomes and the signal intensity rapidly increased. After chromosome segregation, a few signals diffused into the cytoplasm but the main signal remained on the chromosomes. The chromosome signals finally diffused to the cytoplasm after cytokinesis.

The dynamics of GFP-AtCAP-H2 during mitosis of tobacco BY-2 cells is shown in Fig. 2. In interphase, GFP signals were mainly detected in the nucleolus and slightly in the nucleoplasm. Signals were localized in the nucleolus until the end of prophase, moving mainly to the entire chromosomes after disappearance of the nucleolus. The signal intensity on the chromosomes was weaker than that of AtCAP-H. During chromosome segregation, the signals were equally localized on both chromatids, and when the nucleolus reformed in the nucleus, signals once again appeared here. AtCAP-H and AtCAP-H2 are localized in mitotic chromosomes from prometaphase to telophase. However, their localizations during interphase are different; although AtCAP-H is localized in the cytoplasm, AtCAP-H2 is localized in the nucleus, particularly in the nucleolus. These different localizations are indicative of functional differentiation between condensin I and II in *A. thaliana*.

3 Dynamic Analysis of Heterochromatic Protein 1

Heterochromatin corresponds to the relatively gene-poor, late replication, repetitive sequences found near the centromere and telomere. In contrast, euchromatin replicates relatively early in the cell cycle and contains low-copy DNA sequences, including the majority of genes. Heterochromatin and euchromatin show differences in heterochromatin protein 1 (HP1), a highly conserved non-histone chromosomal protein, between yeast and plants and functionally

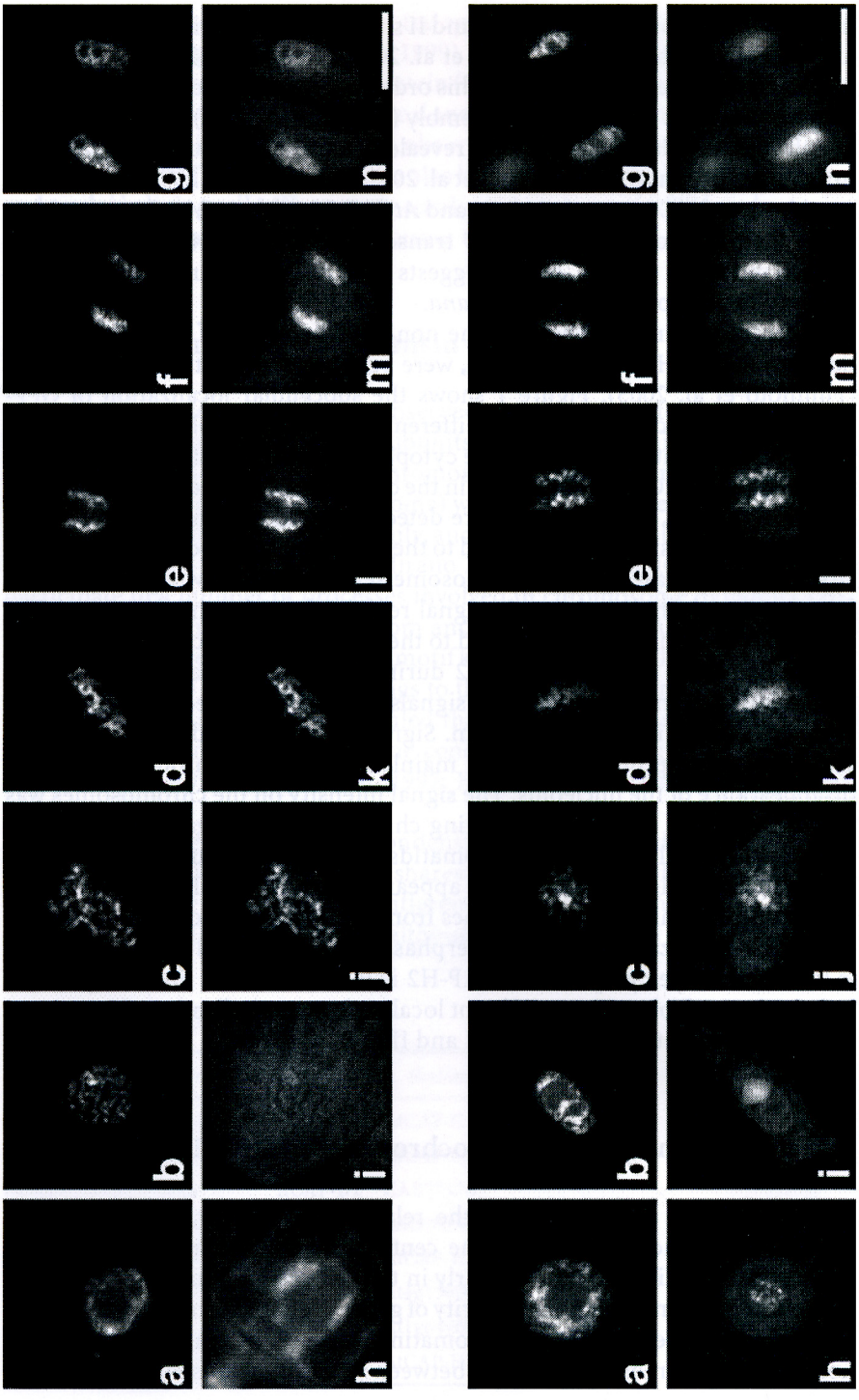
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Fig. 1. Subcellular localization pattern of GFP-AtCAP-H in tobacco BY-2 cells. The *upper row* shows DNA staining with DAPI (4′, 6-diamidino-2-phenylindole) and the *lower* shows GFP fluorescence. Cells at **a, h** interphase, **b, i** prophase, **c, j** prometaphase, **d, k** metaphase, **e, l** anaphase, **f, m** telophase and **g, n** cytokinesis. Scale bar; 10 μm

Fig. 2. Subcellular localization pattern of GFP-AtCAP-H2 in tobacco BY-2 cells. *Upper row* shows DNA staining with DAPI and *lower* GFP fluorescence. Cells at **a, h** interphase, **b, i** prophase, **c, j** prometaphase, **d, k** metaphase, **e, l** anaphase, **f, m** telophase and **g, n** cytokinesis. Scale bar; 10 μm

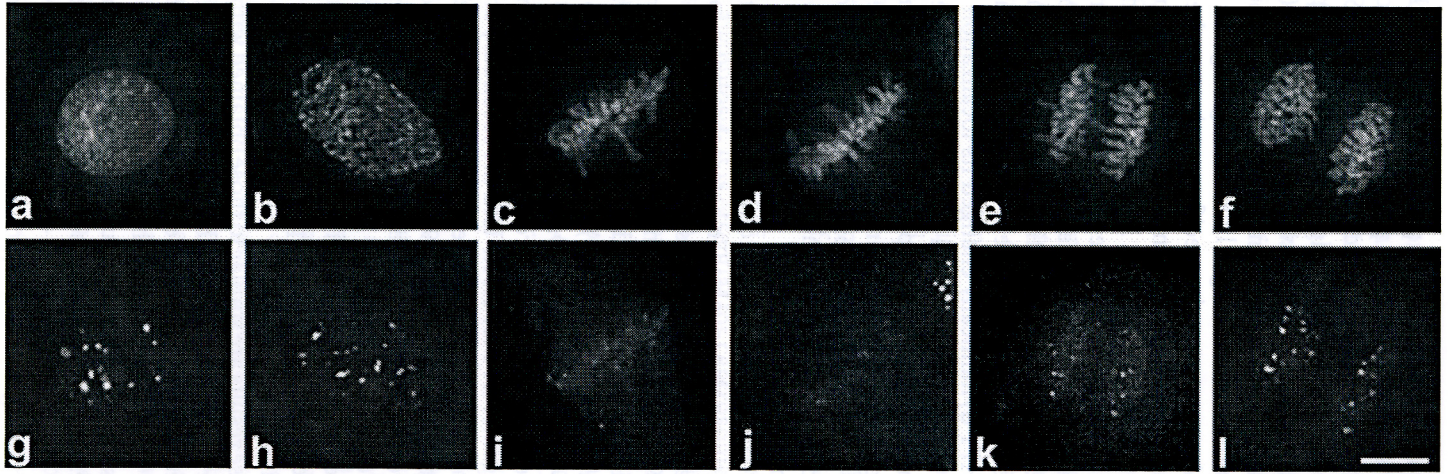

Fig. 3. Subcellular localization pattern of GFP-AtLHP1 in tobacco BY-2 cells. *Upper row* shows DNA staining with DAPI and *lower* GFP fluorescence. Cells at **a, g** interphase, **b, h** prophase, **c, i** prometaphase, **d, j** metaphase, **e, k** anaphase and **f, l** telophase. Scale bar; 10 μm

are involved in chromatin packing and gene silencing (Li et al. 2002). HP1 interacts with the modifier protein of heterochromatin, inducing position effect variegation (PEV), which is encoded by the *Su(var)2-5* gene in *Drosophila* (James and Elgin 1986). The HP1 family is composed of relatively small proteins (15–35 kDa). They have a chromodomain (CD) in the highly conserved N-terminal region, a chromo shadow domain (CSD) in the C-terminal region, and a variable length linker (hinge region) (Eissenberg and Elgin 2000). CENP-A, a centromeric-specific protein, together with HP1 is involved in the association and segregation of sister kinetochores during mitosis in animal cells (Sugimoto et al. 2001). In plants, the *lhp-1* mutant in *Arabidopsis* exhibits alterations in leaf and flower organs, cell size, and flowering time transitions (Gaudin et al. 2001). To obtain insight into the formation and maintenance of heterochromatin, we investigated kinetic binding of GFP-tagged *Arabidopsis* Like Heterochromatin Protein 1 (AtLHP1 in tobacco BY-2 cells.

AtLHP1 dynamically changed its localization during mitosis (Fig. 3). GFP-AtLHP1 foci were detected in interphase nuclei. They then diffused in the cytoplasm during late prophase to anaphase and relocalized on chromatin in telophase. The molecular heterochromatic regions indicated by the localization of AtLHP1 were mostly limited to centromeric regions, although the heterochromatic region is not sharply delimited on tobacco metaphase chromosomes.

Post-transcriptional histone tail modification including methylation and acetylation is also involved in heterochromatin formation (Jackson et al. 2002). Especially, methylation of lysine 9 on Histone 3 by *Su(var)3-9* methyltransferase in *Drosophila* is linked to gene silencing and heterochromatin formation. Colocalization between HP1 and chromatin histone modification was also shown in plant cells (Yu et al. 2004). Chromosome preparations for immunostaining of Met-H3K9 (methylated lysine 9 of histone H3) in GFP-AtLHP1 transformed cells were carried out according to Hasezawa and Kumagai (2002). The antibody against tri-Met-H3K9 and the secondary antibody, anti-rabbit Cy3, were used for immunostaining detection in the BY-2 cells. As a result, the signals of GFP-AtLHP1 and Met-H3K9 were shown to be colocalized in interphase cells. However, AtLHP1 dynamic behavior during chromosome segregation revealed that the foci do not always localize in concert with Met-H3K9. GFP-AtLHP1 localized at specific foci in interphase nuclei, but most GFP-AtLHP1 diffused into the cytoplasm from prophase to anaphase and relocalized at the Met-H3K9 sites in telophase.

These results are similar to those obtained with human and mouse HP1, with release from chromatin to the cytoplasm during prophase to anaphase (Sugimoto et al. 2001). With proceeding cytokinesis, HP1 was predominantly found in the newly formed daughter nuclei, again displaying a heterochromatin-like distribution. These results suggested that, although the majority of HP1 diffuses in the cytoplasm, some populations are retained in the centromeric region and are involved in the association and segregation of sister kinetochores during mitosis (Sugimoto et al. 2002). This is consistent with plant cells,

in which a dynamic change of HP1 on chromosomes is essential for precise association and segregation of chromosomes. These regions are dominated by highly trimethylated histone K9 of histone H3, suggesting formation of the heterochromatin center. AtLHP1 is also thought to be necessary for plant cell division, and histone modification by methylation is believed to play an essential role as a landmark for heterochromatin formation in plant cells.

4 Dynamic Analysis of Aurora Kinases

Aurora kinases belong to the cell-cycle-dependent serine/threonine protein kinase family that regulates several mitotic events (Carmena and Earnshaw 2003). The paralog numbers of Aurora kinases are different among organisms. Yeast has only one Aurora kinase gene in its genome, while animal species have two, identified as Aurora A and B. Mammalian species, such as humans and mice, have an additional Aurora kinase designated as Aurora C, which is specifically expressed in the testis. The Aurora kinase family shows a high sequence similarity of more than 60% at the amino acid level, particularly in the kinase domain; however, Aurora kinases differ in their localization and function.

Aurora A is localized at the centrosomes during interphase and at the spindle poles and mitotic spindle during mitosis. It is essential for centrosome maturation, maintenance, duplication, and segregation, in addition to stabilization of spindle microtubules during mitosis. Moreover, Aurora A phosphorylates centrosome- and mitotic spindle-related proteins.

Aurora B localizes at the centromeres from prophase to metaphase and relocates to the spindle midbody at cytokinesis. Aurora B is a chromosomal passenger protein that interacts with INCENP, Survivin, and Borealin/Dasra B to form a chromosomal passenger complex (Vagnarelli and Earnshaw 2004). These proteins are necessary for localization of Aurora B for regulation of kinetochore formation, chromosome segregation, and cytokinesis. Phosphorylation of histone H3 at Ser10 and Ser28 by Aurora B is considered to be the fundamental role of Aurora B in chromosome segregation and cytokinesis (Goto et al. 2002). Although direct phosphorylation was not reported, the condensin complexes, which are involved in chromosome condensation, could not be localized on chromosomes in Aurora B-depleted cells, resulting in incorrect chromosome condensation (Ono et al. 2003). Aurora B phosphorylates topoisomerase II and ISWI (MacCallum et al. 2002; Morrison et al. 2002), indicating that Aurora B has a role for chromatin organization. Recently, two studies identified plant Aurora kinases in *A. thaliana* (Demidov et al. 2005; Kawabe et al. 2005).

In the *A. thaliana* genome, three deduced amino acid sequences showed high similarity to those of animal Aurora kinase genes. The kinase domain of these three proteins shows more than 60% similarity to those of animal and yeast

Aurora kinases. These three genes were designated *AtAUR1*(*A. thaliana* Aurora kinase)/*AtAurora1*, *AtAUR2*/*AtAurora2*, and *AtAUR3*/*AtAurora3* (Demidov et al. 2005; Kawabe et al. 2005). The AtAUR1 and AtAUR2 sequences shows high similarities with the amino acid sequences of their kinase domains, exhibiting 95% sequence identity. In contrast, the kinase domain of AtAUR3 shares a 65% amino acid sequence identity with the other two AtAURs. Similar to animal Aurora kinases, the N-terminal regions in plants are variable in both length and sequence.

Recently, dynamic analysis of GFP-fused AtAUR proteins was performed using tobacco BY-2 cells (Kawabe et al. 2005). Figure 4 shows dynamic analysis of AtAUR1 during mitosis in tobacco BY-2 cells. AtAUR1 was located on the nuclear membrane at interphase. At prophase, it moved toward peripheral regions of the nucleus near the spindle poles, resembling a cap-like distribution. At prometaphase, microtubules began to project from both poles, forming a mitotic spindle, and the localization of AtAUR1 appeared as fibers. At metaphase, localization appeared on the spindle-like structure. From anaphase to telophase, AtAUR1 was located in the spindle halves moving toward the spindle poles. As the mitotic spindle moved to opposite sides, the signal accumulated in separated spindles. During telophase, AtAUR1 clearly localized in the midzone between the signals in the peripheral region of the two cell nuclei, suggesting localization on the synthesized cell plate. Although localization of AtAUR2 in tobacco BY-2 cells showed almost the same pattern as that of AtAUR1, no significant AtAUR2 signals could be detected around the cell plate in the midzone at telophase. Recently, localization of AtAUR1 in the equatorial plate was also reported (Van Damme et al. 2004). When the mitotic chromosomes began to decondense, AtAUR1 and AtAUR2 gradually returned to the peripheral region of the cell nuclei.

Although the N-terminal regions of AtAUR1 and AtAUR2 show almost no homology, their subcellular localizations are similar, except during cell plate formation. Animal Aurora A kinases localize in the centrosome during interphase, showing strong signals at both spindle poles (Kimura et al. 1997; Sugimoto et al. 2002). Consistent with its localization, Aurora A regulates the stabilization and maturation of centrosome and spindle poles (Giet et al. 1999). Animal cells have centrosomes and fungi have a spindle pole body, while plants have no centrosomes but microtubule organizing centers (MTOCs) in their nuclear membrane during interphase (Staiger and Lloyd 1991). The localizations near the nuclear membrane at interphase, cap-like localization at prophase, and spindle-like localization at metaphase resemble the localizations of γ-tubulin, a component of plant MTOCs. This fact suggests that AtAUR1 and AtAUR2 colocalize with γ-tubulin and function in MTOCs.

Figure 5 shows dynamic analysis of AtAUR3 in tobacco BY-2 cells. AtAUR3 localized at the nuclear periphery during interphase. At prophase, dot-like signals appeared when the chromosomes began to condense. At prometaphase, the signals moved to the metaphase plates along with the condensed chromosomes. At metaphase, the signals aligned in the center of the metaphase

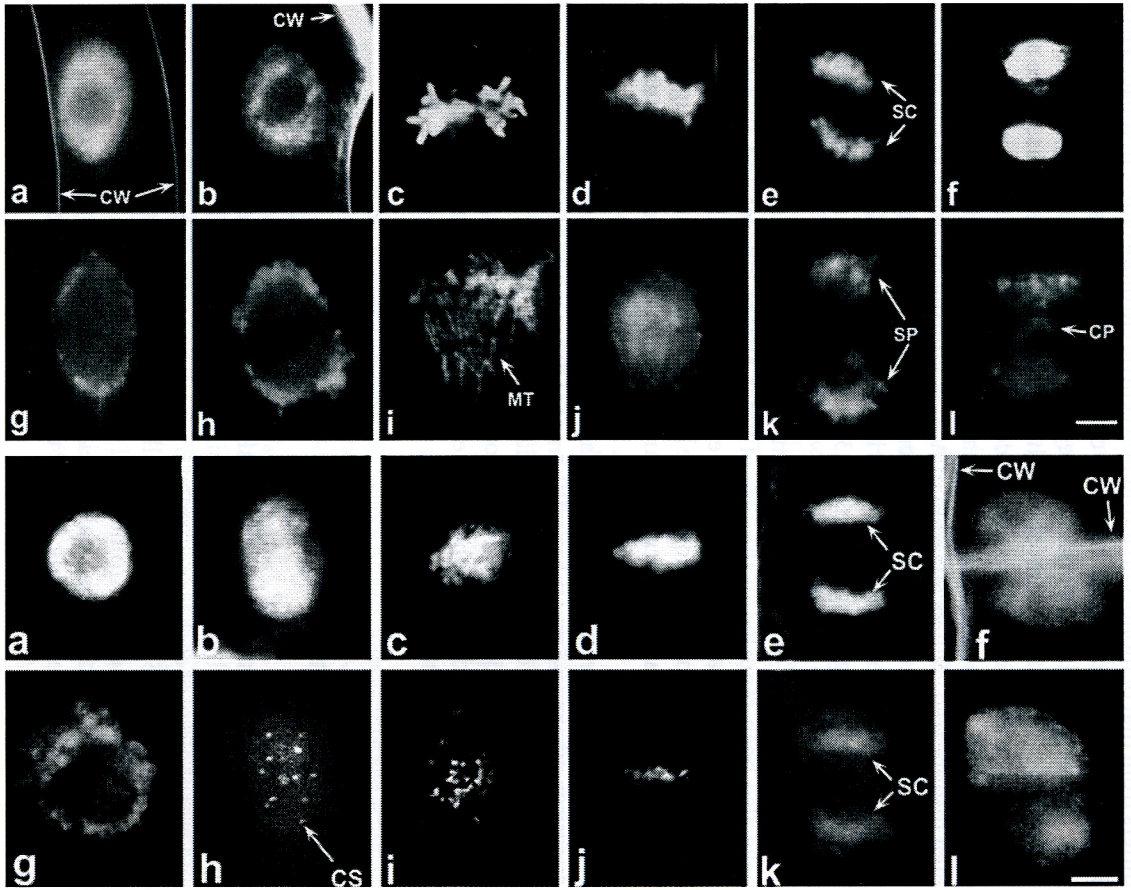

Fig. 4. Localization pattern of AtAUR1 during mitosis of tobacco BY-2 cells. AtAUR1-GFP-overexpressed BY-2 cells were fixed and stained with DAPI. **a–f** DAPI; **g–l** GFP. Cells at **a, g** interphase, **b, h** prophase, **c, i** prometaphase, **d, j** metaphase, **e, k** anaphase, **f, l** telophase. *CW* Cell wall (**a, b**); *MT* microtubule (**i**); *SP* mitotic spindle (**j, k**); *SC* sister chromatid (**e**); *CP* cell plate (**l**). Scale bar; 10 μm

Fig. 5. Localization pattern of AtAUR3 during mitosis of tobacco BY-2 cells. AtAUR3-GFP-overexpressed BY-2 cells were fixed and stained with DAPI. **a–f** DAPI; **g–l** GFP. Cells at **a, g** interphase, **b, h** prophase, **c, i** prometaphase, **d, j** metaphase, **e, k** anaphase, **f, l** telophase. Scale bar; 10 μm

plates. However, at anaphase, in accordance with chromosome segregation, AtAUR3 signals were almost evenly observed on the entire chromosome. After cell division, the AtAUR3 signals returned to the nuclear membrane and the cytoplasm around the nucleus.

The major substrate of Aurora kinases is histone H3 (Adams et al. 2001). In the late G2 phase, phosphorylation of histone H3 at Ser10 is initiated in pericentromeric heterochromatin, spreading throughout the chromosomes by metaphase, with dephosphorylation occurring after anaphase (Hendzel et al. 1997). In plants, the distribution pattern of phosphorylated histone H3 at Ser10 differs from that of monocentric and polycentric chromosomes. In tobacco BY-2 cells, when chromosomes began to condense at prophase, dot-like signals were first detected in the pericentric regions (Kawabe et al. 2005). The dotted signals on the mitotic chromosomes then moved to the metaphase plate and, after segregation to opposite poles was complete, they dispersed along the sister chromatids at late anaphase. At telophase, the signals drastically reduced and then disappeared. The localization of AtAUR3 during mitosis was the same as that of phosphorylated histone H3 at Ser10. All three recombinant AtAURs can phosphorylate histone H3 at Ser10 in vitro (Demidov et al. 2005; Kawabe et al. 2005), strongly suggesting that AtAUR3 plays a major role in phosphorylation of histone H3 at Ser10 in vivo.

Dot-like signals on the chromosomes between prophase and metaphase have also been reported in the case of yeast Aurora kinases and animal Aurora B kinases (Adams et al. 2001; Giet and Glover 2001; Murata-Hori et al. 2002). These patterns were shown to be located on the centromeres, suggesting that Aurora B regulates the formation and cohesion of kinetochores in animals (Kaitna et al. 2002; Murata-Hori et al. 2002). AtAUR3 also located along the metaphase plate, strongly suggesting that AtAUR3 is localized on centromeres during prophase and metaphase. When AtAUR3 is limited at the early mitotic phase, its localization pattern is similar to that of Aurora B. However, after metaphase, AtAUR3 is evenly located on the chromosomes, while animal Aurora B remains at the metaphase plate to regulate cytokinesis (Kaitna et al. 2000; Adams et al. 2001).

5 Conclusion and Perspectives

BY-2 cultured cells are suitable for analyses of chromosome dynamics during mitosis because of their chromosome size and short cell cycle. The growth rate and morphology of each transformed BY-2 cell line were almost the same as the original BY-2 cells. Specifically, BY-2 cells are one of the most suitable materials for dynamic analyses of chromosomal proteins fused with GFP. In fact, even when we analyzed chromosomal proteins of *A. thaliana* in tobacco BY-2 cells, the result was same or highly similar with that in cultured *Arabidopsis* cells (Fujimoto et al. 2005).

Although GFP offers great advantages in plant dynamic analyses, we should pay much more attention to potential artifacts in dynamic analyses of chromosomal proteins with GFP. Dynamic analyses with GFP do not show the real dynamics of proteins in vivo. GFP fusions may lead misfolding of target proteins and masking of specific regions interacted with DNA or other proteins. It is probable that overexpression of GFP fusions may have ceratin effects on the real dynamics or functions of the target protein with its expression under the native promoter. Results of dynamic analyses of chromosomal proteins should be confirmed with immunostaining using an antibody against the target protein.

Recently, the development of GFP variants and novel fluorescent proteins has resulted in dramatic progress in dynamic analyses of chromosomal proteins. Dynamic analyses using multiple fluorescent proteins can be applied to analyses of chromosome dynamics in tobacco BY-2 cells. Moreover, chromosome dynamic analyses using tobacco BY-2 cells is particularly well suited to applications of advanced imaging analyses including fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS), and fluorescence resonance energy transfer (FRET).

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