

Anti-Phosphorylated Histone H2AThr120: A Universal Microscopic Marker for Centromeric Chromatin of Mono- and Holocentric Plant Species

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Key Words

CENH3 · Centromere · *Drosera* · Holokinetic · Meiosis · Mitosis

Abstract

Based on the analysis of 20 different monocot and eudicot species, we propose that the centromeric distribution of the phosphorylated histone H2AThr120 is evolutionary highly conserved across species with mono- and holocentric chromosomes. Therefore, antibodies recognizing the phosphorylated threonine 120 of the histone H2A can serve as a universal marker for the cytological detection of centromeres of mono- and holokinetic plant species. In addition, super-resolution microscopy of signals specific to the centromere-specific histone H3 variant CENH3 and to H2AThr120ph revealed that these histone variants are incorporated into different nucleosomes, which form distinct, partly intermingled chromatin domains. This specific arrangement of both histone variants suggests different centromeric functions during the cell cycle.

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The centromere of monocentric chromosomes is morphologically recognizable as a constriction during the mitotic metaphase. However, due to the small size of the chromosomes or a holocentric chromosome structure, the microscopical detection of centromeres is impaired in some species. Also, in contrast to telomeres, centromeres are not specified by highly conserved DNA sequences, and therefore, centromere detection by in situ hybridization is only possible if centromere-specific probes are available. Contrary to centromeric DNA, structural and functional kinetochore proteins are highly conserved between species [Houben and Schubert, 2003].

Human CREST antisera from patients with a variant of scleroderma have been used to label animal kinetochores [Moroi et al., 1980]. Two CREST sera were the first markers to identify kinetochores of some plant species [Mole-Bajer et al., 1990; Houben et al., 1995]. More recently, besides other kinetochore-specific antibodies (e.g. anti-CENPC [Dawe et al., 1999], anti-MAD2 [Yu and Dawe, 2000], CENPE, and SKP1 [ten Hoopen et al., 2000]), the evolutionarily conserved centromere-specific histone H3-variant CENH3 (in humans called CENPA)

has become a popular centromere-specific marker. CENH3 determines the chromosomal position of kinetochore assembly and forms a link between the centromeric DNA and the proteinaceous kinetochore in eukaryotes [Blower et al., 2002]. Anti-CENH3 antibodies were frequently used for various centromere-related studies in plants [Nagaki et al., 2004]. Dependent on the epitope used for the generation of the antibody, some of the CENH3 antibodies cross-react with CENH3 of related species (e.g. anti rice-CENH3 [Nagaki et al., 2004] recognizes *Aegilops markgrafii* [Schubert, 2010], *Coix lacrym-jobi* [Han et al., 2010], maize [Jin et al., 2004], sugarcane [Nagaki and Murata, 2005], wheat [Liu et al., 2008; Zhang et al., 2010], barley [Nasuda et al., 2005; Houben et al., 2007; Zhang and Dawe, 2012], and rye [Houben et al., 2011] or only the species used for the generation of the antibody e.g. *A. thaliana* [Talbert et al., 2002] and barley [Sanei et al., 2011]). However, no universal plant centromere-specific marker has been reported so far. Only antibodies specific for post-translational modifications of histones, namely phosphorylated histone H3 at residues serine 10 [Houben et al., 1999] and serine 28 [Gernand et al., 2003], were used as a marker for active mitotic pericentromeres in a wide range of plant species.

Another cell-cycle dependent histone mark involved in centromere function has been identified recently. Phosphorylated histone H2A at serine 121 in fission yeast (equivalent to human H2A Thr120) promotes the recruitment of shugoshin, a protein which protects cohesin at centromeres from cleavage by separase during mitosis and meiosis [Kawashima et al., 2010; Wang et al., 2011; Gutierrez-Caballero et al., 2012]. The conserved spindle assembly checkpoint protein kinase Bub1 (budding uninhibited by benzimidazole 1) phosphorylates H2A at this position [Kawashima et al., 2010].

Dong and Han [2012] identified that some members of the H2A gene family of maize contain a threonine residue 133 in the evolutionarily conserved LLPKKT/S-region that undergoes phosphorylation in a cell cycle-dependent manner. During mitosis and meiosis, H2AThr133 phosphorylation in maize (equivalent to human H2AThr120) is enriched in metaphase specifically at centromeric regions. Then it decreases during late anaphase and telophase. Throughout meiosis, phosphorylation of H2A at Thr133 persists at the centromeric regions. Immunostaining of a dicentric maize chromosome revealed that only the active centromere is marked by this histone modification. Also, cross-reactivity of this antibody with the centromeres of wheat and rice was reported [Dong and Han, 2012].

In the present study we demonstrate that antibodies recognizing the phosphorylated threonine 120 of histone H2A in humans can serve as a universal marker for the cytological detection of centromeres in mono- and polycentric plant species. In addition, we show that CENH3 and H2AThr120ph are components of different nucleosomes composing distinct intermingled centromeric chromatin domains.

Materials and Methods

Plant Material

The following plant species have been used: eudicots: *Arabidopsis thaliana*, *Vicia faba* (field bean), *Lens culinaris* (lentil), *Gossypium hirsutum* (cotton), *Daucus carota* (carrot), *Beta vulgaris* (sugar beet), *Brachycome dichromosomatica*, *Solanum lycopersicum* (tomato), *Nicotiana tabacum* (tobacco), and the sundew species *Drosera binata*, *D. alicia*, and *D. rotundifolia*; monocots: *Zingiber biebersteiniana*, *Luzula luzuloides*, *L. elegans*, *Secale cereale* (rye), *Hordeum vulgare* (barley), *Triticum aestivum* subsp. *spelta* (dinkel wheat), *Allium fistulosum* (Welsh onion), and *Cyperus alternifolius* (umbrella papyrus).

Chromosome Preparation and Indirect Immunofluorescence

For immunodetection, root tips from all species, staged anthers from *S. cereale*, and flower buds from *A. thaliana* were isolated and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) as previously described [Manzanero et al., 2000]. *S. cereale*, *H. vulgare*, and *V. faba* root tips were digested with 2% pectinase, 2% cellulase 'Onozuka R-10', and 2% pectoylase Y-23, and squashes were made in PBS. To avoid non-specific antibody-binding, slides were blocked for 1 h in 4% BSA plus 0.1% Triton X-100 in PBS at room temperature, prior to 2 × 5 min washes in PBS, and incubated with the primary antibodies in a humid chamber for 12 h at 4°C. The following primary antibodies were used: rabbit anti-phosphorylated histone H2AThr120 (Abcam, ab111492, diluted 1:200), guinea pig anti-barley α CENH3 (diluted 1:100 [Sanei et al., 2011]), and monoclonal mouse anti-H3Ser10ph (Abcam, ab14955, diluted 1:1,000). After washing for 15 min in PBS, the slides were incubated in rhodamine-conjugated anti-rabbit IgG (Dianova) and FITC-conjugated anti-mouse IgG (Dianova), diluted 1:200 in PBS, 3% BSA for 1 h at 37°C. After final washes in PBS, the preparations were counterstained with 4',6-diamidino-2-phenylindole hydrochloride (DAPI) in antifade. The slides were analyzed with an Olympus BX61 microscope equipped with an ORCA-ER CCD camera. Deconvolution microscopy was employed to reduce out-of-focus blur. To achieve an optical resolution of ca. 100 nm, we applied structured illumination microscopy using a C-Apo 63x/1.2W Korr objective of an Elyra microscope system and the software ZEN (Zeiss).

Phosphatase Treatment of Meristematic Cells

To demonstrate phospho-specificity of the H2AThr120ph-antibody, rye chromosomes were incubated with a mix of 100 units alkaline phosphatase (Sigma-Aldrich) and 4,000 units lambda protein phosphatase (NEB) in 100 μ l lambda phosphatase buffer (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 1 mM MnCl₂, 0.01%

Brij-35, pH 7.5) at 30°C overnight. Slides were then washed 3 × 5 min in 1× PBS and processed for immunofluorescence as described above.

Chromosome Preparation and Immunogold Labeling for Scanning Electron Microscopy

T. aestivum chromosomes were prepared for scanning electron microscopy (SEM) as described by Schubert et al. [1993]. Briefly, root tips were fixed with 2% formaldehyde (in 10 mM Tris, 10 mM Na₂EDTA, 100 mM NaCl), mechanically dispersed (Polytron® 5 mm mixer, Kinematica, Switzerland) in isolation buffer (15 mM Tris, 2 mM Na₂EDTA, 0.5 mM Spermin, 80 mM KCl, 20 mM NaCl, 15 mM Mercaptoethanol, 1% Triton-X-100), and spun ('swing out' centrifugation) onto carbon coated glass slides. Specimens were washed in PBS buffer, blocked in 0.1% bovine serum albumin + 0.1% Tween 20 in PBS for 30 min, and incubated with the H2AThr120ph antibody (1:100 diluted in blocking solution) for 2 h. After washing, specimens were incubated with anti-rabbit-Alexa488-Nanogold® (Nanoprobes) for 2 h. Specimens were washed and post-fixed with 2.5% glutaraldehyde in PBS and subsequently silver enhanced (HQ Silver, Nanoprobes) according to the manufacturer's instructions. Specimens were washed in PBS, followed by distilled water, dehydrated in acetone, critical point dried from CO₂, cut to size, and mounted onto aluminum stubs. Specimens were examined uncoated with a Zeiss Auriga Cross-Beam Workstation. Micrographs were taken at accelerating voltages from 1 kV (SE-signal; chamber SE-detector) and 30 kV (BSE-signal; chamber QBSD-semiconductor detector).

Protein Extraction and Western Blot Analysis

Nuclear proteins from 200 mg of flower buds and leaves of *A. thaliana* were isolated according to Gendrel et al. [2005]. A total of 10 µg of protein was separated on 15% SDS/PAGE gels and subsequently blotted on polyvinylidene fluoride membranes. Membranes were incubated first with primary antibodies (anti-histone H3, ab1791, Abcam, diluted 1:2,500; anti-H2AThr120ph, ab111492, Abcam, diluted 1:2,000) and then with a secondary antibody (anti-rabbit IgG: IRDye800CW conjugated, LI-COR, diluted 1:5,000). Fluorescence signals were detected by a LI-COR Odyssey imaging system.

Results and Discussion

To test whether the amino acid sequence of human histone H2A flanking the phosphorylation site of threonine 120 is evolutionarily conserved, a sequence comparison was performed with the deduced protein sequence of histone *H2A* genes of 17 different lower and higher plant species. The comparison revealed that a large proportion of the H2A region used for the generation of the H2AThr120ph-specific antibody is conserved across species (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000360018). However, in some species, threonine 120 of histone H2A is replaced by serine. Serine is the most common replace-

ment for threonine which is also capable of being phosphorylated by kinases [Betts and Russell, 2007]. We would like to note that the histone H2A family in many eukaryotes typically contains different isoforms with either threonine or serine at this position. Further, anti-H2AThr120ph recognized a protein of the histone H2A-type, molecular mass of ~15 kDa on Western blots, of *A. thaliana* nuclear protein extracts (online suppl. fig. 2). Since the histone H2A amino acid sequences of many plants are highly similar, the antibody employed is likely able to recognize histone H2AThr120ph modification in different species.

The localization of an anti-H2AThr120ph epitope during the cell cycle of *A. thaliana* was analyzed by indirect immunofluorescence. Mitotic chromosomes revealed centromeric signals with the onset of chromosome condensation (fig. 1A). To confirm that the antibody recognizes the phosphate moiety on the Thr120-residue of H2A, we treated fixed rye root tips with phosphatases followed by incubation with the antibodies. Absence of specific immunofluorescence signals demonstrated that only the phosphorylated variant of H2A is recognized by anti-H2AThr120ph. In untreated controls, strong immunostaining signals were detected at all centromeres (online suppl. fig. 3).

To see whether the position of H2AThr120ph and that of the centromeric histone H3 variant (CENH3) coincide as suggested by Dong and Han [2012], the large somatic metaphase chromosomes of *H. vulgare* were analyzed. Double labeling with anti-αCENH3 and anti-H2AThr120ph revealed the localization of both histones in the primary constriction of all chromosomes. Consistent with the proposed function of H2AThr120ph as an interactor of the sister chromatid cohesion protector sughoshin [Kawashima et al., 2010; Wang et al., 2011; Gutierrez-Caballero et al., 2012], phosphorylated H2A localized more towards the inner domain of the centromere. CENH3 appeared mainly at the periphery of the sister chromatids. To ascertain the detailed arrangement of both centromere components, we applied structured illumination microscopy to achieve a resolution below the limit of light microscopy (~100 nm) (fig. 2; online suppl. video 1). Both immunosignals form distinct, partly intermingling structures. This suggests that CENH3 and H2AThr120ph are components of different nucleosomes forming chromatin subdomains which localize close to each other. These subdomains are likely responsible for different centromeric functions, namely centromere formation and control of centromere cohesion.

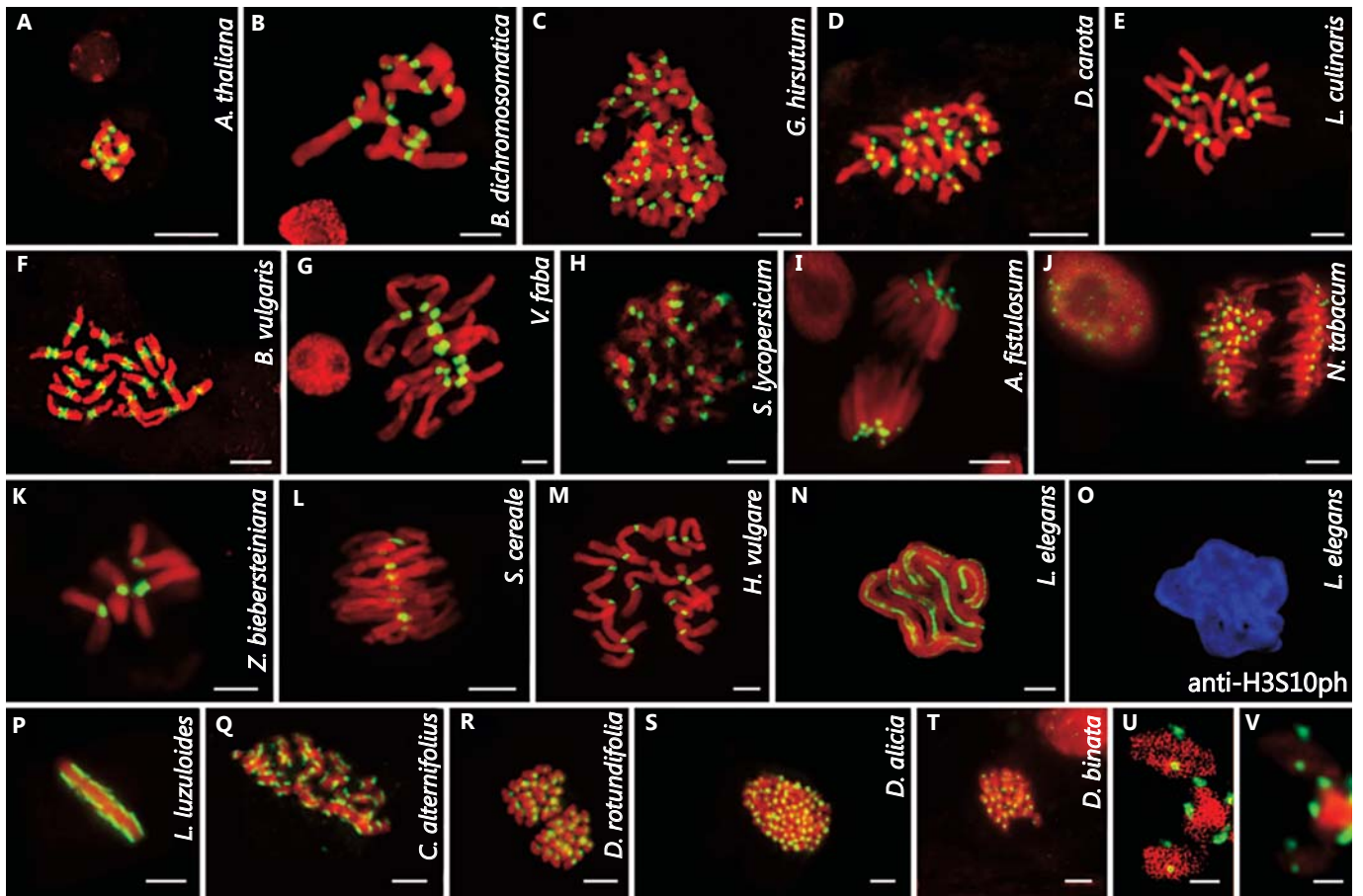


Fig. 1. Anti-histone H2AThr120ph recognizes centromeres of mono- and holocentric metaphase chromosomes of mono- and eudicot species. Fluorescent lightmicrographs of **A** *Arabidopsis thaliana*, **B** *Brachycome dichromosomatica*, **C** *Gossypium hirsutum*, **D** *Daucus carota*, **E** *Lens culinaris*, **F** *Beta vulgaris*, **G** *Vicia faba*, **H** *Solanum lycopersicum*, **I** *Allium fistulosum*, **J** *Nicotiana tabacum*, **K** *Zingeria biebersteiniana*, **L** *Secale cereale*, **M** *Hordeum vulgare*, **N** *Luzula elegans*, **P** *Luzula luzuloides*, **Q** *Cyperus alternifolius*, **R** *Drosera rotundifolia*, **S** *Drosera alicia*, and **T–V** *Drosera binata*

mitotic cells after immunostaining with antibodies recognizing the phosphorylated threonine 120 of histone H2A (in yellow). Only *L. elegans* chromosomes were additionally labeled with anti-H3Ser10ph (in blue) (**O**). To demonstrate the monocentric-like distribution of H2AThr120ph signals, selected chromosomes of *D. binata* in **U** and **V** were visualized by structural illumination microscopy and epifluorescence microscopy, respectively. Size bars represent 5 μm in **A–T** and 1 μm in **U** and **V**.

Adequate structural preservation of mitotic chromosomes in combination with immunogold labeling allowed a comparative investigation of the distribution of phosphorylated histone H2AThr120 with high resolution SEM. *T. aestivum* metaphase chromosomes are ultrastructurally characterized by densely-packed chromomeres and a centromeric region characterized by fewer chromomeres and parallel fibrils (fig. 3A). Intense H2AThr120ph-specific signals were evenly distributed over the primary constriction (fig. 3B). Weak immunosignals were found along the chromosome arms. Distribution of CENH3 labels previously analyzed by SEM re-

vealed a comparable distribution in the primary constriction [Houben et al., 2007]. In contrast, pericentromere-specific histone H3 modification H3Ser10ph, although also distributed along the chromosome arms, exhibited a maximum density in the pericentric regions and a minimum, namely a signal gap, at the primary constriction [Schroeder-Reiter et al., 2003].

Anti-phosphorylated histone H2AThr120 cross-reacts with the centromeric chromatin of mono- and holocentric chromosome species. To test whether the antibody recognizing phosphorylated H2AThr120 can serve as a universal marker for the cytological detection of plant centro-

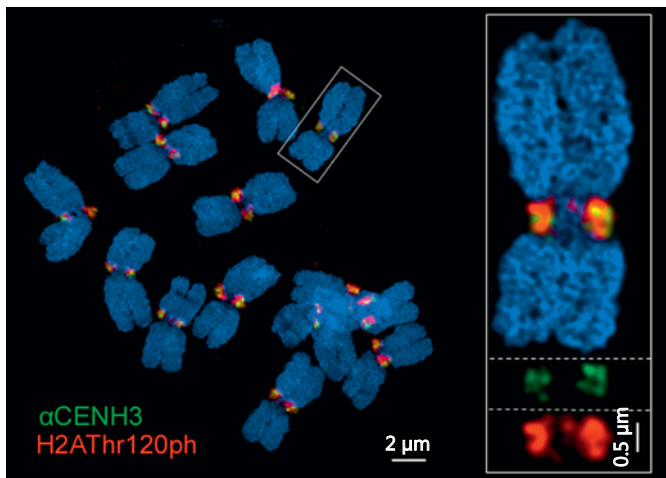


Fig. 2. Structured illumination microscopy resolves the arrangement of α CENH3 and H2AThr120ph at barley centromeres. Both histone variants are incorporated into different nucleosomes which form distinct, partly intermingling chromatin structures. The **inset** shows a further enlarged chromosome (top) and individual immunosignals (below) (see also online suppl. video 1).

meres, we immunolabeled diverse mono- and eudicot species possessing monocentric chromosomes with different genome sizes. Anti-H2AThr120-specific signals colocalized with the position of the centromere of mitotic chromosomes in all 13 species analyzed (fig. 1A–M). In addition, in a number of species with monocentric chromosomes (tomato, barley, rye, field bean, tobacco) centromeric signals were found in meristematic interphase nuclei. Also, rye chromosomes displayed phosphorylated H2AThr120 at centromeres during meiosis I and II (fig. 4).

To investigate whether the type of centromere affects the distribution of phosphorylated H2A, mitotic chromosomes of the holocentric species *L. luzuloides* and *L. elegans* were investigated. Both *Juncaceae* species show a continuous distribution of CENH3 along almost the entire length of mitotic metaphase chromosomes [Nagaki et al., 2005; Heckmann et al., 2011]. A similar centromere-like distribution was found for anti-H2AThr120ph at metaphase (fig. 1N, P). In contrast, the pericentromere-specific mark H3Ser10ph revealed a uniform labeling of the condensed mitotic chromosomes (fig. 1O) as previously demonstrated by Gernand et al. [2003]. This implies that the centromeric distribution of phosphorylated H2AThr120 is evolutionary conserved across species with mono- or holocentric chromosomes.

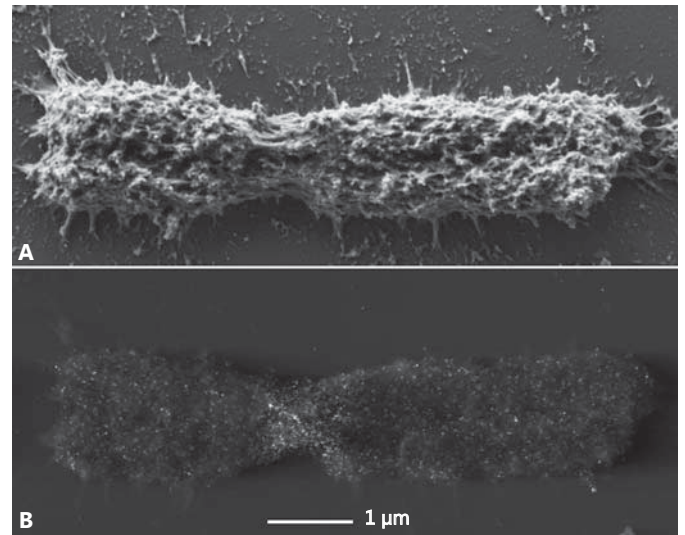


Fig. 3. Scanning electron micrographs of a topographic secondary electron image (**A**) and backscattered electron signal (**B**) of the anti-H2AThr120ph label distribution at the primary constriction of a single *T. aestivum* mitotic metaphase chromosome. The primary constriction is ultrastructurally characterized by exposed parallel fibrils (**A**). H2AThr120ph exhibits a high density of labels (white dots) in the primary constriction (**B**).

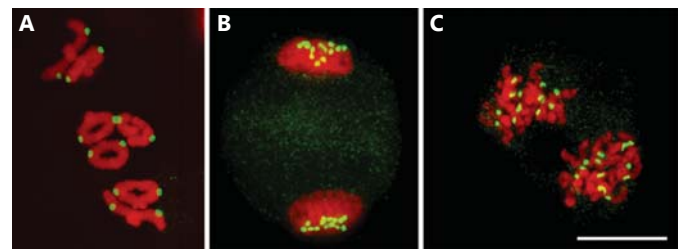


Fig. 4. Immunolabeling of meiotic *S. cereale* cells at **A** metaphase I, **B** interkineses, and **C** metaphase II with anti-H2AThr120ph (yellow). The size bar represents 10 μ m.

To evaluate whether species, which were categorized earlier as holocentrics, possess indeed holocentric chromosomes, we immunostained mitotic metaphase cells of *C. alternifolius* and of different *Drosera* species with anti-H2AThr120ph. Shirakawa et al. [2011] postulated the occurrence of mono- (*D. regia*) and holocentric (*D. arcturi*) chromosomes in *Drosera* due to presence or absence of a primary constriction at mitotic metaphase, respectively. As expected for *Cyperus* based on early electron microscopic studies by Braselton [1971], chromosomes of *C. alternifolius* displayed a holocentric-like distribution of

immunofluorescence signals (fig. 1Q). In contrast, all *Drosera* species analyzed displayed a typical monocentric-like distribution of anti-H2AThr120ph immunosignals (fig. 1R–V). This result was unexpected for *Drosera* species, since previous studies indicated the occurrence of holocentric chromosomes in the genus based on their chromosome morphology (no primary constriction evident), the mitotic anaphase behavior (chromatids move as parallel linear bars), and the stable transmission of chromosome fragments [Kondo and Lavarack, 1984; Furuita and Kondo, 1990; Sheikh and Kondo, 1995; Sheikh et al., 1995; Hoshi, 2002]. Hence, it is likely that at least the analyzed species *D. binata*, *D. alicia*, and *D. rotundi-*

folia possess monocentric chromosomes and not holocentric ones.

In summary, we have identified the first universal antibody-based marker which can be used to visualize centromeric chromatin of mono- and holocentric plant species.

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