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ARTICLE

Heterochromatin distribution and histone modification patterns of H4K5 acetylation and H3S10 phosphorylation in *Capsicum* L.

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Abstract: *We evaluated domesticated Capsicum pepper species from Brazil using classical and molecular cytogenetic techniques, and describe here for the first time their immunostaining patterns using anti-H4K5ac and anti-H3S10ph antibodies. All accessions showed 2n = 24 chromosomes with metacentric and submetacentric morphologies. CMA_3 patterns ranged from four terminal bands in most accessions to 18 variable $CMA^{++}/DAPI^{-}$ and $CMA^{+}/DAPI^{0}$ bands. Anti-H4K5ac signals were detected in the decondensed terminal euchromatin of most chromosome arms, indicating gene-rich open chromatin regions. Anti-H3S10ph signals were restricted to pericentromeric regions and were associated with sister chromatid cohesions and/or the condensation of all mitotic chromosomes. These karyological analyses constitute valuable diagnostic tools for identifying cultivated peppers, will be important to Capsicum breeding programs and will provide additional cytogenetic features that can be used for the conservation of capsicum genetic resources.*

Key words: *Cytogenetics, domesticated peppers, heterochromatic blocks, epigenetics.*

INTRODUCTION

Understanding the genomic organization and chromosomal evolution of socioeconomically important crops is essential to characterizing their genetic diversity, and represents an important initial step in plant breeding programs (Scaldaferro et al. 2013). The genus *Capsicum* L. (Solanaceae) comprises approximately 35 species that are mostly classified as wild or semi-domesticated, with five taxa being cultivated worldwide: *C. annuum* L. var. *annuum* (bell pepper), *C. baccatum* L. var. *pendulum* (Willd.) Eshbaugh (ají), *C. chinense* Jacq. (habanero), *C. frutescens* L. (tabasco) and *C. pubescens* Ruiz and Pavon (rocoto) (Carrizo García et al. 2013).

Capsicum is native to the tropical and subtropical Americas, with Brazil being considered a secondary center of its genetic diversity (Mongkolporn and Taylor 2011). Peppers constitute part of the Brazilian cultural and genetic heritage and are cultivated throughout that country with a variety of local names, types, shapes, colors, flavors, pungencies and aromas (Reyes-Escogido et al. 2011). Capsaicin (8-methyl-N-vanillyl-6-nonanamide) is an alkaloid exclusive to

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the capsicums, and their protein, vitamin and fiber contents lend them high nutritional value as well as antimicrobial, antioxidant, anti-inflammatory and anti-carcinogenic qualities (Singh and Jain 2016).

Despite their known socio-economic importance, our cytological knowledge of peppers is still incipient and further studies based on biochemical, molecular and cytogenetic techniques are needed (Moscone et al. 1993). Karyotype analysis is an important tool for characterizing *Capsicum* species (Moscone et al. 2007) and complements morphological, phytogeographical and molecular approaches. Cytogenetic data, associated with cytomolecular techniques and studies of karyotype evolution have aided in the correct delimitations of *Capsicum* – a monophyletic group with a complex taxonomy and numerous unresolved interspecific relationships (Sehr et al. 2013, Carrizo García et al. 2016).

There are two different basal chromosome numbers known for the genus: $x = 12$ and $x = 13$. Although no consensus exists yet concerning the ancestral chromosome number of that group, the presence of more symmetrical karyotypes among species showing $x = 12$ has led to the assumption that it is the probable ancestral number for the genus, and that the more asymmetrical karyotypes with $x = 13$ are derived. While $2n = 2x = 24$ species have symmetrical karyotypes, low DNA amount and simple heterochromatic banding patterns, $2n = 2x = 26$ species have asymmetric karyotypes and higher DNA and heterochromatin contents. Different inter- and intra-specific cytotypes are common and indicate very active processes of chromosome diversification in that group (Moscone et al. 2007, Teodoro-Pardo et al. 2007, Scaldaferro et al. 2013, Moreira et al. 2017).

The packing status of DNA associated with proteins can provide a better understanding of genetic and epigenetic regulation in eukaryotes, including vegetables (Heslop-Harrison and Schwarzacher 2011). Post-translational modifications on the N-terminal tails of histones play essential and distinct roles in chromosome organization (Marcon-Tavares et al. 2014). The acetylation of lysine 5 on histone H4 (one of the most well-studied modifications) is generally associated with actively transcribed open chromatin, and is directly involved in the control of chromatin replication (Zhang et al. 2015). The phosphorylation of serine 10 on histone H3 during mitosis and meiosis, on the other hand, seems to be associated with cell division control. The temporal and spatial distribution of H3S10ph is currently known only for few angiosperms, and immunostaining data is almost nonexistent for *Capsicum*.

Thus, in light of the need to provide more cytogenetic information concerning the genomic structures of peppers to aid breeding programs, we characterized accessions from the *Capsicum* Germplasm Active Bank at the Federal University of Piauí (BAGC-UFPI) using Giemsa staining, CMA/DAPI banding techniques and, for first time, immunostaining techniques using anti-H4K5ac and H3S10ph antibodies. Those immunostaining techniques should allow the identification and localization of modified histones associated with gene-rich regions (characterized by open chromatin; anti-H4K5ac) and regions of chromatid cohesion and/or chromosome condensation (anti-H3S10ph) to provide detailed maps of those two markers in mitotic chromosomes of *Capsicum* and better define pepper karyotypes.

MATERIAL AND METHODS

Plant materials

Capsicum seeds were obtained from accessions held at BAGC-UFPI (Table 1).

Chromosome preparation

Root tips obtained from germinated seeds were pretreated with p-dichlorobenzene (0.015 g mL^{-1}) for 2 hours at room temperature and then fixed in *Carnoy* (ethanol: acetic acid v/v) for at least 12 hours at -20 °C.

Conventional staining

Slide preparation followed the methodology described by Guerra (2002). Root tips were hydrolyzed in 5N HCl for 20 minutes at room temperature and then squashed in 45% acetic acid. The slides were subsequently frozen in liquid nitrogen, stained with Giemsa for 10-15 min and mounted with Entellan (Merck).

CMA/DAPI fluorochrome staining

The protocol described by Schweizer and Ambros (1994) was followed, with minor modifications. Root tips were

Table 1. Scientific name, Commom name, Accession, Provenance, Diploid chromosome number, Range of Chromosomal Size (RCS), Ratio between long and short arms (R) of each chromosome pair, Karyotype Formula (KF), Total Chromosomal Length (TCL), Chromosomal Medium Length (CML), Haploid Karyotype Length (HKL) and number of CMA/DAPI bands (CMA_3/DAPI). $^{++}$ represents CMA bands more strongly stained and $^{+}$ CMA bands slightly stained. ^{0}represents AT neutral and $^{-}$AT reduced bands. Accessions followed by the same superscript letter were grouped by Tukey's test at the 5% significance level

Scientific Name	Commom name	Accession	Provenance	2*n*	RCS µm	R	KF	TCL µm	CML µm	HKL µm	CMA_3/DAPI
C. annuum	Ornamental pepper	BAGC 99	Teresina - PI	24	2.34 – 4.92	1.12	12 M	89.27 ^{ab}	3.72 ^{ab}	44.63 ^{ab}	2 CMA^{++}/$DAPI^{-}$
C. baccatum var. *pendulum*	Red pepper	BAGC 104	Fortaleza - CE	24	2.58 – 5.48	1.26	11 M + 1 SM	100.47 ^{a}	4.18 ^{a}	50.23 ^{a}	2 CMA^{+}/$DAPI^{0}$ 2 CMA^{+}/$DAPI^{-}$ 10 CMA^{+}/$DAPI^{-}$
C. baccatum var. *pendulum*	Dedo-de-moça	BAGC 110	Teresina - PI	24	3.61 – 5.49	1.27	11 M + 1 SM	111.69 ^{a}	4.65 ^{a}	55.84 ^{a}	4 CMA^{++}/$DAPI^{-}$ 6 CMA^{+}/$DAPI^{0}$
C. baccatum var. *pendulum*	Dedo-de-moça	BAGC 125	Natal – RN	24	3.24 – 5.85	1.2	11 M + 1 SM	117.56 ^{a}	4.9 ^{a}	58.78 ^{a}	-
C. baccatum var. *pendulum*	Dedo-de-moça	BAGC 194	Recife – PE	24	2.22 – 4.87	1.29	10 M + 2 SM	87.87 ^{ab}	3.66 ^{ab}	43.93 ^{ab}	6 CMA^{++}/$DAPI^{-}$ 12 CMA^{+}/$DAPI^{0}$
C. chinense	"Cheiro" pepper	BAGC 105	Fortaleza - CE	24	2.80 – 4.75	1.25	12 M	94.82 ^{a}	3.95 ^{a}	47.41 ^{a}	2 CMA^{++}/$DAPI^{-}$ 2 CMA^{+}/$DAPI^{-}$
C. chinense	Murici	BAGC 87	Codó -MA	24	2.21 – 4.57	1.29	11 M + 1 SM	83.03 ^{ab}	3.46 ^{ab}	41.48 ^{ab}	-
C. chinense	Murici	BAGC 111	Teresina - PI	24	2.04 – 4.27	1.2	11 M + 1 SM	70.75 ^{ab}	2.95 ^{ab}	35.38 ^{ab}	2 CMA^{++}/$DAPI^{-}$ 2 CMA^{+}/$DAPI^{0}$
C. chinense	Bhut jolokia	BAGC 139	Teresina - PI	24	3.56 – 5.94	1.23	11 M + 1 SM	110.85 ^{a}	4.62 ^{a}	55.43 ^{a}	-
C. chinense	Red bode	BAGC 193	Recife – PE	24	2.08 – 5.09	1.27	11 M + 1 SM	83.25 ^{ab}	3.47 ^{ab}	41.63 ^{ab}	2 CMA^{++}/$DAPI^{-}$ 2 CMA^{+}/$DAPI^{0}$
C. frutescens	Chilli pepper	BAGC 106	Fortaleza - CE	24	2.27 – 4.49	1.24	12 M	80.14 ^{ab}	3.34 ^{ab}	40.07 ^{ab}	-
C. frutescens	Chilli pepper	BAGC 126	Natal – RN	24	2.34 – 5.05	1.28	11 M + 1 SM	90.80 ^{ab}	3.78 ^{ab}	45.40 ^{ab}	4 CMA^{+}/$DAPI^{0}$
C. frutescens	Chayenne's pepper	BAGC 191	Recife – PE	24	1.96 – 3.63	1.27	12 M	66.55 ^{b}	2.77 ^{b}	33.27 ^{b}	2 CMA^{++}/$DAPI^{-}$ 4 CMA^{+}/$DAPI^{0}$

digested with 2% cellulase (Onozuka R-10) and 20% pectinase (Sigma). The slides were stained with 10 µL of CMA (0.5 mg mL^{-1}) for 1h, counterstained with 10 µl of DAPI (2 mg mL^{-1}) for 30 min, mounted in glycerol/McIlvaine (1:1) and stored for three days before analysis.

Immunostaining with H4K5ac and H3S10ph antibodies

The immunostaining technique followed the protocol of Feitoza and Guerra (2011a). Roots tips fixed in 4% paraformaldehyde were washed in 1 × PBS and incubated overnight at 4 °C with the primary anti-H4K5ac and anti-H3S10ph antibodies (rabbit polyclonal IgG-Upstate Biotechnology, USA) diluted 1:300 in 1× PBS containing 3% BSA. To detect the primary antibodies, 15 µL of secondary antibody FITC-conjugated goat anti-rabbit IgG (Sigma) diluted 1:60 in 3% BSA was added, and the root tips held in a dark chamber at room temperature for 3 hours. The preparations were then mounted in DAPI (2 mg mL^{-1}): Vectashield (1:1 v/v) and immediately photographed.

Image analyses and morphometry

The best five metaphases of each accession were photographed using a DFC345Fx digital camera coupled to a Leica DM2500 microscope. The images were adjusted and optimized for brightness and contrast using *Adobe Photoshop CS3.* Chromosome sizes were determined using the *Micromeasure 3.3* program supplemented by Microsoft Excel 2010 software. Idiograms were constructed using *Corel Draw X7* and chromosome morphologies were classified according to Guerra (1988).

RESULTS AND DISCUSSION

Conventional staining allowed a detailed description of the interphase nuclei, prophase condensation patterns, chromosome numbers, as well as chromosome morphologies and sizes (Figure 1, Table 1). Giemsa staining revealed semi-reticulate nuclei (Figure 1a) and Solanum-type prophase condensation pattern (CP) with early condensed proximal regions and late condensing terminal chromatin (Feitoza Costa and Guerra 2017). All accessions showed 2*n* = 24 chromosomes, with metacentric and submetacentric morphologies. As reported by Moscone et al. (2007), all domesticated *Capsicum* taxa have a chromosome base number x = 12 with symmetrical karyotypes, while other semi-domesticated and several wild species (such as *C. campylopodium, C. villousum* and *C. buforum*) have x = 13 with asymmetrical karyotypes that are probably derived from centric fissions (Robertsonian).

Most accessions (BAGC 87, 104, 110, 111, 125, 126, 139, and 193, see table 1) showed the karyotype formula 11M + 1SM, while BAGC 99 (*C. annuum*), 105 (*C. chinense*) and 106 and 191 (*C. frutescens*) showed 12M; only the accession BAGC 194 (*C. baccatum* var. *pendulum*) showed 10M + 2SM. Chromosome sizes (RCS) ranged from 1.96 µm in *C. frutescens* (BAGC 191) to 5.94 µm in *C. chinense* (BAGC 139), while total chromosomal lengths (TCL) ranged from 66.55µm in *C. frutescens* (BAGC 191) to 117.56 µm in *C. baccatum* var. *pendulum* (BAGC 125) Previous workers (Moscone et al. 1996) reported TCL variations from 122.62 µm in *C. chinense* to 148.62 µm in *C. baccatum* var. *pendulum*. Similarly, other *C. annuum* accessions from the *Capsicum* germplasm bank in Mexico showed high TCL polymorphisms, demonstrating the high inter- and intraspecific chromosome diversity found in *Capsicum* (Teodoro-Pardo et al. 2007). The observed differences in chromosome sizes of the different accessions could be related to unequal degrees of chromosome contraction during cell division (Moscone 1990), differences in pretreatment and chromosome condensation (Pozzobon et al. 2006), and/or to different classes of repetitive DNA sequences as evolutionary components of pepper genome structures based on constitutive heterochromatin expansions (Shcherban 2015, Scaldaferro et al. 2016).

The double-staining technique with CMA and DAPI fluorochromes allowed the identification of three different heterochromatic banding patterns: highly GC-rich and AT-reduced ($CMA^{++}/DAPI^{-}$); moderately GC-rich and AT-reduced ($CMA^{+}/DAPI^{-}$); moderately GC-rich and AT-neutral ($CMA^{+}/DAPI^{0}$) (Table 1, Figure 2). Differences in the numbers, patterns, and intensities of heterochromatic bands were observed. Four CMA bands were observed in two chromosome pairs in *C. annuum* (BAGC 99) and *C. chinense* (BAGC 111 and 193), with at least one pair of larger CMA^{++} bands and one pair of small CMA^{+} bands. *C. frutescens* (BAGC 126) showed four CMA^{++} bands, while BAGC 191 exhibited six bands (two $CMA^{++}/DAPI^{-}$ and four smaller additional $CMA^{+}/DAPI^{0}$ bands). All of the accessions that showed variable numbers of heterochromatic bands (10, 12 and 18 $CMA^{++}/DAPI^{-}$ and $CMA^{+}/DAPI^{0}$ bands) were *C. baccatum* var. *pendulum* accessions (BAGC 110, 104 and 194 respectively). None of the accessions showed visible $DAPI^{+}$ bands. The idiograms in Figure 3 present graphic karyotype representations of each accession.

GC-rich heterochromatin is universal in *Capsicum*, appearing in all taxa with *x* = 12; although located mainly in terminal regions, it may also occur in variable numbers of small intercalary and distal bands that are difficult to detect (as in *C. chacoense, C. annuum* var. *glabriusculum, C. recurvatum, C. villosum* and *C. flexuosum*) (Scaldaferro et al. 2013). Heterochromatin distribution is not always homogeneous within or among species, and polymorphisms have been reported (reviewed by Guerra 2000). Moscone et al. (1993) observed centromeric, distal, and intercalary bands unequally distributed in both chromosome arms of peppers. Those authors classified domesticated species into two groups based on their C-band contents: the white-flowered group (*C. annuum*, *C. chinense,* and C. *frutescens*) with low heterochromatin contents, small terminal bands and lacking intercalary bands; and the purple-flowered group (which includes *C. campylopodium* and *C. pubescens*) with high heterochromatin contents, large telomeric bands and intercalary blocks. Although *C. baccatum* belongs to the white-flowered subgroup, it differs from the other three domesticated species by showing a complex heterochromatin pattern and longer chromosome lengths. We note here that *C. baccatum* var. *pendulum* exhibits higher numbers of heteromorphic GC-rich bands as compared to *C. annuum, C. chinense,* and *C. frutescens.*

For a better understanding of chromatin structural patterns and cell cycles in *Capsicum*, we performed

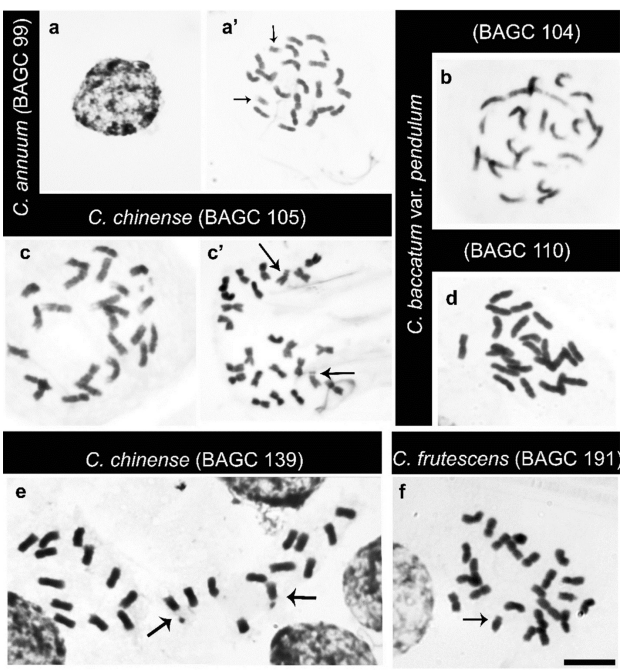

Figure 1. Chromosome number of prometaphase and metaphase in *Capsicum* sp. by Giemsa staining. a- interphase nucleus; a'- prometaphase with arrows indicating chromosomic pair with decondensed chromatin in terminal region; b, c - prometaphase; c', d, e and f – metaphase. Arrows in c', e and f indicate NORs. Bar = 10 µm.

immunostaining using antibodies against histones modified by the acetylation of lysine 5 of histone H4 (H4K5ac) and the phosphorylation of serine 10 of histone H3 (H3S10ph).

Anti-H4K5 patterns were classified as Solanum-type (Feitoza Costa and Guerra 2017), with labeling restricted to diffuse chromatin of the nuclei and in the decondensed terminal euchromatin of most mitotic chromosomes, from prophase-metaphase (Figure 4); the chromocenters of the interphase nuclei (and some terminal heterochromatic regions) were not stained. The acetylation patterns we observed showed labeling tightly associated with terminal open chromatin in the mitotic chromosomes of *C. baccatum* var. *pendulum* and *C. chinense*. Similar labeling has been reported in other plants with small chromosomes and well-established condensation pattern, such as *Costus spiralis, Eleutherine bulbosa* (Feitoza and Guerra 2011b) and *P. vulgaris* (Fonseca et al. 2014). The fact that anti-H4K5ac markings were present in all of the cell cycle phases analyzed suggests their close relationships with chromatin dynamic states that may change during the cell cycle and become restricted to specific chromosome sites during meiosis (Feitoza and Guerra 2011b).

Additionally, hyperacetylated regions (immunostaining marking) coincided with late condensing euchromatic regions (conventional staining), while hypoacetylated or non-acetylated regions were directly linked to early condensing prophase chromatin and to CMA^{++} or CMA^{+} blocks (CMA/DAPI staining). As suggested by Dhar et al. (2009), acetylation patterns are inversely related to heterochromatic patterns: late replicating euchromatin (including heterochromatin) is early condensing, whereas early replicating chromatin (gene-rich euchromatin) is late condensing.

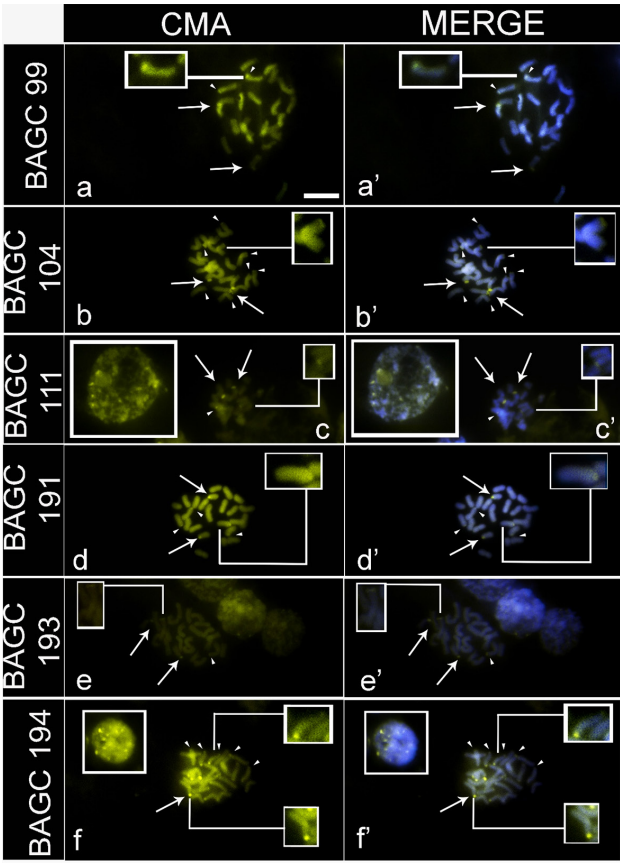

Figure 2. Double staining with CMA and DAPI fluorochromes in *Capsicum* accessions. a – *C. annuum* (BAGC 99); b and f - *C. baccatum* var. *pendulum* (BAGC 104 and 194); c and e - *C. chinense* (BAGC 111 and 193); d - *C. frutescens* (BAGC 191). Arrows indicate large CMA^{++} blocks. Arrowheads indicate small CMA^{+} blocks in terminal region difficult to detect, enlarged in all inserts. Bar = 10µm.

Since H4K5 acetylation is universally associated with gene expression and open chromatin (as observed in tomatoes [Cigliano et al. 2013], *Silene* and *Allium cepa* [Chang et al. 2008]), we believe that its labelling in uncondensed euchromatin is related to potentially active regions in the chromosomes of *Capsicum.*

Anti-H3S10ph signals, on the other hand, were restricted to the pericentromeric regions of all of the chromosomes during prometaphase-metaphase, while interphase nuclei were not stained (Figure 5). This pattern is associated with mitotic condensation and/or chromatid cohesion regardless of eu- and heterochromatin regions involved (unlike the pattern observed for anti-H4K5 markings). That phosphorylation marking only occurred in pericentromeric regions of the A chromosomes in *Cestrum strigilatum* (another Solanaceae species), although an exception was observed in its B chromosomes (Fernandes et al. 2008).

These results, associated with previous work performed in other socio-economically important crops, reinforce the idea that the hyperacetylation of H4K5 can be used as a chromosome marker for detecting active euchromatin associated with gene expression. H3S10 phosphorylation seems to be involved in regulating sister chromatid cohesion, although further studies will be needed. Our results demonstrated the feasibility of using immunostaining to detect gene-rich and pericentromeric regions during the cell cycle of *Capsicum* that could be used in future studies involving cytomolecular mapping and contribute to the genetic improvement of peppers and their preservation in Brazil.

Figure 3. Idiograms representing size, morphology and distribution of CMA^{++} terminals bands (larger yellow bands) and CMA^{+} bands (smaller yellow bands) on chromosome of *Capsicum*'s accessions.

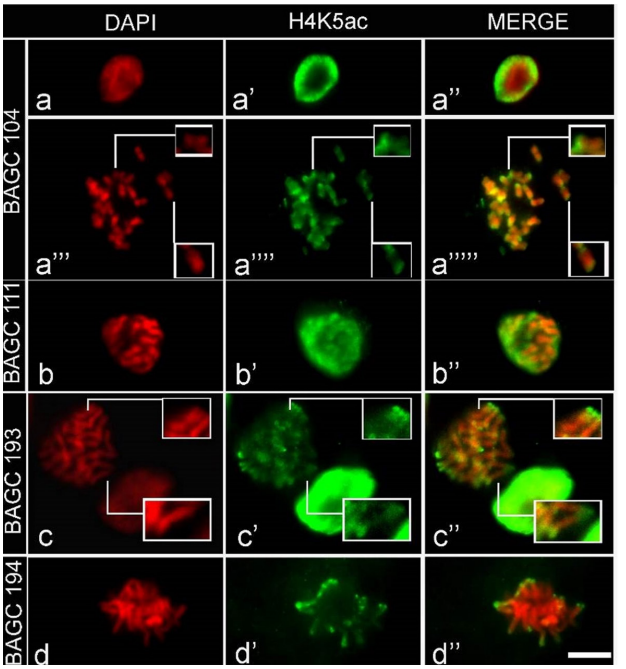

Figure 4. Immunostaining with anti-H4K5ac antibody in *C. baccatum* var. *pendulum* (BAGC 104 and 194) and *C. chinense* (BAGC 111 and 193). a, -a''– interphase nucleus; a'''- a''''' – metaphase; b – prophase and c-d'' – prometaphase showing terminal chromatin strongly marked (green) compared with condensed chromatin (red). Upper inserts indicate chromosome with terminal region strongly acetylated on one terminal arm, while lower inserts indicate chromosome with terminal regions strongly acetylated on both arms (green). DAPI pseudocolored in red and anti-H4K5ac in green. Bar = 10 µm.

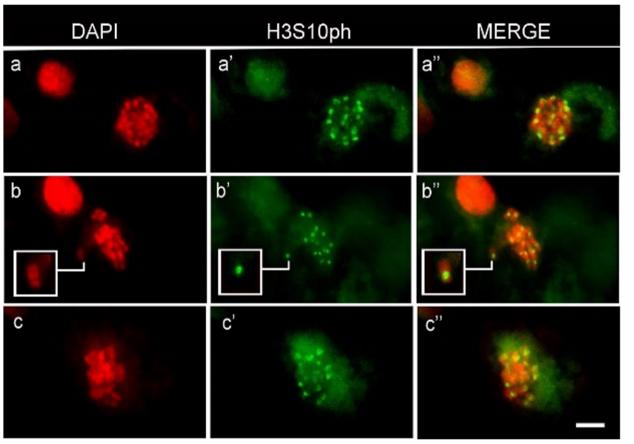

Figure 5. Immunostaining with anti-H3S10ph antibody in prometaphase chromosome of: a – *C.annuum* (BAGC 99), b - *C. chinense* (BAGC 111) and c - *C. baccatum* var. *pendulum* (BAGC 125). Observe intense staining of antibody (green) in pericentromeric region (insert in b). Interphase nuclei in the left corner on a and b were not marked. Bar = 10 µm.

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