RESEARCH ARTICLE

Distribution pattern of histone H3 phosphorylation at serine 10 during mitosis and meiosis in *Brachiaria* species

C. M. P. PAULA[1], V. H. TECHIO[2,*], F. SOUZA SOBRINHO[3] and A. S. FREITAS[4]

[1]*Genetics and Plant Breeding, Universidade Federal de Lavras (UFLA), Campus Universitário, Caixa Postal 3037, CEP: 372000-000 Lavras-MG, Brazil*
[2]*Universidade Federal de Lavras (UFLA), Campus Universitário, Caixa Postal 3037, CEP: 372000-000 Lavras-MG, Brazil*
[3]*Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Embrapa Gado de Leite (CNPGL), Rua Eugênio do Nascimento, 610, Bairro Dom Bosco, CEP: 36038-330 Juiz de Fora-MG, Brazil*
[4]*Biological Sciences, Universidade Federal de Lavras (UFLA), Campus Universitário, Caixa Postal 3037, CEP: 372000-000 Lavras-MG, Brazil*

Abstract

Histones are the major eukaryotic DNA-binding proteins. Posttranslational modifications on N-terminal tails of histones that form nucleosomes are often associated with distinct biological functions. Some theories suggest that one of these modifications, the phosphorylation of histone H3 at serine 10 (H3S10ph) plays a role in both chromosome condensation and sister chromatid cohesion. Although histones and some of their modifications are highly conserved, studies have shown that role and distribution of H3S10ph may differ between species. We evaluated the pattern of H3 phosphorylation using immunodetection during mitosis and meiosis in both diploid and tetraploid genotypes of *Brachiaria* species. Results revealed differences in chromosome distribution of H3S10ph when mitosis and meiosis were compared. Whole chromosomes were phosphorylated during meiosis I, whereas phosphorylation was restricted to the pericentromeric region in both meiosis II and mitosis. There was no variation in phosphorylation patterns between *Brachiaria* species and diploid and tetraploid genotypes. Regarding spatiotemporal coordination in the *Brachiaria* species evaluated, H3S10ph is related to maintenance of sister chromatid cohesion during cell divisions.

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Introduction

Eukaryotic chromatin is organized into basic units, called nucleosomes, which consist of approximately 147 base pairs (bp) of DNA wrapped around a histone octamer comprising two molecules of histones H2A, H2B, H3 and H4. Each histone forming the nucleosome octamer has a core and a terminal amino acid chain, called N-terminal tail. Both are subject to posttranslational modifications, although occurring more frequently in the N-terminal tail (Kouzarides 2007; Jin *et al.* 2008; Bannister and Kouzarides 2011).

These modifications play a fundamental role in most biological processes involved in DNA expression, such as the epigenetic control that regulates gene activation and chromatin modifications during cell cycle (Bannister and Kouzarides 2011). At least eight different types of alterations are found in histones, among which the most studied are acetylation, methylation and phosphorylation (Kouzarides 2007; Chen *et al.* 2010). Phosphorylation may have important consequences for chromatin packing due to change in histone load, which consequently influences chromatin structure (Bannister and Kouzarides 2011). Histone H3 phosphorylation at serine 10 (H3S10ph) is a major alteration where spatiotemporal distribution during cell cycle may vary between species (Hans and Dimitrov 2001); however, its function during cell divisions has not yet been completely defined. Some authors report that H3S10ph is involved not only in chromosome condensation but also in cohesion maintenance and segregation of sister chromatids (Hendzel *et al.* 1997; Kaszás and Cande 2000; Manzanero *et al.* 2000; Johansen and Johansen 2006; Houben *et al.* 2007).

*For correspondence. E-mail: vhtechio@dbi.ufla.br.

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It is important to evaluate the histone H3S10ph distribution pattern and to determine its function during cell divisions in *Brachiaria* species (A. Rich.) Stapf [(syn. *Urochloa* Hochst.ex A.Rich.) RDWebster], since current knowledge about specific roles of histone modifications in plants, especially grasses, is still insufficient to clarify divergent issues related to their regulatory role. In the specific case of *Brachiaria*, there is special interest in evaluating different ploidy levels to find possible modifications resulting from polyploidization, as such processes may be accompanied by epigenetic changes (Li *et al.* 2011).

Thus, our study determined the distribution pattern of histone H3 phosphorylation at serine 10 in *Brachiaria* species with different ploidy levels in order to investigate the dynamics of this posttranslational modification and to correlate it with regulatory function during mitosis and meiosis.

Material and methods

Plant material

We used immunodetection in *Brachiaria ruziziensis* (cultivar Common, $2n = 2x = 18$ and tetraploid population, $2n = 4x = 36$, obtained at Embrapa Gado de Leite, Juiz de Fora - Minas Gerais, Brazil), in *B. brizantha* (cultivar Marandu, $2n = 4x = 36$), and in *B. decumbens* (cultivar Basilisk, $2n = 4x = 36$).

Immunodetection of H3S10ph

The immunodetection technique followed the method described by Manzanero *et al.* (2000) with some modifications.

Roots and anthers were collected, fixed in 4% paraformaldehyde solution and washed in phosphate buffered saline (PBS) buffer. Roots were digested with 2% cellulase, 2% pectinase at 37° C for 2 h 30 min. Slides were prepared with squash technique and incubated in 50 μL PBS containing BSA 3% and Triton X-100; 0.1% for 20 min at room temperature. Subsequently we applied 25 μL of primary antibody (Rabbit polyclonal IgG, Santa Cruz Biotechnology, USA) per slide at 1:100 dilution. The slides were kept in a moist chamber for at least 16 h at 4°C, then washed in PBS and detected with the secondary antibody (Goat anti-rabbit IgG-FITC, Santa Cruz Biotechnology) at 1:100 dilution. The slides were then kept in a moist chamber for 3 h at room temperature under aphotic conditions. After being washed in PBS, the slides were counterstained and mounted in DAPI solution (4′, 6-diamidino-2-phenylindole)/Vectashield H-1000 (1:100).

Image capture and processing

Samples were examined under epifluorescence microscope Olympus BX60, photographed with digital camera (Sony Cyber-shot, 10.1 mega pixels) and processed with Adobe Photoshop CS3 (for brightness and contrast only). The distribution pattern of H3 S10ph was based on both occurrence and nonoccurrence of immunodetection in at least 100 slides and approximately 1200 cells of each species evaluated during mitosis and meiosis.

Results and discussion

A summary of the immunodetection pattern obtained with antibody against histone H3 phosphorylation at serine 10 during different phases of mitosis and meiosis in *Brachiaria* species is shown in table 1 and figure 1.

Histone H3 phosphorylation at serine 10 during mitosis and meiosis showed specific coordination pattern in space and time, i.e. location and time of phosphorylation during cell divisions were the same for *B. ruziziensis* (diploid and tetraploid), *B. decumbens* and *B. brizantha* (table 1). However, the immunodetection model obtained for these species was different when H3S10ph chromosome distribution in mitosis and meiosis were compared, suggesting that this posttranslational modification could play different roles through cell division cycle. This variation has also been observed in other grasses such as *Secale cereale* and *Triticum aestivum* (Manzanero *et al.* 2000), and *Zea mays* (Kaszás and Cande 2000).

In contrast, phosphorylation was absent in interphase during both mitosis and meiosis in the four genotypes (figure 2), thus confirming the observation by Schroeder-Reiter *et al.* (2003) that this posttranslational modification is cell cycle-dependent. Studies of various organisms have shown that H3S10 phosphorylation levels that decrease in interphase substantially increase at the beginning of cell division and ultimately decrease during telophase (Hendzel *et al.* 1997; Wei *et al.* 1998; Houben *et al.* 1999). Thus, as already described by Germand *et al.* (2003), cell cycle progression depends on histone H3 phosphorylation at Ser 10 and this event is conserved in eukaryotes.

As the model of H3S10 mitotic phosphorylation was similar in *B. ruziziensis* chromosomes (diploid and tetraploid), *B. decumbens* and *B. brizantha*, we conclude that variation in ploidy levels was not accompanied by changes in H3 phosphorylation pattern, thus confirming its important role in cell cycle progression.

The distribution of H3S10 phosphorylation during mitosis in *Brachiaria* was restricted to the pericentromeric region (table 1; figures 1 and 3) thus agreeing with Hans and Dimitrov (2001) and Zhang *et al.* (2005), who state that the mitotic pattern of H3 phosphorylation correlates with the pericentromeric chromatin in plants.

Phosphorylation begins late in prophase (figure 3a), continues on the metaphase plate (figure 3b) where all chromosomes show H3 phosphorylation, and in the pericentromeric chromatin. It then extends to anaphase (figure 3c) and gradually disappears in telophase (figure 3d).

Table 1. Distribution of H3S10ph in mitosis and meiosis of *Brachiaria* species.

Material	Mitosis	First meiotic division								Second meiotic division			
		L	Z	Pa	D	Dc	MI	AI	TI	PII	MII	AII	TII
B. ruziziensis (2x)	P	+	+	+	+	+	+	+	−	P	P	P	−
B. ruziziensis (4x)	P	+	+	+	+	+	+	+	−	P	P	P	−
B. brizantha (4x)	P	+	+	+	+	+	+	+	−	P	P	P	−
B. decumbens (4x)	P	+	+	+	+	+	+	+	−	P	P	P	−

P, immunodetection in the pericentromeric region; +, immunodetection visible along the chromosome; −, not detectable. Division phases: L, leptotene; Z, zygotene; Pa, pachytene; D, diplotene; Dc, diakinesis; MI, metaphase I; AI, anaphase I; TI, telophase I; PII, prophase II; MII, metaphase II; AII, anaphase II; TII, telophase II.

The distribution model of H3S10 phosphorylation found in mitosis in *Brachiaria* species was similar to that of plant species already reported in literature, such as *Secale cereal* and *Triticum aestivum* (Manzanero *et al.* 2000), *Hordeum vulgare* and *Vicia faba* (Houben *et al.* 1999), and *Cestrum strigilatum* (Fernandes *et al.* 2008). In our experiment, H3 phosphorylation beginning in already condensed chromosomes in prophase along with immunodetection in the pericentromeric region provide a strong argument against linking this posttranslational modification to chromosome condensation during mitosis in the species under study.

A recent study of the species *Eleutherine bulbosa* by Feitoza and Guerra (2011) reports no causal relationship between H3S10ph and mitotic condensation in plants, as the species shows a distinct pattern of well-defined early condensation in prophase, which is restricted to the chromosome pair 1. However, these chromosomes are neither prematurely

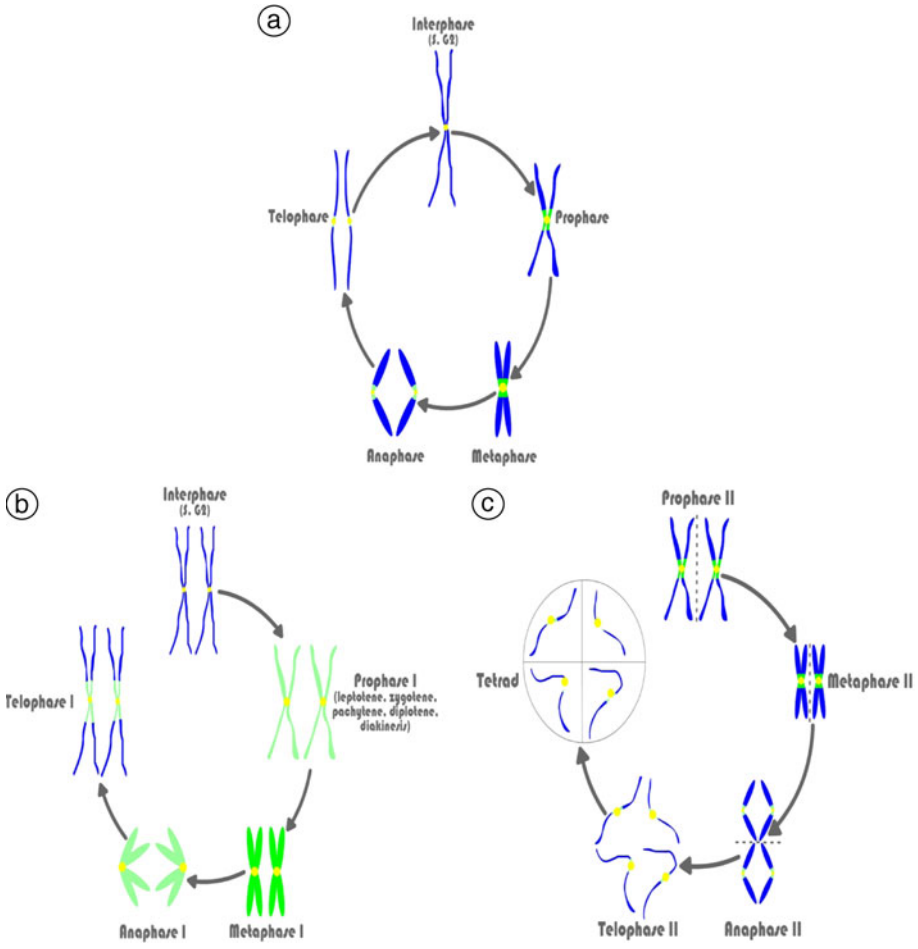

Figure 1. Immunodetection (green) of histone H3S10 phosphorylation in mitosis (a) and meiosis (b and c).

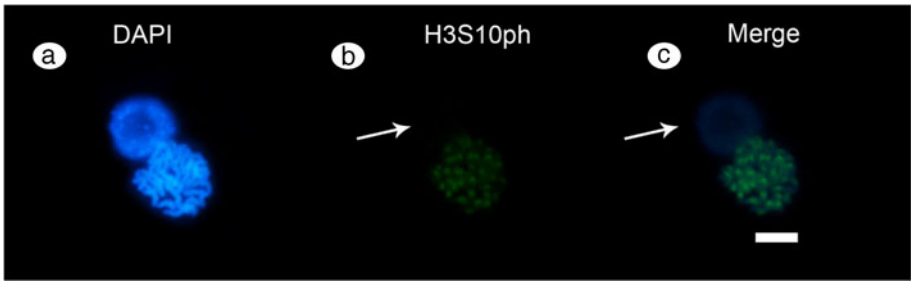

Figure 2. Absence of histone H3S10 phosphorylation in the interphase nucleus (arrow) of *B. brizantha*. (a) Observed with DAPI. (b) Observed with FITC. (c) Overlapping of DAPI and FITC images. The bar represents 10 μm.

phosphorylated nor more extensively phosphorylated than the others.

Studies of species with monocentric centromeres such as *Brachiaria* compared to species with holocentric centromeres such as *Luzula luzuloides* (Germand *et al.* 2003) and *Rhynchospora tenuis* (Guerra *et al.* 2006) showed the same temporal pattern of H3S10 phosphorylation. However, in contrast to chromosomes of *Brachiaria* species, chromosomes of holocentric species are uniformly marked through their length in metaphase and anaphase. This characteristic confirms that distribution of H3 phosphorylation correlates with distribution of active centromeres, thus the entire length of holocentric chromosomes would function as an extended centromeric region.

While observing H3 hyperphosphorylation in centromeric chromatin regions in plants, Houben *et al.* (1999) suggested that this modification is essentially related to the centromere and kinetochore structure, which can provide the mechanical stability necessary to compete with forces generated in kinetochores during chromosome movement.

H3S10 phosphorylation occurring at the end of mitotic prophase, associated with the arguments mentioned above, suggest that this modification is unlikely to play an important role in mitotic chromosome condensation in *Brachiaria* species, although it is directly related to the maintenance of sister chromatid cohesion in the pericentromeric region.

The immunodetection pattern with antibody against H3S10 phosphorylation was the same for both diploid and tetraploid species of *Brachiaria* in all meiosis stages. Polyploidy or duplication of the entire genome, as occurred in artificially tetraploidized *B. ruziziensis,* is often accompanied by potentially reversible epigenetic changes or post-translational modifications. According to Chen (2007), such epigenetic changes provide an efficient and flexible way for

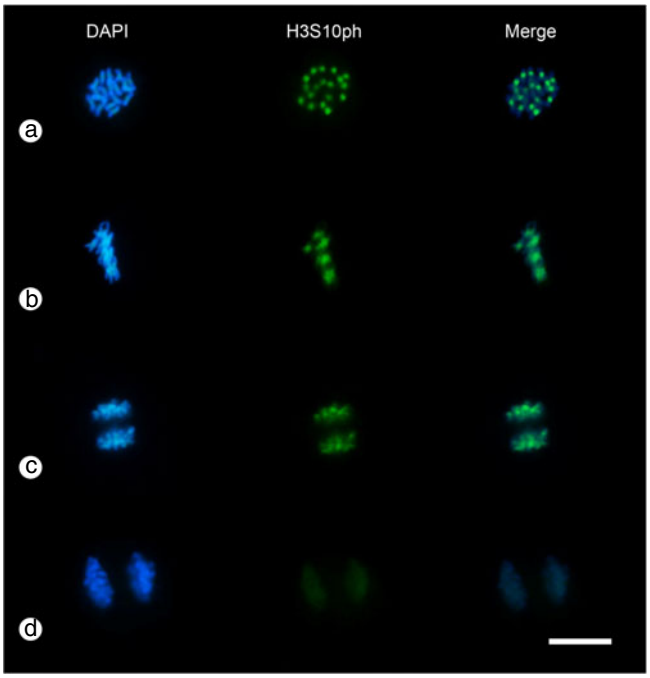

Figure 3. Immunodetection of histone H3S10ph during mitosis in *B. ruziziensis* diploid. (a) Prophase, (b) metaphase, (c) anaphase, and (d) telophase. The bar represents 10 μm.

cell to respond to both polyploidy and genomic conflict. However, as well as in mitosis, difference in ploidy levels in *Brachiaria* species was not accompanied by changes in the distribution pattern of this posttranslational modification (table 1).

In *B. ruziziensis*, *B. decumbens* and *B. brizantha*, H3S10ph distribution varied between the first and second meiotic division, similarly to the distribution in meiocytes of *S. cereale* and *T. aestivum* reported by Manzanero *et al.* (2000). During the first division, whole chromosomes were marked and H3 phosphorylation ended near telophase I. In the second meiotic division, however, only the pericentromeric regions of prophase II through anaphase II were marked, analogous to mitosis (table 1; figures 1, 4 and 5).

Cell cycle progression in the early stages of prophase I (leptotene, zygotene and pachytene) occurred with H3S10 phosphorylation. Immunodetection signals gradually increased from the diplotene stage on, reaching intense detection in the diakinesis stage. Thus, the most intense immunodetection of phosphorylation was found at the end of prophase I, when chromosomes are packed and congressing to the metaphase plate (table 1; figures 1 and 4).

The temporal model of H3S10 phosphorylation in *Brachiaria* species was similar to that observed in *S. cereale* and *T. aestivum*, where the first immune signals were detected during transition from leptotene to zygotene stage (Manzanero *et al.* 2000). However, the model was different from that found in *Z. mays* (Kaszás and Cande 2000), where the transition from leptotene to zygotene stage occurred without H3 phosphorylation.

The study demonstrated that changes in H3 phosphorylation in meiosis stages in *Z. mays* correlated with maintenance of sister chromatid cohesion rather than chromosome condensation. Phosphorylation occurs in the transition from diakinesis to metaphase I (when chromosomes are already packed) and coincides with rupture of the nuclear membrane (Kaszás and Cande 2000).

In *Brachiaria* species, bivalents were already fully and strongly marked in metaphase I. These chromosomes migrated to cell poles in anaphase I still homogenously marked, although with reduced intensity (table 1; figures 1, 4 and 5).

When only H3S10ph spatiotemporal distribution in meiosis I in *Brachiaria* genotypes is considered, it seems to have a correlation with chromosome condensation. However, although cohesins are enriched around centromeres, they also bind to sites along chromosome arms during meiosis I (Kaszás and Cande 2000; Eckert *et al.* 2007). These

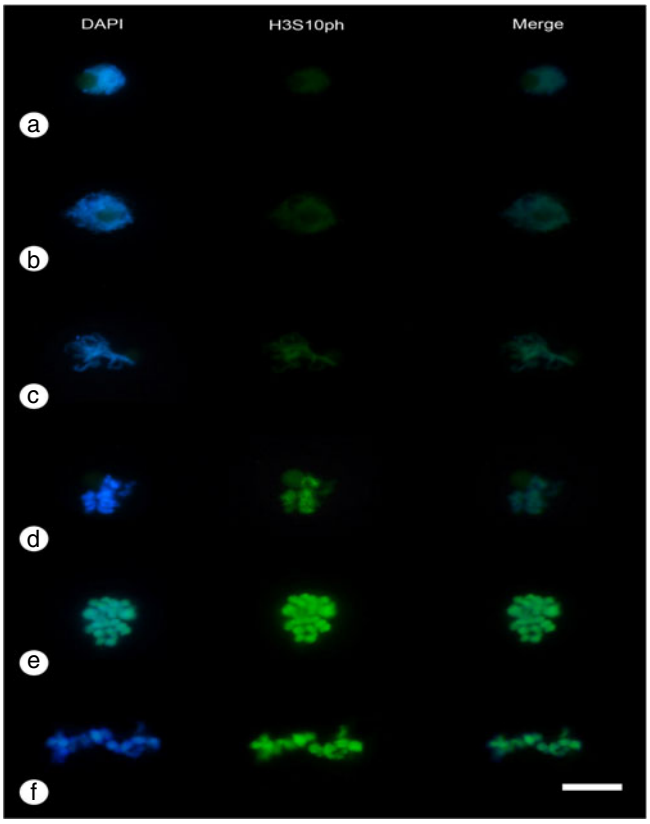

Figure 4. Immunodetection of histone H3S10ph during meiosis in *B. brizantha*. (a) Leptotene, (b) zygotene, (c) pachytene, (d) diplotene, (e) diakinesis, and (f) metaphase I. The bar represents 10 μm.

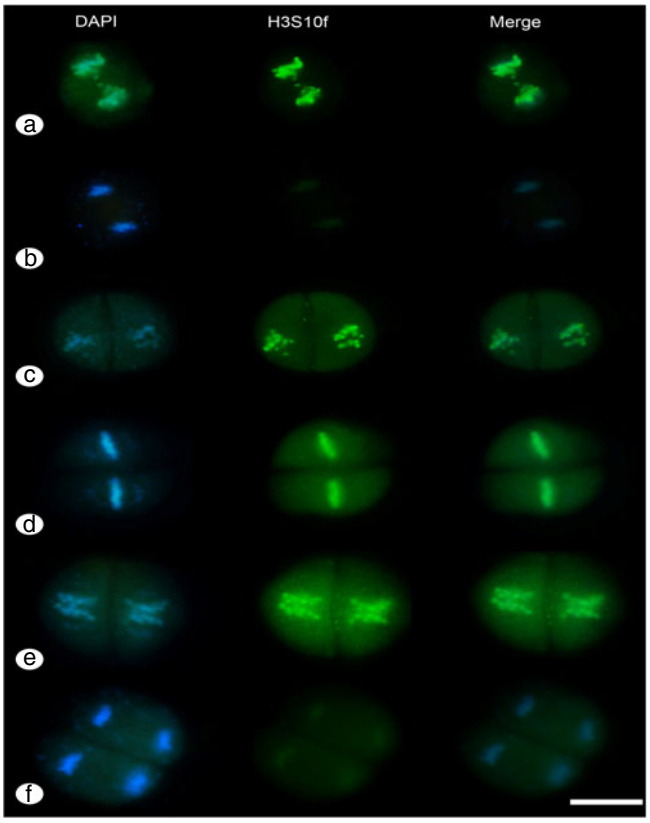

Figure 5. Immunodetection of histone H3S10ph during meiosis in *B. brizantha*. (a) Anaphase I, (b) telophase I, (c) prophase II, (d) metaphase II, (e) anaphase II, and (f) telophase II. The bar represents 10 μm.

cohesin proteins bind primarily to regions near the centromere. Then, they either diffuse or are actively moved through the chromosome arms (Eckert *et al.* 2007) in order to keep bivalents intact. In meiosis progression this binding along chromosome arms disappears in anaphase I, thus allowing homologous chromosomes to segregate to opposite cell poles (Nasmyth and Haering 2009). These characteristics can explain why chromosomes of *Brachiaria* species appear fully marked in immunodetection during prophase I through metaphase I and then decrease in signal intensity in anaphase I.

Exclusion of causal relationship of H3S10ph with chromosome condensation was also found in H3S10ph immunodetection in haploid wheat meiocytes. In the study (Manzanero *et al.* 2000; Germand *et al.* 2003) the authors found that single chromatids resulting from the equational division of univalents in anaphase I showed no H3 phosphorylation. In addition, regardless of their low phosphorylation level, prematurely separated chromatids showed normal condensation and kinetochore–microtubule interactions.

This hypothesis was also supported by observations in a mutant genotype of *Z. mays* (adf1) with deficient cohesion between sister chromatids in metaphase II. In this mutant, univalents in metaphase I were strongly phosphorylated only in the pericentromeric region. Meiosis II showed no marking, thus suggesting association between H3S10 phosphorylation and sister chromatid cohesion (Kaszas and Cande 2000).

In *Brachiaria* species, histone H3 became dephosphorylated in both telophase I and interkinesis stages and was phosphorylated again during prophase II (table 1; figures 1 and 5). According to Houben *et al.* (2007), this behaviour indicates that this posttranslational modification is reversible and can occur independently of DNA replication process.

H3 phosphorylation in the second meiotic division followed the same distribution pattern found in mitosis of *Brachiaria* genotypes, with immunodetection limited to the pericentromeric region. In prophase II, chromosomes were condensed again and accompanied by H3S10ph in the pericentromeric region. Phosphorylation remained in metaphase II with reduction of immune signals in anaphase and telophase II (table 1; figures 1 and 5). There was no H3S10ph marking in tetrad formation (figure 6).

Like in mitosis, cohesion restricted to the pericentromeric region in meiosis II is crucial for correct chromosome orientation. In addition, this cohesion stabilizes the connection between sister chromatids through forces generated by spindle fibers (Nasmyth and Haering 2009).

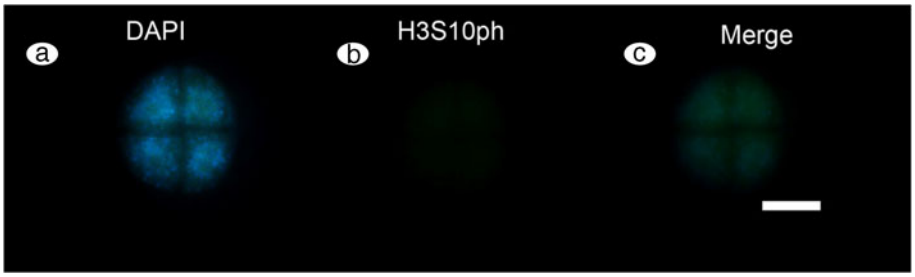

Figure 6. Absence of phosphorylation of histone H3S10 in tetrads of *B. brizantha*. (a) Observed with DAPI. (b) Observed with FITC. (c) Overlapping of DAPI and FITC images. The bar represents 10 μm.

The cohesin complex (SMC1, SMC3 and SCC3 SCC1/REC8/SYN1/Rad21) is the central component responsible for cohesion between sister chromatids during mitosis and meiosis (Nasmyth and Haering 2009; Qiao *et al.* 2011). The establishment and maintenance of cohesion are complex processes, which need to be coordinated with other functions, including replication and DNA repair. Genetic studies have identified a large number of proteins that potentially interact with the cohesin subunits and participate in such processes (Hirano 2002), however, cytologically their interactions were not studied in detail, and it is still unclear whether the behaviour of cohesins in plants compared to other organisms (Qiao *et al.* 2011).

The results presented here and in literature suggest that H3S10 phosphorylation pattern in plant cells is more related to maintenance of sister chromatid cohesion than to chromosome condensation. However, as discussed by Manzanero *et al.* (2000), correlation between sister chromatid cohesion and H3S10 phosphorylation is not perfect since the processes do not begin and end at the same time, as sister chromatids are physically united by cohesin proteins since their formation in S phase (Bardan 2010). In this stage, however, no H3S10ph immune signals were found.

Based on studies of *Z. mays*, Kaszás and Cande (2000) suggest the existence of at least two possible models for the role of H3S10ph in sister chromatid cohesion. First, phosphorylation can protect sister chromatid cohesion site, possibly providing stable binding of cohesin to chromatin during metaphase. This adjustment would be necessary since most cohesins dissociate from chromosomes during prophase/prometaphase (Losada *et al.* 1998). Alternatively, phosphorylation could prepare chromosomes for cohesion destruction during the transition from metaphase to anaphase, and signals remaining in anaphase would imply that endogenous phosphatases had not yet acted on the phosphorylated sites. Although many factors involved in cohesin requirement have already been identified, the exact mechanism by which it is required at specific genomic sites is not yet well understood. Studies suggest that posttranslational modifications of histones may be involved in these mechanisms (Ünal *et al.* 2004; Nasmyth and Haering 2009).

H3S10 phosphorylation in *Brachiaria* species was detected late in mitosis and meiotic prophase rather than in chromosomes immediately after DNA replication, when cohesion is established. Thus, histone H3 phosphorylation is probably not involved in the onset of sister chromatid cohesion; however, it may be related to cohesion maintenance. Nasmyth and Haering (2009) state that some factors are only required to establish sister chromatid cohesion while others are required to maintain cohesion through the process. An alternative possibility is that H3S10 phosphorylation may serve as a signal for recruitment of specific proteins that maintain sister chromatid cohesion during cell divisions.

The results shown in our study, along with previous studies of other grasses such as *T. aestivum* (Manzanero *et al.* 2000), *S. cereale* (Houben *et al.* 1999) and *Z. mays* (Kaszás and Cande 2000) support the hypothesis that H3 phosphorylation at serine 10 is associated with regulation of maintenance of sister chromatid cohesion in mitosis and meiosis in *B. brizantha*, *B. decumbens*, and *B. ruziziensis*. In chromosome condensation, however, other posttranslational modifications might be involved or could depend on phosphorylation to occur through trans-histone regulation. Nevertheless, it should be emphasized that the precise role of H3S10 phosphorylation mechanism is still unclear in most organisms and also not restricted only to the maintenance of sister chromatid cohesion. This modification of histones has been studied in many organisms and may be involved in transcription as well as cell division. Thus, further studies are needed for better understanding of the mechanisms driving these complex alterations.

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