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Effects of hybridization and polyploidy on the histone H3 phosphorylation at serine 10 (H3S10ph) in *Pennisetum* spp. Rich. (Poaceae)

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Abstract

Post-translational modifications on N-terminal tails of histones form nucleosomes, which are often associated with distinct biological functions. Some theories suggest that one of these changes, the phosphorylation of histone H3 at serine 10 (H3S10ph) plays an important role in chromosome condensation and sister chromatid cohesion. Although the histones are highly conserved, studies have been shown that the role and distribution of H3S10ph may differ between species. This study evaluated the effects of interspecific hybridization and polyploidization on the histone H3 phosphorylation at serine 10 (H3S10ph), using two model species *Pennisetum purpureum* Schum (elephant grass; 2n=4x=28) and *Pennisetum glaucum* (L.) R. Br. (Millet; 2n=2x=14). These species differ in ploidy levels and their hybrids are sterile. The fertility is restored by inducing chromosome doubling with colchicine. The effectiveness of inducing polyploidy and genomic behavior of duplicated hybrids were verified by meiotic analysis, chromosome counting in mitotic metaphase, cell cycle analysis and estimates of DNA content by flow cytometry. The H3S10 phosphorylation pattern was evaluated by immune-detection technique during the cell cycle in *Pennisetum purpureum*, *Pennisetum glaucum* and their triploid and hexaploid hybrids. First, roots were fixed in 4% paraformaldehyde. We primarily applied antibody (rabbit polyclonal IgG), which was detected with a secondary antibody (goat anti-rabbit IgG-FITC). The pattern of H3S10ph during mitosis showed coordination in space and time. The pattern was similar in parents and hybrids chromosomes. Phosphorylation was coincident with cohesion restricted to pericentromeric region. In triploid and partial hexaploid hybrids, bridges in anaphase and telophase were found, which showed signals of phosphorylation of H3S10, suggesting loss of chromosome fragments, mainly due the process of hybridization and polyploidization. In the hybrid hexaploid, metaphases with non-oriented chromosomes and anaphases with delayed and lost chromosomes with and without immune signal were found. This suggests that, after polyploidization, even chromosomes with functional centromeres, identified by phosphorylation of H3S10ph, are eliminated in hybrid *Pennisetum*, indicating that inactivation of the centromere is not a factor that contributes to chromosome elimination in *Pennisetum* partial hexaploid hybrids.

Keywords: Centromere; H3S10ph; interspecific hybrids; post translational modification; *Pennisetum glaucum*; *Pennisetum purpureum*.
Abbreviations: BAGCE_Active Germplasm Bank of elephant grass; BSA_bovine serum albumin; CENH3_centromere-specific histone; DAPI_4',6-diamidino-2-phenylindole; FITC, _fluorescein isothiocyanate; H3S10ph_histone H3 phosphorylation at serine 10; IgG, _immunoglobulin G; PBS_Phosphate Buffer Saline; PFA_paraformaldehyde.

Introduction

An alternative to study the genome plasticity is the analysis of species/genotypes, exhibiting their evolutionary history events of interspecific hybridization and polyploidization. It is including the assessment of parents with different ploidy and respective hybrids. *Pennisetum purpureum* Schum. (elephant grass) and *Pennisetum glaucum* (L.) R. Br. (pearl millet) are species that meet these requirements and can become a model for studying this area, since the group contains favorable traits such as: (i) species with different ploidy (*P. purpureum* with 2n=4x=28, genomes A´A´BB and *P. glaucum* with 2n=2x=14, AA genome); (ii) genetic proximity that allows to obtain triploid hybrids (2n=3x=21, genome A´AB); (iii) possibility of obtaining synthetic hexaploid hybrid (2n=6x=42, genome A´A´AABB) and (iv) mixoploid plants generated by artificial polyploidization (Jauhar, 1981; Campos et al., 2009;). Hybridization can lead to several intergenomic conflicts (Jones and Pasakinskiené, 2005) due to differences in composition and size genome, regulatory mechanisms and cell cycle duration (Jones and Hegarty, 2009). Such changes can be associated with changes in DNA sequence as well as with epigenetic mechanisms that trigger changes in gene expression to short and long term (Grant-Downton et al., 2006). Also after polyploidization, there may be post-translational modifications that modulate the chromatin and affect gene expression (Grant-Downton, et al., 2006; Chen, 2007; Li et al., 2011). However, to date there is no information on the effects of epigenetic mechanisms from hybridization and polyploidization in *Pennisetum* hybrids.

Studies have linked chromosome elimination to epigenetic changes, involving the centromeric activity and affecting the orientation and segregation of chromosomes (Gernand et al., 2003; Mochida, 2004; Ishii et al., 2010). Although there are specific centromere markers, studies with various species such as *Brachiaria brizantha* (Hochst.) Stapf, *Brachiaria*

decumbens Stapf and *Brachiaria ruziziensis* R. Germ & Evrard (Paula et al., 2013), *Costus spiralis* (Jacq.) Roscoe (Feitoza and Guerra, 2011), *Zea mays* L. (Kaszás and Cande, 2000) demonstrated the relationship of phosphorylation of serine 10 on H3 (H3S10ph) with the maintenance of cohesion and segregation of sister chromatids, notably by pericentromeric markings. Cohesion restricted to the pericentromeric region is essential for the correct chromosome orientation (Paula et al., 2013) and stabilizes the connection between sister chromatids through the forces generated by spindle fibers (Nasmyth and Haering, 2009).

Additionally, analysis of dicentric chromosomes revealed hyperphosphorylation of H3S10ph only in the functional centromere (Han et al., 2006; Fu et al., 2012), indicating that this is an epigenetic mark for active (peri) centromeres (Houben et al., 2013). However, most studies with H3S10ph performed no comparative analysis on the distribution in space and time of that post-translational modification in parents and their hybrids. Therefore, the evaluation of cells with abnormalities related to chromosome elimination will demonstrate the effect of this post-translational modification on chromosome elimination in hybrids of elephant grass and pearl millet. The way these *Pennisetum* hybrids eliminate the genetic material has serious implications for the establishment of plants, including their potential use in plant breeding, especially for obtaining lines of addition and/or replacement. Furthermore, the pattern of spatiotemporal distribution of H3S10ph in mitosis will contribute to the understanding of mechanisms modulated by epigenetic factors involved in the evolution of groups that exhibit hybridization and polyploidization in their evolutionary history.

Results and Discussion

For all genotypes of *Pennisetum* Rich. evaluated, regardless of ploidy, interspecific hybridization or polyploidization induced the spatio-temporal distribution pattern of phosphorylation of histone H3 at serine 10 in the cell cycle, which was similar to pericentromeric location, beginning in prophase (Fig. 1a-d), continuing in prometaphase (Fig. 1e-h) and in metaphase, when the phosphorylation reached its apex (Fig. 1 i-l). In anaphase, the phosphorylation signal still remained, but less intense (Fig. 1m-p), disappearing gradually in telophase (Fig. 1q-t). This pattern of phosphorylation of H3 at serine 10 is consistent with that observed in plants with monocentric chromosomes both in mitosis and in meiosis II, since in meiosis I, the immunolocalization has been observed over the entire chromosome length (Kaszas and Cande, 2000; Manzanero et al., 2000; Manzanero et al., 2002; Gernand et al., 2003; Paula et al., 2013). In mitosis of mammalian cells, phosphorylation of H3 at serine 10 and 28 is dependent on cell-cycle, starting in the pericentromeric region and spreading across the chromosome at G2/M transition, suggesting that these post-translational modifications are involved in chromosome condensation (Van Hooser et al., 1998; Goto et al., 1999; Goto et al., 2002; Houben et al., 2013).

Although several authors agree that the phosphorylation of H3 at serine 10 is involved with condensation (Fuchs and Schubert, 2012), studies have shown that the events of this post-translational modification in plants are more directly related to maintaining cohesion between sister chromatids (Manzanero et al., 2000; Gernand et al., 2003; Topp and Dawe, 2006), even for species with different ploidy, as demonstrated for diploid and tetraploide *Brachiaria ruziziensis* (R. Germ & Evrard) (Paula et al., 2013). This is explained by the fact that holocentric chromosomes are marked throughout their length in mitosis when treated with anti-H3S10ph and anti-H3S28ph (Gernand et al., 2003; Guerra et al., 2006). According to Houben et al. (2013), phosphorylation of histone H3S10/S28 occurs consistently while sister chromatids remain cohesive until the onset of anaphase. In polycentric chromosome, cohesion occurs along the chromatid arm; and in monocentric, only in the centromeric region. In addition, single chromatid of B chromosomes and univalent in haploid resulting from equational division at anaphase I showed no phosphorylation at serine 10 of H3 at anaphase II, even maintaining normal condensation, with kinetochores interacting with microtubules (Manzanero et al., 2000; Gernand et al., 2003). The functionally related H3S10ph to chromatid cohesion was also supported by the observation of a maize mutant, which is defective in chromatid cohesion, whose univalent at metaphase I and shows intense phosphorylation only in the pericentromeric region (Kaszás and Cande 2000).

In many organisms during the cell cycle, pericentromeric chromatin is phosphorylated on 10 and 28 serine residues of histone H3 (H3S10ph and H3S28ph) by aurora kinase (Topp and Dawe, 2006). According to these authors, these are key regulators of the transition from metaphase to anaphase and for chromatid separation.

In interphase nuclei of all genotypes, there were no signs of phosphorylation (Fig. 1a, b, c, d). Since these cells are in a division cycle, the absence of signal in interphase nuclei during the cell cycle confirms what is suggested for meiosis, the fact that H3 histone becomes dephosphorylated in interkinesis and is phosphorylated again during prophase II. This indicates that the phosphorylation of H3 is reversible and occurs regardless of DNA replication (Manzanero et al., 2000; Houben et al., 2013.). The observed number of centromeric signals corresponds to the number of chromosomes in the cells of elephant grass (Fig. 1e), pearl millet (Fig. 1f), triploid hybrid (Fig. 1g), with 28, 14 and 21 chromosomes, respectively. In cells of the hexaploide hybrid, we observed cells with 42 signals (Fig. 1h), and lower numbers as 14 and 21 (Fig. 2a, 2b). These results confirm the somatic chromosome stability of the 3x hybrid and the mixoploid condition of the artificially doubled hybrid, as shown in previous researches (Barbosa et al., 2003; Abreu et al., 2006; Barbosa et al., 2007; Andrade-Vieira et al., 2013).
In the triploid and partial hexaploid hybrids, we also evaluated cells with abnormalities in cell cycle to examine the relationship of H3S10ph with the elimination of genetic material (Fig. 2). In the polyploid hybrid, we registered metaphases with non-oriented chromosomes (Fig. 2c) and anaphases with lost/delayed chromosomes (Fig. 2d), with and without immunosignal. Chromosome bridges in anaphase and telophase were observed (Fig. 2d-2h), which are usually multiple in anaphases (Fig. 2e, 2g). Some anaphase and telophase bridges of both hybrids showed signals of H3 phosphorylation at serine 10, suggesting loss of centromeric sequences with subsequent rupture of the bridge involving the (peri) centromeres (Fig. 2e - 2h).

Although the cohesion complex is the central component responsible for cohesion between sister chromatids during mitosis and meiosis (Nasmyth and Haering, 2009; Qiao et al.,

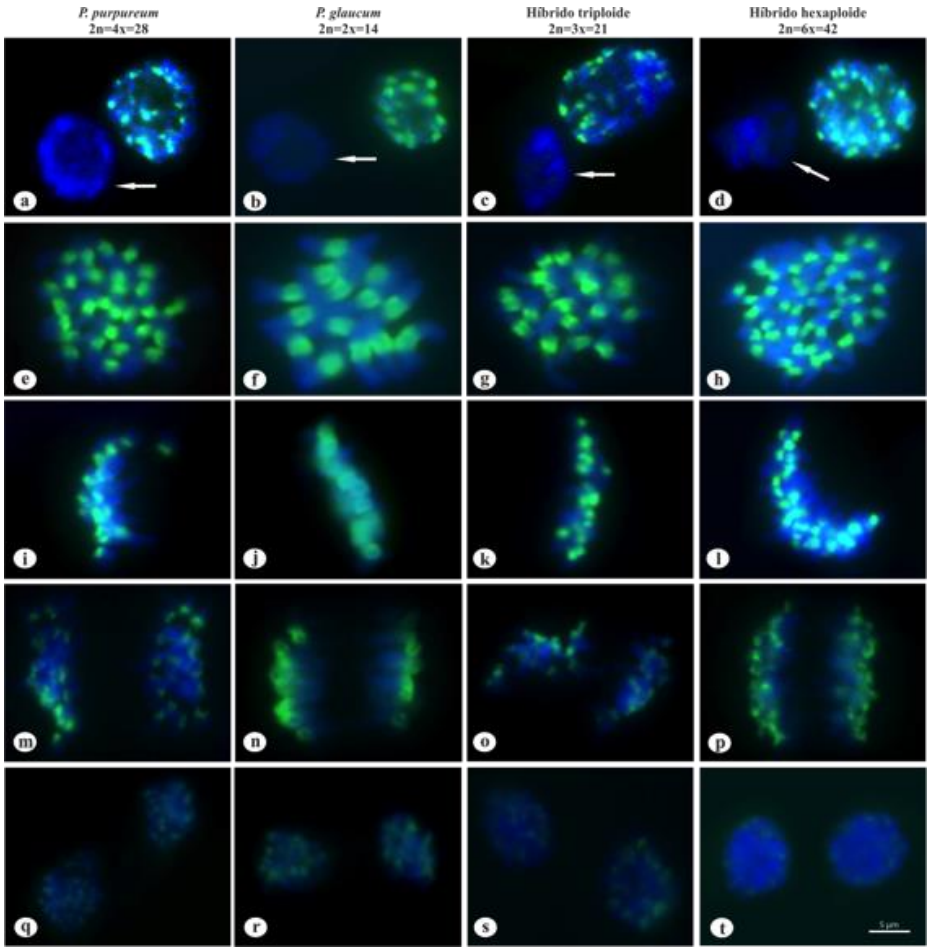

Fig 1. Immunodetection of histone H3 phosphorylation at serine 10 (H3S10ph) during the cell cycle in *Pennisetum* spp. Rich: (a, b, c, d) interphase nuclei with no sign of phosphorylation (white arrows) and prophase nuclei; (e, f, g, h) pro-metaphases; (i, j, k, l) metaphases; (m, n, o, p) anaphases and (q, r, s, t) telophases. (a, e, i, m, q).

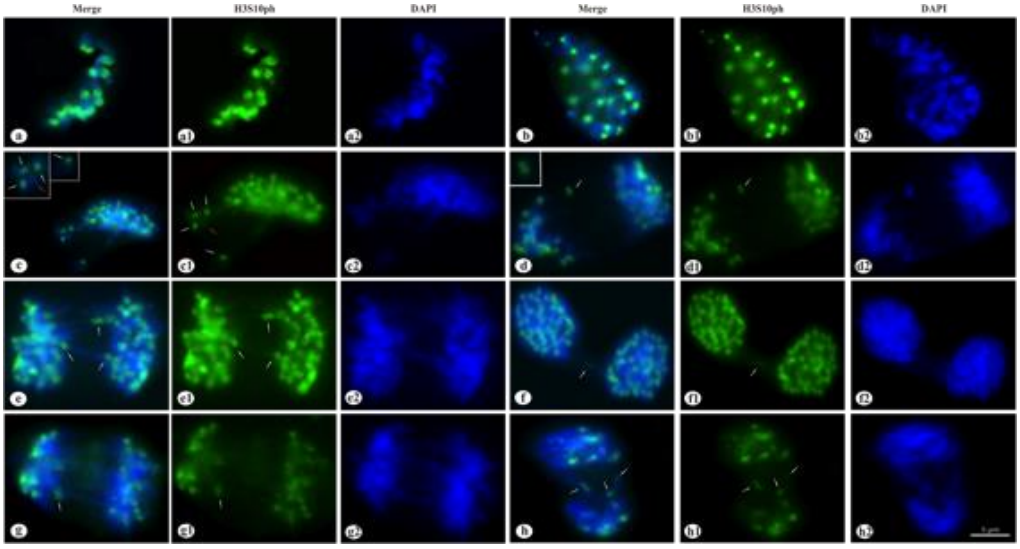

Fig 2. Immunodetection of histone H3 phosphorylation at serine 10 (H3S10ph) during the cell cycle abnormalities in *Pennisetum* spp. Rich. Meristematic cells of the synthetic hexaploid hybrid: (a) metaphase with 14 and (b) 21 chromosomes, (c) metaphase with non-oriented chromosomes and (d) anaphase with lost/delayed chromosome, both with and without immunosignal; (e) anaphase and (f) telophase with chromosome bridges immunosignal. Meristematic cells of the triploid hybrid: (g) anaphase and (h) telophase with chromosome bridges with immunosignal. a1 to h1: Signal of H3S10ph observed with FITC. a2 to h2: DAPI. White arrows show chromosomes or fragments with signals of H3S10ph and red arrows indicate no phosphorylation.

2011). There is evidence that in the kinetochore structure, the CENH3 is intercalated with the canonical H3 (Jiang et al., 2003) and when the canonical H3 is phosphorylated at serine 10 it has used as a marker of active centromere in plants. Thus, as the hyperphosphorylation of H3 at serine 10 occurs only in the functional centromere (Han et al., 2006; Fu et al., 2012; Houben et al., 2013), it is presumed that in the polyploid hybrid of *Pennisetum* the genetic material without signal of H3S10ph corresponds to acentric fragments and/or chromosomes with inactive centromeres in elimination process. Those presenting H3S10ph signals indicate that chromosomes/fragments with active centromeres are probably being eliminated. Chromosomes with and without imunosignal were also observed in micronucleus of meiocytes of the hybrid partial hexaploid (unpublished data).
These abnormalities in cell cycle of the triploid and partial polyploid hybrids result from genomic rearrangements, which are frequent in hybridization and polyploidization, especially in recent polyploids (Riddle and Birchler, 2003; Tiwari et al., 2010). The results for both hybrids corroborate previous reports demonstrating the somatic stability of the triploid hybrid for chromosome number, in which only the elimination of DNA sequences occurs, compared to the polyploid hybrid which show elimination of both chromosome and DNA sequences (Barbosa et al., 2003; Campos et al., 2009; Nunes et al., 2013).

For hybrids between *Hordeum vulgare* L. x *Hordeum bulbosum* L., Sanei et al. (2011) reported that the uniparental elimination of *H. bulbosum* chromosomes is due to the inactivity of centromeres in consequence of the loss of the centromeric protein CENH3. According to the authors, due to asynchronous cell cycle between the two parental genomes, chromosomes of *H. bulbosum* did not incorporate CENH3, leading to the delay of these chromosomes during anaphase, due to centromere inactivation and, consequently, the formation of micronuclei. In the case of hybrids of *Pennisetum* studied herein, the stable chromosome number of the triploid seems to justify the elimination of chromosomes even with active centromeres in the polyploid hybrid. These results confirm that the biparental elimination of chromosomes in these hybrids results from abnormalities in chromosome pairing (Paiva et al., 2012) and combination of the high degree of ploidy and high homology between the genomes(Andrade-Vieira et al., 2013; Reis et al., 2014).

Materials and Methods

Plant materials

The experiments were conducted with genotypes of elephant grass– BAG65 (2n=4x=28, *Pennisetum purpureum* Schumach) and pearl millet – BN2 (2n=2x=14, *Pennisetum glaucum* (L.) R. Br.), both used as parental of the triploid hybrid (2n=3x=21), and of a synthetic partial hexaploid hybrid from chromosome duplication of triploid hybrid with chromosome number ranging from 14 to 42. All the genotypes were obtained from the Active Germplasm Bank of elephant grass (BAGCE), Embrapa Dairy Cattle, experimental field José Henrique Bruschi, in Coronel Pacheco, Minas Gerais State, Brazil.

Immunolocalization of the histone H3 phosphorylation at serine 10 (H3S10ph)

The evaluations were carried out in meristematic cells fixed for 45 minutes in 4% paraformaldehyde containing 1x PBS (Phosphate Buffer Saline) at room temperature. After fixation, roots were washed in 1x PBS and enzymatically digested with pectinase/cellulase/pectolyase (100U/200U/5%), for 4h30min. Slides were prepared by the technique of Fukuy and Nakayama (1996).

Phosphorylated histone H3 at serine 10 was detected in mitotic cells with the method described by Manzanero et al. (2000) with some modifications. The prepared slides were pretreated with blocker (PBS, BSA and Triton X-100) for 20 minutes in a humid chamber at room temperature. Then, anti H3S10ph primary antibodies (Rabbit polyclonal IgG, Santa Cruz Biotechnology, USA) were applied to the chromosome preparations and incubated for at least 16 hours at 4°C in humid chamber. Subsequently, the material was washed with 1x PBS and incubated with secondary antibodies (Goat anti-rabbit IgG-FITC, Santa Cruz Biotechnology, USA) at 37°C for 2h in humid chamber. Chromosome preparations were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in Vectashield. The material was analyzed and photographed with an Olympus BX60 fluorescence microscope equipped with an Axiocam HRM digital camera (Zeiss). The images were captured with the software Axio vision Rel. 4.8. The pattern of H3S10phdistribution was examined by the presence/absence of the immunolocalization signal in approximately 1,000 cells of each genotype during mitosis.

Conclusion

Based on the spatiotemporal location in the species of *Pennisetum*, the phosphorylation of histone H3 at serine 10 is related to the maintenance of cohesion between sister chromatids during mitosis without changes in this pattern from the processes of interspecific hybridization and artificial polyploidization. In this way, in normal cell cycle, (peri) centromeres remained active and functional, allowing a correct segregation of sister chromatids during the mitotic cell cycle. Nevertheless, after the polyploidization, even chromosomes/fragments with functional centromeres, identified by phosphorylation of H3S10ph, are probably eliminated in *Pennisetum* hybrids, pointing out that centromere inactivation does not contribute to chromosome elimination in partial hexaploid hybrids of *Pennisetum*.

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