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Research Article

Hypomethylation of cytosine residues in cold-sensitive regions of *Cestrum strigilatum* (Solanaceae)

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Abstract

Heterochromatin comprises a fraction of the genome usually with highly repeated DNA sequences and lacks of functional genes. This region can be revealed by using Giemsa C-banding, fluorochrome staining and cytomolecular tools. Some plant species are of particular interest through having a special type of heterochromatin denominated the cold-sensitive region (CSR). Independent of other chromosomal regions, when biological materials are subjected to low temperatures (about 0 °C), CSRs appear slightly stained and decondensed. In this study, we used *Cestrum strigilatum* (Solanaceae) to understand some aspects of CSR condensation associated with cytosine methylation levels, and to compare the behavior of different heterochromatin types of this species, when subjected to low temperatures.

Key words: anti-5-methylcytosine, *Cestrum*, chromosomes, CMA/DAPI banding, heterochromatin, immunolabeling.

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Introduction

The major portion of plant genomes is composed of repetitive DNAs, which are organized in families, according to base-pair length, mechanisms of accumulation/reduction, dispersion and their role in genome behavior. The most important site of satDNA occurrence is heterochromatin, and the genomes of some plant species exhibit large portions of heterochromatin, in addition to other repetitive families, for example, *Scilla* (Deumling and Greilhuber, 1992) and *Citrus* (Silva *et al.*, 2010). Furthermore, heterochromatin itself can also be associated with various repetitive DNA families (Grewal and Jia, 2007; Gaeta *et al.*, 2010), which can be revealed after Giemsa C-banding, differentiated as AT-rich ($DAPI^+$) or GC-rich (CMA^+) by fluorescence staining (Guerra, 2000), and recognized by molecular techniques (Chang *et al.*, 2008). A good example of this is in a study by Urdampilleta *et al.* (2009), who isolated and located a satDNA (AT-rich) with a length of 725 bp, in the chromosomes of *Urvillea chacoensis* (Sapindaceae).

The genomes of species of *Cestrum* (Solanaceae) also possess numerous and diverse repetitive DNA families, many of which associated with heterochromatin. These, encountered as dots or large blocks, can be classified into i) C-Giemsa$^+$ bands, ii) CMA^+ bands associated or not with NORs, (iii) $DAPI^+$ bands associated or not with CSRs (cold-sensitive regions), and (iv) $CMA^+/DAPI^+$ bands (Berg and Greilhuber, 1992, 1993a,b; Fregonezi *et al.*, 2006; Fernandes *et al.*, 2009). Cold-sensitive regions are revealed when meristematic tissues are exposed to low temperatures (about 0 °C). They appear as decondensed chromatin, weakly stained after exposure to a nonspecific dye, such as Giemsa or orcein. CSRs have been described in approximately 50 plant-species belonging to different families (see Punina *et al.*, 2001). Eight species of *Cestrum* revealed CSRs. *Cestrum parqui*, *C. strigilatum, C. fasciculatum*, *C. elegans* and *C. aurantiacum* were studied by Berg and Greilhuber (1992, 1993a,b). Fregonezi *et al.* (2006), when studying *C. amictum*, *C. intermedium*, *C. sendtnerianum* and *C. strigilatum*, showed that, in these species, the principal CSRs are $DAPI^+$. Even so, in the case of *C. strigilatum*, not all $DAPI^+$ heterochromatin corresponds to CSRs. Although, according to Punina *et al.* (2001), most CSRs are composed of $DAPI^+$ heterochromatin, there are species of the genus *Sambucus* and *Viburnum* with CMA^+/CSRs (Benko-Iseppon and Morawetz, 1993).

To better understand the decondensed behavior of $DAPI^+$/CSRs in *Cestrum strigilatum*, we examined root tips treated at low temperatures (= cold treatment) using se-

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quential fluorescence banding (CMA/DAPI) and immunolabeling with an anti-5-methylcytosine antibody.

Material and Methods

Seedlings of *Cestrum strigilatum* (10) were collected in five different localities at Londrina, Paraná, Brazil, for subsequent cultivation in tubes in the Laboratório de Biodiversidade e Restauração de Ecossistemas (LABRE), Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Paraná, Brazil. Vouchers were deposited at the FUEL herbarium. Samples were pre-treated in two ways: i) with 0.05% colchicine at room temperature for 4 h to obtain conventional chromosomes and ii) with distilled water at 0 °C for 25 h to obtain CSRs. In both cases, samples were fixed in a solution of ethanol/acetic acid (3:1, v:v), for up to 24 h and stored at -20 °C. For conventional staining, samples were softened in 4% cellulase plus 40% pectinase (w: v) at 37 °C for 1 h, hydrolyzed in 1 N HCl for 10 min at 60 °C, dissected in a drop of 60% acetic acid, and then squashed. After removal of the coverslips by freezing in liquid nitrogen, samples were stained in 2% Giemsa and mounted with Entellan (Merck).

Chromosome banding was according to Schweizer (1976). For both pre-treatments, root tips were first softened in an enzyme solution, dissected and squashed, as previously described. After removal of the coverslips by freezing in liquid nitrogen, samples were aged for 3 days, stained with 0.5 mg/mL of chromomycin A_3 (CMA) for 90 min and 2 μg/mL of 4-6-diamidino-2-phenylindole (DAPI) for 30 min, and mounted in 1:1 glycerol/McIlvaine buffer (pH 7.0) plus 2.5 mM $MgCl_2$.

Immunodetection was performed in root tips previously treated with colchicine and with low temperatures. Samples were softened in 4% cellulase plus 40% pectinase (w: v) in 1x PBS buffer at 37 °C for 2 h, dissected and squashed, as previously described. After removal of the coverslips by freezing in liquid nitrogen, slides were kept in 1x PBS for 5 min. The material was blocked in a solution of 3% BSA (Inlab) in 1x PBS plus 0.2% Tween 20 at room temperature for 10 min. Afterwards, samples were incubated in a moist chamber at 4 °C for 12 h with 25 μL of a solution (1:100, v:v) of mouse anti-5-methylcytosine (Eurogentec) and 3% BSA (Inlab) in 1x PBS plus 0.2% Tween 20. After three washes in 1x PBS for 5 min each, samples were incubated with the FITC-conjugated secondary goat anti-mouse IgG, diluted 1:100 (v:v) in 3% BSA in 1x PBS plus 0.2% Tween 20, at 37 °C for 1 h. Washes were carried out in 1x PBS at room temperature. Slides were mounted with 25 μL of a solution composed of glycerol (90%), DABCO (2.3%), 20 mM Tris-HCl, pH 8.0 (2%), 2.5 mM $MgCl_2$ (4%), and distilled water (1.7%), plus 1 μL of 2 μg/mL DAPI.

Images were acquired separately in grayscale mode using a Leica DM 4500 B microscope equipped with a DFC 300FX camera, and overlapped using a Leica IM50 4.0 software, with the colors blue for DAPI, yellow for CMA_3, and greenish-yellow for FITC.

Results and Discussion

Conventional Giemsa staining and CMA/DAPI banding showed that the samples of *Cestrum strigilatum* examined here had a chromosome number of $2n = 16$, with five metacentric and three submetacentric pairs (Figure 1A), as well as large AT-rich blocks in pairs 2, 3, 4, 6, 7 and 8 (Figure 1C) and fewer GC-rich blocks in pairs 7 and 8 (Figure 1E), as previously reported (Berg and Greilhuber, 1993a; Fregonezi *et al.*, 2006). Cold treatment showed that the $DAPI^+$/CSRs of pairs 2, 4, 6, 7 and 8 were negatively stained with CMA (Figures 1B, D, F and 2), as reported by Berg and Greilhuber (1993a) and Fregonezi *et al.* (2006). Notwithstanding, some fine AT-rich blocks that appeared as CMA^- (Figure 1D and F, indicated by arrowheads), and also in pair 4 (Figure 2), were not cold-sensitive. The decondensed appearance of $DAPI^+$/CSRs was more evident when compared to adjacent GC-rich blocks (Figures 2 and 3, pair 8) and non-adjacent blocks (Figures 2 - pair 7 and 3 - pair 6). This difference in chromatin condensation certainly depends on epigenetic control, which basically consists of the modification of histones and/or DNA methylation (Fuks, 2005).

In general, when compared to other chromosome regions, heterochromatic regions have high levels of cytosine methylation, as also occurs in non-cold-treated chromosomes of *C. strigilatum* (Figure 4A-F). The use of the anti-5-methylcytosine antibody in plants has been effective in differentiating highly methylated cytosine residues in chromosomes (Cremonini *et al.*, 2003). Differences in methylation levels between euchromatic and heterochromatic regions have been reported by immunolabeling with anti-5-methylcytosine antibody in several plant groups, such as *Haplopappus gracilis* (Castiglione *et al.*, 2008), and *Costus spiralis* and *Eleutherini bulbosa* (Feitosa and Guerra, 2011).

Immunodetection using anti-5-methylcytosine antibodies in cold-treated samples revealed hypomethylation in the $DAPI^+$/CSR regions (Figure 5). The differences in methylation levels can be better visualized, on comparing both $DAPI^+$ regions of non-cold-treated samples (Figure 4) and the terminal CMA^+ block on the short arm of pair 7. Notwithstanding also showing hypermethylation (Figure 5G-H, head and arrowhead), thereby indicating that AT-rich sequences of *C. strigilatum* are more inclined to hypomethylation than the GC-rich. It is assumed here that this relationship is due to DAPI preferentially binding to AT-rich and CMA to GC-rich regions (see Guerra, 2000). Interestingly, Cremonini *et al.* (2003) obtained CSRs in those chromosomes of *Zingeria biebersteiniana* that were not fully compatible with the heterochromatic regions revealed by chromosome banding. Our results with *C. strigilatum*

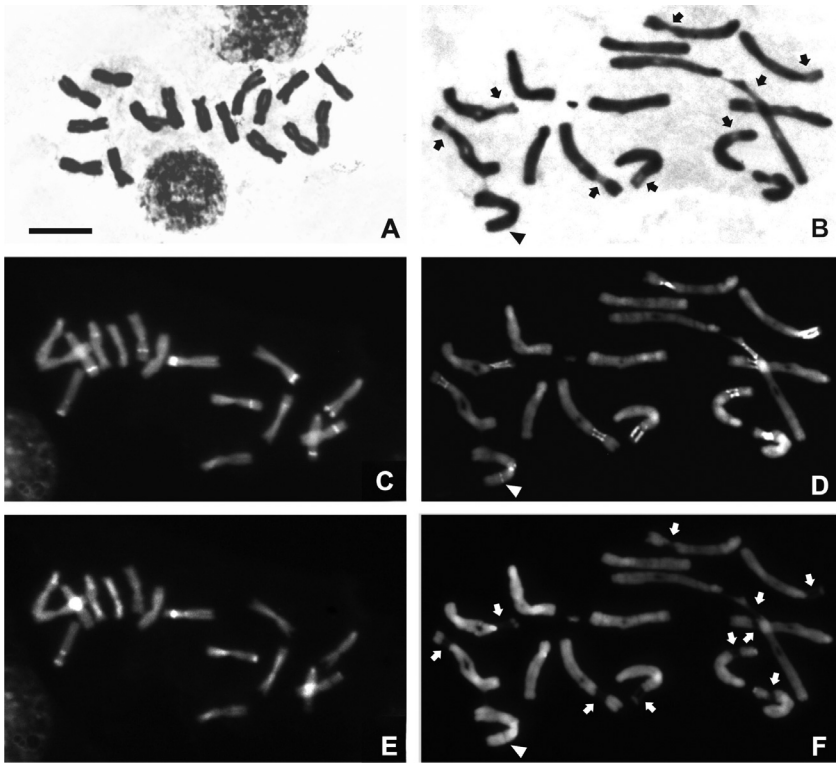

Figure 1 - Conventional staining and CMA/DAPI banding in *Cestrum strigilatum.* Metaphases in A, C and E correspond to non-cold-induced material: A) 2% Giemsa, C) DAPI-banding and E) CMA-banding. Prometaphases in B, D and F correspond to cold-induced material: B) 2% Giemsa. Arrows indicate the CSRs. D and F correspond to DAPI- and CMA-banding, respectively. Arrows in F indicate the CMA^-CSRs, Arrowheads in B, D and F indicate a small $DAPI^+/CMA^-$ band not CSR. Note that all cold-sensitive regions are $DAPI^+$ (D). Bar represents 10 μm.

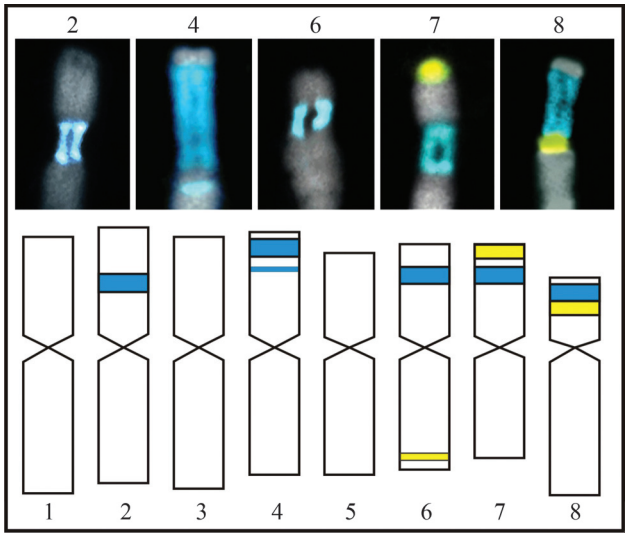

Figure 2 - Partial karyogram and idiogram showing the locations of $DAPI^+$/CSRs, $DAPI^+$/ not CSR and CMA^+/ not CSR. Note that all the CSRs are from interstitial to terminal regions in short arms. Also note a small $DAPI^+$/ not CSR in pair 4. The CMA^+ bands of pairs 7 and 8 are associated with NOR. The subterminal CMA^+ band in pair 6 (in this idiogram) is shown in Figure 3.

seem to be more consistent with the hypothesis proposed by Punina *et al.* (2001) that cold-sensitive regions are AT-rich heterochromatic regions that decondense in response to a

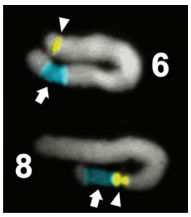

Figure 3 - Two chromosomes representative of pairs 6 and 8, which show differences in condensation levels between $DAPI^+$/CSR (arrows) and CMA^+ bands (arrowheads). The CMA^+ band in pair 8 is associated with NOR.

strong cold-shock. It is proposed that cold treatment could interfere in the stability of $DAPI^+$/CSRs in *C. strigilatum*, thereby provoking modifications in normal heterochromatin condensation. According to Avramova (2002), DNA methylation is important in stabilizing heterochromatin structures.

Species of *Cestrum* show karyotypical differences in the number of sites and amount of CSRs. In spite of this discrepancy, it is important to note that in our results, as well as for $DAPI^+$/CSRs bands in species of *Cestrum* (Berg and Greilhuber, 1993a; Fregonezi *et al.*, 2006), and CMA^+/CSRs bands in species of *Sambucus* and *Viburnum* (Benko-Iseppon and Morawetz, 1993), only larger heterochromatic blocks are not only more easily visualized, but

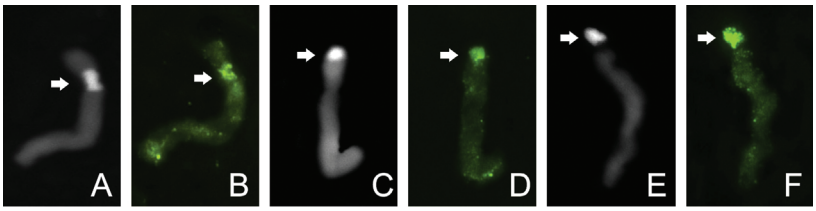

Figure 4 - Immunolabeling with an anti-5-methylcytosine antibody in the prometaphasic chromosomes of *C. strigilatum* obtained from control experiments (non-cold-induced). A, C and E correspond to pairs 2, 4 and 8, respectively, after DAPI staining. B, D and F correspond to pairs 2, 4 and 8, respectively, after immunolabeling and detection with an FITC-conjugated secondary antibody. Arrows indicate hypermethylated DAPI$^+$ bands. For comparison, observe the decondensed and hypomethylated regions in Figure 5.

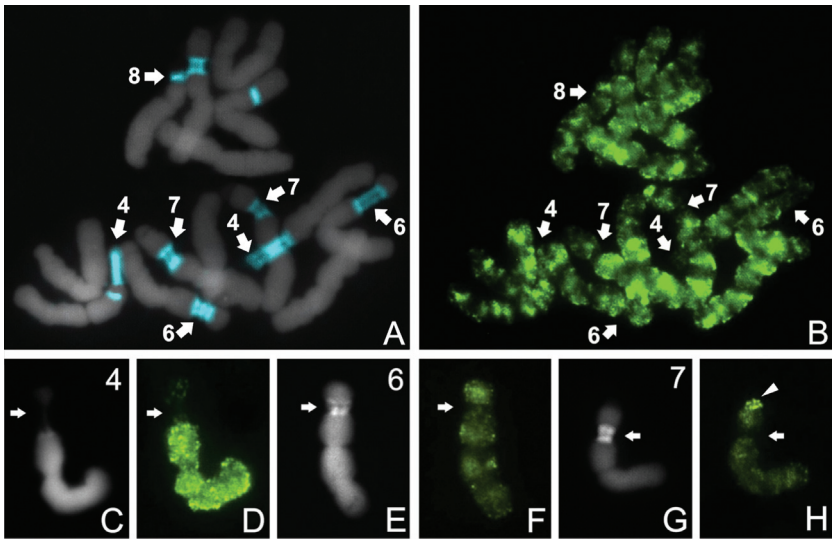

Figure 5 - Immunolabeling with an anti-5-methylcytosine antibody in the prometaphase and metaphasic chromosomes of *C. strigilatum* obtained from cold-induction experiments. A) DAPI stained partial prometaphase. Arrows indicate CSRs. This image was acquired in grayscale format, whereupon the DAPI bands were converted to blue. B) The same prometaphase after immunolabeling and detection with an FITC-conjugated secondary antibody. Arrows indicate CSRs. C-D, E-F and G-H show pairs 4, 6 and 7, respectively. Note the hypomethylation status of the DAPI$^+$/CSR (arrows). For comparison, observe the terminal region of the short arm in pair 7, where there is a hypermethylated NOR-associated CMA$^+$ band, which shows negative DAPI staining.

appear evidently decondensed, when samples are treated at low temperatures.

Thus, there is possibly a minimum size for heterochromatin blocks capable of detecting changes in methylation levels, when samples are exposed to cold-treatment. This, together with the examination of small and hypomethylated heterochromatic regions being difficult through technical limitations, could possibly be alternative explanations for the occurrence of cold-sensitive regions containing both DAPI$^+$ and CMA$^+$. However, it is important to mention that anti-5-methylcytosine detection has not been undertaken in *Sambucus* and *Viburnum* species.

To better understand the behavior of CSRs, as well as the evolutionary significance, epigenetic role and molecular drive of these DAPI$^+$/CSRs in *Cestrum*, it will be necessary, not only to determine the sequences of these satDNA regions, but also test other antibodies against proteins capable of controlling heterochromatin dynamics, such as post-translational modifications associated with histone phosphorylation, acetylation, ubiquitylation and/or methylation.

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References

Avramova ZV (2002) Heterochromatin in animals and plants: Similarities and differences. Plant Physiol 129:40-49.

Benko-Iseppon AM and Morawetz W (1993) Cold-induced chromosome regions and karyosystematics in *Sambucus* and *Viburnum*. Bot Acta 106:183-192.

Berg C and Greilhuber J (1992) Cold-sensitive chromosome regions and their relation to constitutive heterochromatin in *Cestrum parqui* (Solanaceae). Genome 35:921-930.

Berg C and Greilhuber J (1993a) Cold-sensitive chromosome regions and heterochromatin in *Cestrum* (Solanaceae): *C. strigilatum, C. fasciculatum* and *C. elegans*. Plant Syst Evol 185:133-151.

Berg C and Greilhuber J (1993b) Cold-sensitive regions and heterochromatin in *Cestrum aurantiacum* (Solanaceae). Plant Syst Evol 185:259-273.

Castiglione MR, Frediani M, Venora G and Cremonini R (2008) Cytological investigation of *Haplopappus gracilis* (Nutt.) Gray: 5-methylcytosine rich regions, fluorochrome banding and chromatin sensitivity to DNase I digestion. Protoplasma 233:107-113.

Chang S, Yang T, Datema E, Vugt J, Vosman B, Kuipers A, Meznikova M, Szinay D, Lankhorst RK, Jacobsen E, *et al.* (2008) FISH mapping and molecular organization of the major repetitive sequences of tomato. Chromosome Res 16:919-933.

Cremonini R, Ruffini Castiglione M, Grif VG, Kotseruba VV, Punina EO, Rodionov AV, Muravenko OV, Popov KV, Samatadze TE and Zelenin AV (2003) Chromosome banding and DNA methylation patterns, chromatin organization and nuclear DNA content in *Zingeria biebersteiniana* (Claus) P. Smirnov. Biol Plant 46:543-550.

Deumling B and Greilhuber J (1992) Characterization of heterochromatin in different species of the *Scilla siberica* group (Liliaceae) by *in situ* hybridization of satellite DNAs and fluorochrome banding. Chromosoma 84:535-555.

Feitosa L and Guerra M (2011) Different types of plant chromatin associated with modified histones H3 and H4 and methylated DNA. Genetica 139:305-314.

Fernandes T, Rego LNAA, Nardy M, Yuyama PM and Vanzela ALL (2009) Karyotype differentiation of four *Cestrum* species (Solanaceae) revealed by fluorescent chromosome banding and FISH. Genet Mol Biol 32:320-327.

Fregonezi JN, Torezan JMD, Vieira AOS and Vanzela ALL (2006) Karyotype differentiation of four *Cestrum* species (Solanaceae) based on physical mapping of repetitive DNA. Genet Mol Biol 29:97-104.

Fuks F (2005) DNA methylation and histone modifications: Teaming up to silence genes. Curr Opin Genet Dev 15:1-6.

Gaeta ML, Yuyama PM, Sartori D, Fungaro MHP and Vanzela ALL (2010) Occurrence and chromosome distribution of retroelementos and NUPT sequences in *Copaifera langsdorffii* Desf. (Caesalpinioideae). Chromosome Res 18:515-524.

Grewal SI and Jia S (2007) Heterochromatin revisited. Nat Rev Genet 8:35-46.

Guerra M (2000) Patterns of heterochromatin distribution in plant chromosomes. Genet Mol Biol 23:1029-1041.

Punina EO, Rodionov AV, Myakoshina YA and Grif VG (2001) Nucleotide composition of the cold-sensitive heterochromatic regions in *Paris hainanensis* Merrill. Russ J Genet 37:776-782.

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58:307-324.

Silva AEB, Marques A, dos Santos KGB and Guerra M (2010) The evolution of CMA bands in *Citrus* and related genera. Chromosome Res 18:503-514.

Urdampilleta JD, Souza AP, Schneider DRS, Vanzela ALL, Ferrucci MS and Martins ERF (2009) Molecular and cytogenetic characterization of an AT-rich satellite DNA family in *Urvillea chacoensis* Hunz. (Paullinieae, Sapindaceae). Genetica 136:171-177.

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