



Dynamics of 45S rDNA sites in the cell cycle: fragile sites and chromosomal stability in *Lolium* and *Festuca*

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ABSTRACT. Analyses carried out with fluorescence *in situ* hybridization (FISH) in C-metaphases of the *Lolium-Festuca* complex have shown the occurrence of spontaneous fragile sites (FSs) in 45S rDNA regions. FSs are expressed as gaps but they do not result in breaks or chromosomal fragments in these species. These gaps have high DNA condensation observed as thin chromatin fibers that connect the apparent segments of the fragile chromosome, allowing for genomic stability. Assessing the behavior of these regions in the cell cycle of *Lolium* and *Festuca* species may lead to a better understanding of the dynamics that preserve stability during cell division. Furthermore, it is interesting to track the dynamics of chromosomes bearing 45S rDNA sites in the cell cycle as well as to observe the expression of FSs with no effect of the mitotic block. We observed variation in both the number and

size of 45S FISH signals from the S/G2 phases of interphase and from prophase to anaphase where gaps in 45S rDNA sites also were observed. The change in the degree of condensation of the 45S site begins in the S/G2 phase and appears to be related to the transcriptional demand. Taking into account that the number of 45S rDNA sites tends to be re-established when cells reach telophase, we suggest that the chromatin fiber goes back to the normal condensation level to the anaphase (after segregation), allowing for the approximation of chromosome segments and ensuring dynamics that favor the genomic stability of these species.

Key words: Genetic stability; Chromosomal fragments; Fragile site; Gene activity-dependent decondensation; Chromatin fiber; High decondensation;

INTRODUCTION

Fragile sites (FSs) are chromosome regions prone to breaks or narrowing with the subsequent formation of gaps with reduced coloration due to the lower degree of chromatin compaction in the chromosome or even due to chromosome breakages (Richards, 2001; Glover, 2006). In humans, FSs are well-studied and related to epigenetic modifications, as well as replicative and transcriptional stress (Wang, 2006; Leman and Noguchi, 2013).

In some plants, FSs are expressed in 45S rDNA sites (Huang et al., 2008, 2009, 2012; Waminal et al., 2013; Bustamante et al., 2014; Grabowska-Joachimciak et al., 2015; Rocha et al., 2015, 2016; Lan et al., 2016). In *Lolium multiflorum* Lam. and *Lolium perenne* L., FSs are randomly distributed in 45S rDNA sites and promote changes in the number and position of fluorescence *in situ* hybridization (FISH) signals. The predominant number of described 45S rDNA sites is six and seven for *L. multiflorum* and *L. perenne*, respectively (Thomas et al., 1996; Rocha et al., 2015). However, in C-metaphases with FSs, the number of 45S FISH signals range from 6 to 13 in *L. multiflorum* and from 7 to 13 in *L. perenne*. In intact chromosomes, invariably, the signals are located at interstitial chromosome positions, whereas in chromosomes with FSs, the variable-sized signals are located in the terminal regions. Initially, it was proposed that the occurrence of breakages or gaps in these regions resulted in the formation of chromosomal fragments (Huang et al., 2008; Bustamante et al., 2014; Rocha et al., 2015).

Recently, it was reported that FSs are also present in *Festuca arundinacea* and *Festulolium* hybrids (Rocha et al., 2016). In the same study, it was shown that FSs are not expressed through chromosomal breakages but rather as gaps covered by a thin DNA fiber, not detected by 4',6-diamidino-2-phenylindole (DAPI), connecting parts of the fragile chromosomes. The presence of these DNA fibers in FSs allows the maintenance of chromosomal integrity and an amount of nuclear DNA with a low percentage of micronuclei. This suggests that there is no loss of chromosome fragments in these genotypes. Rocha et al. (2016) suggested that the chromatin fiber in FSs of *Lolium*, *Festuca*, and *Festulolium* hybrids have a high decondensation process related to an increased genic activity, thus, supporting the findings by Huang et al. (2012), Bustamante et al. (2014), and Rocha et al. (2015).

So far, reports on FSs in *Lolium* and *Festuca* have been made based on metaphasic chromosomes under the effect of thermal shock (0°C) used as a mitotic block. Observing the behavior of 45S rDNA sites in the cell cycle may provide information on the spontaneous

occurrence of FSs, thus, allowing for monitoring of the spatial/temporal expression patterns as well as observation of the dynamics that contribute to the genetic stability.

MATERIAL AND METHODS

The experiments were carried out with *L. multiflorum* (access No. LOL 161) and *L. perenne* ('Ellet'), both $2n = 2x = 14$ (Pereira et al., 2014; Rocha et al., 2015), in addition to two *F. arundinacea* cultivars ('Rizomat' and 'Quantum II') $2n = 4x = 42$. The *Lolium* species came from Banco Ativo de Germoplasma de Plantas Forrageiras (Active Germplasm Bank) and Embrapa Recursos Genéticos e Biotecnologia. Seeds from *F. arundinacea* cultivars were commercially obtained.

Roots were collected and fixed in Carnoy (3:1 ethanol:acetic acid) and stored at -4°C for at least 24 h. To prepare slides, roots were subjected to enzymatic maceration in pectinase/cellulose solution (2/4%) (Sigma-Aldrich™, St Louise, MO, USA) for 130 min for *L. multiflorum* and *L. perenne*, and 105 min for *F. arundinacea* in an oven at 37°C . Slides were then flame-dried (Dong et al., 2000). For FISH, a 45S rDNA probe (pTa 71 *Triticum aestivum* L.) marked with digoxigenin through a nick translation reaction was used. Slides were denatured in 70% formamide in SSC 2X for 1 min and 40 s at 85°C and then dehydrated in an alcohol series. The mixture containing the probe (50% formamide SSC 2X, 10% dextran sulfate, and approximately 50 ng marked probes) was denatured at 95°C for 8 min. The hybridization was conducted at 37°C in a moistened chamber for at least 16 h. The detection was carried out with TRITC (tetramethylrhodamine) in TNB (sodium chloride-Tris buffer) for 1 h at 37°C in a moistened chamber. Slides were mounted on Vectashield with DAPI and evaluated on an epifluorescence microscope with 358/461 (DAPI) and 550/575 (TRITC) excitation/emission wavelengths. Images digitalized using a cooled monochrome camera AxioCamHRm (Zeiss, Oberkochen, Baden-Württemberg, Germany) were processed by using the Axio Vision Release 4.8.2 (Zeiss) software and Adobe Photoshop CS6.

RESULTS

In the three species evaluated, the S/G2 interphase nuclei had the highest number of 45S rDNA probe signals in comparison to the G1 nuclei (Figure 1). The size of the signals at interphase ranged from 0.3 to 0.8 μm (Figures 2A and B, 3A and B, 4A and B, and 5A and B).

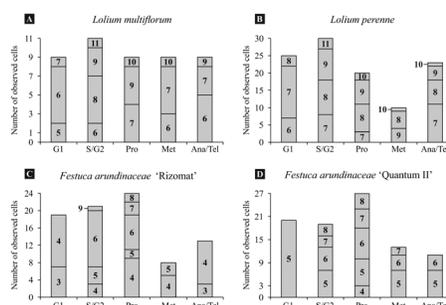


Figure 1. Number of cells analyzed in different phases of the cell cycle of *Lolium multiflorum* (A), *Lolium perenne* (B), *Festuca arundinacea* 'Rizomat' (C), and *Festuca arundinacea* 'Quantum II' (D). Within columns numbers represent the variability in number of 45S rDNA signals found within each phase of the cell cycle. G1 and S/G2 represent subphases of interphase and the abbreviations correspond to prophase (Pro), metaphase (Met), and anaphase and telophase (Ana/Tel).

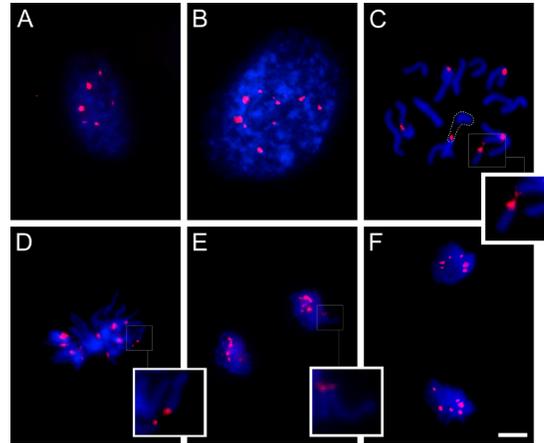


Figure 2. Cell cycle of *Lolium multiflorum* ($2n = 2x = 14$), in red: 45S rDNA signals. **A.** Interphase G1 with six 45S rDNA signals, **B.** Interphase S/G2 with eleven 45S rDNA signals, **C.** Prometaphase: the dotted line is an FS in which the 45S rDNA signal is only found in one of the chromosome parts and the enlargement shows a gap in the 45S rDNA site, **D.** Metaphase: the enlargement shows 45S rDNA signals on both sides of the fragile chromosome, **E.** Late anaphase: the enlargement shows a gap in the 45S rDNA site, **F.** Late anaphase with six 45S rDNA signals on each pole. Scale bar: 5 μm .

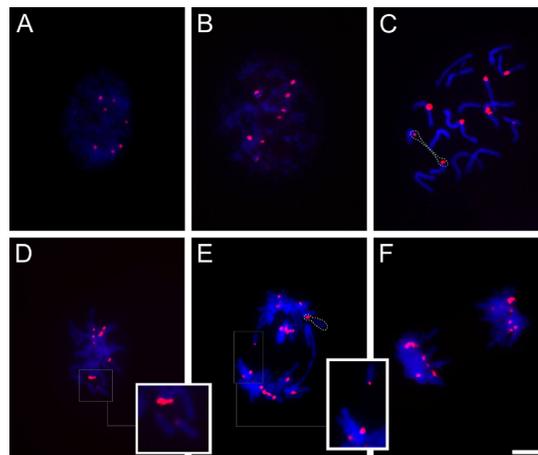


Figure 3. Cell cycle of *Lolium perenne* ($2n = 2x = 14$), in red: 45S rDNA signals. **A.** Interphase G1 with seven 45S rDNA signals, **B.** Interphase S/G2 with nine 45S rDNA signals, **C.** Prometaphase: the dotted line shows 45S rDNA signals in both parts of the fragile chromosome, **D.** Metaphase: the enlargement shows two fragile chromosomes, **E.** Early anaphase: the dotted line shows the 45S signal in only one of the fragile parts and the enlargement shows a fragile chromosome with distant segments and 45S rDNA signals on both sides, **F.** Late anaphase with seven 45S signals in one of the poles. Scale bar: 5 μm .

Differences in the number and size of the 45S signals were observed throughout mitosis (Figures 1-5). The largest, intact signals were on average 0.94 μm , the mean signals 0.48 μm , and the smallest signals were 0.2 μm . During prophase, metaphase, and anaphase gaps were evidenced in 45S rDNA sites supporting the maintenance of the expression of FSs in the cell cycle in both *Lolium* species as well as in *F. arundinacea* (Figures 1, 2C-E, 3C-E, 4C-E and 5C-E).

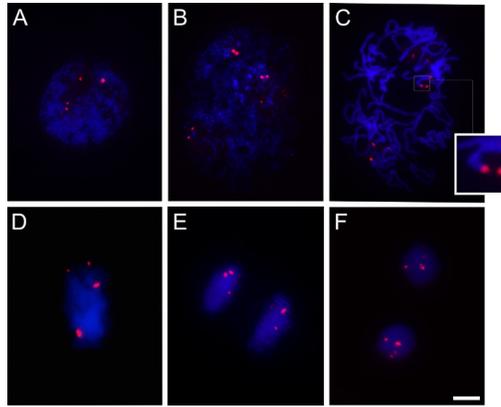


Figure 4. Cell cycle of *Festuca arundinacea* 'Rizomat' ($2n = 6x = 42$), in red: 45S rDNA signals, **A.** Interphase G1 with five 45S rDNA signals, **B.** Interphase S/G2 with nine 45S rDNA signals, **C.** Prometaphase: the amplification shows a small chromosome segment co-located with a 45S signal, **D.** Metaphase with four 45S rDNA signals, **E.** Anaphase with three and four 45S rDNA signals, **F.** Telophase with four 45S rDNA signals. Scale bar: 5 μm .

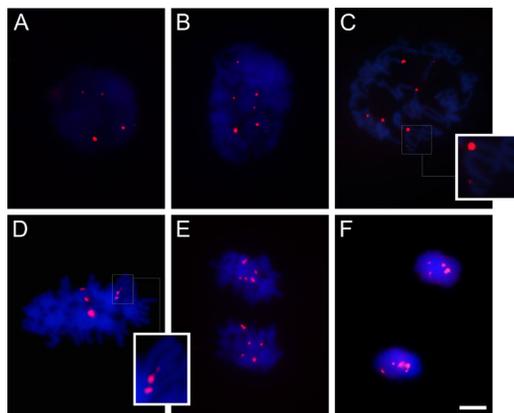


Figure 5. Cell cycle of *Festuca arundinacea* 'Quantum II' ($2n = 6x = 42$), in red: 45S rDNA signals, **A.** Interphase G1 with four 45S rDNA signals, **B.** Interphase S/G2 with seven 45S rDNA signals, **C.** Prophase: the enlargement shows a chromosome with 45S rDNA signals of different sizes on both sides of the fragile chromosome, **D.** Metaphase: the enlargement shows a chromosome with a gap in the 45S rDNA site, **E.** Anaphase with six 45S signals on each pole, **F.** Telophase with four and five 45S rDNA signals. Scale bar: 5 μm .

Structurally, the chromosome appeared to be segmented and the 45S rDNA probe sometimes marked this region (Figures 2C and D, 3C-E, 4C and 5C). In the studied *Lolium* species, such segments corresponded to about 1/3 of the chromosome size, due to interstitial 45S sites with FSs (Figures 2C-E and 3C-E). In contrast, in *F. arundinacea* small chromosome segments always co-located with 45S signals along its length due to 45S sites being located in terminal positions (Figure 4C) and larger segments (Figure 5C and D).

Only at the early anaphases of *L. perenne* it was possible to observe one or two segments spaced from the chromatid of origin, which had already been segregated (Figure 3E). Nevertheless, in all studied species, the late anaphases and telophases had close to the expected number of 45S rDNA signals (Figures 1, 2E and F, 4F, and 5F). In *L. perenne* and *L.*

multiflorum, the segmented part, whether followed by 45S rDNA signals or not, was located only at the proximal region of the chromosome (Figures 2E and 3F).

DISCUSSION

In interphasic G1 nuclei, the number of 45S signals measured was similar to the descriptions for mitotic chromosomes with six, seven, and four/five sites for *L. multiflorum*, *L. perenne*, and *F. arundinacea*, respectively (Thomas et al., 1996, 1997; Bustamante et al., 2014; Rocha et al., 2015). In S/G2 nuclei, the number of 45S signals was higher for the three species studied here owing to the occurrence of FSSs. This indicates that the fragility of sites, apparently splitting the 45S site into two, coincides with the DNA replication, as has been suggested for human chromosomes (Wang, 2006; Leman and Noguchi, 2013). It has been shown that one of the causes of the formation of FSSs in humans is replicative stress that promotes a delay in the replication fork and/or collisions between the replication and transcription machinery that maintains the DNA strand unzipped, which might result in breakages of the double-stranded DNA (Wang, 2006; Leman and Noguchi, 2013).

Additional evidence suggesting that FSSs are expressed during interphase was provided by Huang et al. (2009). In their study, the 45S rDNA sites of *L. perenne* were highly decondensed in more than 90% of the G2 nuclei and the authors suggested that failure in the chromatin folding complex of these sites could be responsible for the spontaneous formation of FSSs (Huang et al., 2009). In another study, following induction with actinomycin D (a transcription inhibitor) and aphidicolin (a replication inhibitor), the interphasic nuclei of maize, rice, barley, shorgum, and ryegrass were highly decondensed and had fiber-like thread after FISH 45S in metaphasic chromosomes (Huang et al., 2012). Thus, it is likely that the replicative and transcriptional processes are involved with the spontaneous FSSs in *Lolium* and *Festuca*.

The chromosome fragility in 45S rDNA sites in *Lolium* and *Festuca* detected by the high chromatin decondensation during the cell cycle is supported by a recent review by Dvořáčková et al. (2015). They suggest that the rDNA is a region prone to fragility or instability due to the high transcriptional activity that may take place throughout the cell cycle, including the S phase of interphase (Dvořáčková et al., 2015). The intense transcription may delay the replicative process, cause collision between these two machineries in the rDNA, and interfere with the cohesion between sister chromatids (Ide et al., 2010; Dvořáčková et al., 2015). As Huang et al. (2012), Bustamante et al. (2014), and Rocha et al. (2015) have already demonstrated, the 45S fragile sites in *Lolium* species have high and variable transcriptional activity as evidenced by silver-staining nucleolar organizer regions (AgNOR).

One of the explanations for the stability of FSSs in *Lolium* and *Festuca* is that as the cell cycle is a continuous process in which cells maintain the exceeding number of 45S rDNA signals, as well as the decondensed status in this region up to anaphase (Figure 6) with no breakages. The replication of 45S rDNA sites, so far delayed due to the collision with the transcription machinery, likely occurs during or even after segregation, as proposed by Chan et al. (2009). The end of the replication reaches its peak with the resumption of the condensation of FSSs of *Lolium* and *Festuca* at a level similar to the other chromatid regions, restoring the number of 45S signals in late anaphases, telophases, and favoring the approximation of chromosome segments (Figure 6). In this case, it is also possible that a minimum condensation status required to assure the segregation of the remaining parts of the fragile chromosomes is maintained, therefore, retaining genome stability. Such behavior may explain the stability

of the DNA content found by means of flow cytometry in *L. multiflorum*, *L. perenne*, and *F. arundinacea* (Bustamante et al., 2015; Rocha et al., 2016) and the low percentage of micronuclei (<1%) observed by Rocha et al. (2016) in the same species.

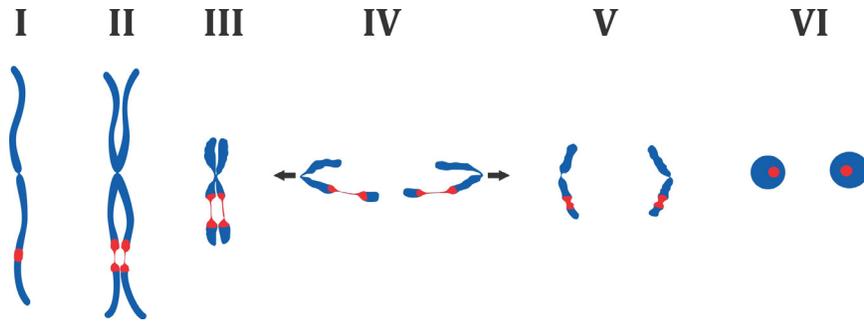


Figure 6. Schematic of the dynamic condensation in 45S rDNA signals during the cell cycle. I: Phase G1, II: Phase S/G2 with decondensation in 45S rDNA sites, III: Prophase/Metaphase chromosomes with decondensed 45S, even after the condensation of the remaining of the chromosome, IV and V: 45S rDNA signals in chromatids go back to a compaction level similar to the remaining of the DNA after segregating, VI: In telophase, 45S rDNA sites have the number of signals expected, with no leftover.

In FSSs, the DNA fibers are generally not visible when contrasted with conventional staining, with DAPI, and sometimes with techniques such as FISH. The methods used to prepare slides and the pre-hybridization might break the fine DNA fibers, seeming to form chromosome fragments. Although the chromatin fibers were not evident in all FSSs in the present study, they are responsible for the chromosome stability observed in the evaluated *Lolium* and *Festuca* species. In a previous study, Rocha et al. (2016) did not find evidence for the hypothesis that the neocentromere activity in presumable chromosome fragments would be responsible for the stability of the genome of *Lolium*, *Festuca*, and *Festulolium* hybrids.

The delay in the replication, as well as other possible damages in the chromatin, may be restored during the cell cycle as described by Chan et al. (2009). In their study, the authors reported the role played by replication factors (helicases) and DNA repairing (FANCD2) after the segregation of chromatids in human fibroblasts treated with a replication inhibitor (aphidicolin) that promotes the expression of FSSs. In their study, they also found a co-location of FANCD2 marks, DNA repairing protein, along with 45S rDNA sites from prometaphase to late anaphase of fibroblasts, which suggests that the repairing mechanism and replication completion are active even during segregation (Chan et al., 2009). Interestingly, only 10% of the FANCD2 marks seen in metaphases were present in anaphases. This suggests that FANCD2 is efficient in repairing the DNA structure during the early phases of cell division or even after the beginning of anaphase. Thus, the presence of repair during cell division could be a potential mechanism to maintain FSSs, compensating for any failures of replication checkpoints in interphase, and concluding the replication in 45S rDNA sites that had not been finished in interphase, which is one of the causes of the expression of fragility.

We observed that FSSs occur regularly and non-induced during the cell cycle of *L. perenne*, *L. multiflorum*, and *F. arundinacea*. The method by which FSSs are expressed does not promote any genome/chromosome instability, since there is no formation of fragments or loss of genetic material that might result in deleterious effects for the studied species.

Studies on FSs conducted so far have described the spontaneous expression in C-metaphases of *Lolium* and *Festuca* (Huang et al., 2008; Bustamante et al., 2014; Rocha et al., 2015, 2016). In these studies, C-metaphases are in general obtained through a block with cold water for 24 h. The suspicion that FSs could be due to the effect of heat shock was supported by Guarido et al. (2012), who demonstrated that some regions of plant chromosomes belonging to the genus *Solanum* are prone to undergo decondensation when exposed to low temperatures and these regions are denominated cold sensitive. Results obtained with analysis of the cell cycle in the present study confirmed that the expression of FSs is spontaneous and independent of the treatment of *Lolium* and *Festuca*. Signals of the 45S rDNA probe and the site of FS occurrence were observed in variable numbers and sizes in the three studied species. Furthermore, gaps were observed, which are typical of the occurrence of FSs.

Future epigenetic studies will allow for further examination of which events are involved in the high decondensation of 45S rDNA sites, an extremely important multigene family for the maintenance of the cell metabolism. Future studies will also allow us to understand the dynamics that promote fragility and allow gene stability, in addition to elucidating the feasible evolutionary and biological meaning of the peculiar behavior observed in 45S rDNA sites in *Lolium* and *Festuca*.

Conflicts of interest

The authors declare no conflict of interest.

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