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Heterochromatic knobs gene-*like* effects on flowering time, and the seed aging epigenetic-genetic program in maize

Renata Flávia de Carvalho

Thesis presented to obtain the degree of Doctor in Science. Area: Genetics and Plant Breeding

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Renata Flávia de Carvalho
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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To my family, my safe haven

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Start over

“When life hits hard and your soul bleeds.
When that heavy world hurts you, crush you.
It's time to start over. ***Start fighting again.***
When everything is dark and nothing illuminates.
When everything is uncertain and you just doubt.
It's time to start over. ***Start believing again.***
When the road is long and your body weakens.
When there is no path or place to reach.
It's time to start over. ***Start walking again.***
When evil is evident and love is hidden.
When the chest is empty and the hug is missing.
It's time to start over. ***Start loving again…****"*

Braulio Bessa

SUMMARY

RESUMO

Efeitos gene-*like* dos knobs heterocromáticos no tempo de florescimento e o programa epigenético-genético de envelhecimento de semente em milho

O milho (*Zea mays* spp. *mays*) é uma espécie cultivada conhecida por sua ampla variabilidade genética. Grande parte da diversidade observada no tamanho dos genomas é devido a presença de sequências repetitivas, as quais podem compor até 90% de DNA em uma espécie. De modo especial, knobs são regiões de heterocromatina constitutiva presentes no genoma do milho e devido à sua natureza repetitiva, nenhuma função específica foi atribuída até o momento. Por ser uma região citológica visivelmente observada ao microscópio, os knobs dão ao genoma do milho uma variabilidade cariotípica única. Apesar de nenhuma função atribuída, há evidências de uma possível influência dos knobs sobre características fenotípicas do milho. O envelhecimento de sementes é uma área igualmente relevante, pois pode subsidiar práticas de armazenamento de sementes, as quais são primordiais para a conservação de recursos genéticos, incluindo a cultura do milho. Existe uma relação entre o envelhecimento de sementes e o impacto sobre rendimento das culturas e isto tem sido demonstrado por meio de estudos citogenéticos e fisiológicos. No entanto, um entendimento mais aprofundado sobre as consequências do envelhecimento sobre o epigenoma das plantas ainda permanece obscuro. Nesse contexto, estrutura-se os dois capítulos desta tese, os quais apresentam objetivos bem distintos, mas que são complementares no que diz respeito à contribuição científica para as áreas de genética e melhoramento do milho. O primeiro capítulo discutiu as hipóteses da contribuição dos knobs heterocromáticos para o tamanho do genoma e o tempo de florescimento em milho. Neste estudo foram utilizadas famílias de linhagens isogênicas de milho (JD 1-3, JD 2-1 e JD 4-4) e os híbridos obtidos entre elas, mapeadas para os knobs em posições específicas nos cromossomos 3, 5, 7 e 9. Todo este material foi caracterizado quanto ao tempo de florescimento masculino e feminino e o tamanho do genoma. Os dados foram analisados por meio de uma adaptação do Genome-Wide Association Study (GWAS), onde os knobs foram utilizados em lugar dos marcadores moleculares de polimorfismo de nucleotídeo único (SNPs). Estas análises mostraram uma associação significativa entre o knob no braço curto do cromossomo 9 e o tempo de florescimento, no qual a presença do knob em homozigose ou heterozigose pode levar a uma redução no tempo de florescimento do milho, tornando-o mais precoce. O segundo capítulo teve como objetivo avaliar os efeitos do envelhecimento natural de sementes e seus impactos na germinação, comportamento dos cromossomos e da cromatina do milho. Foram utilizados neste estudo genótipos da família de linhagens JD 4-1, os quais foram mantidos armazenados por 24 anos em câmara fria. Sementes envelhecidas e não-envelhecidas foram analisadas comparativamente quanto aos parâmetros de índice mitótico, alterações cromossômicas, porcentagem de germinação, análises meióticas e metilação do DNA. Houve diferenças significativas entre os tratamentos para todos os parâmetros analisados. O resultado mais expressivo foi observado no nível de metilação, no qual as sementes envelhecidas apresentaram valores médios de metilação do DNA maiores do que as sementes não envelhecidas. Entre as sementes envelhecidas também houve diferenças quanto a metilação, mostrando que há uma resposta genótipo-específico ao envelhecimento. A ação combinada entre o componente genético e a indexação epigenética contribuíram para manutenção da viabilidade das sementes envelhecidas após longos anos de armazenamento.

Palavras-chave: *Zea mays*, Knobs heterocromáticos, Tempo de florescimento, Tamanho do genoma, Envelhecimento de sementes, Alterações cromossômicas, Epigenética, Tolerância ao envelhecimento

ABSTRACT

Heterochromatic knobs gene-like effects on flowering time, and the seed aging epigenetic-genetic program in maize

Maize (*Zea mays* spp. *mays*) is a cultivated species known for its wide genetic variability. A large part of the diversity observed in the genome size is due to the presence of repetitive sequences, which can compose up to 90% of DNA in a species. In particular, knobs are regions of constitutive heterochromatin present in the maize genome and due to their repetitive nature, no specific function has been assigned until this moment. Because it is a cytological region visibly observed under a microscope, knobs give the maize genome a unique karyotype variability. Despite no assigned role, there is evidence of a possible influence of the knobs on maize phenotypic traits. Seed aging is an equally relevant area, as it can subsidize seed storage practices, which are essential for the conservation of genetic resources, including the maize crop. There is a relationship between seed aging and the impact on crop yield and this has been demonstrated through cytogenetic and physiological studies. However, a deeper understanding of the consequences of aging on the plants epigenome remains unclear. In this context, the two chapters of this thesis are structured, which have very different objectives, but which are complementary with regard to the scientific contribution to the areas of genetics and maize breeding. The first chapter discussed the hypotheses of the heterochromatic knobs contribution to the genome size and maize flowering time. In this study, maize inbred line families (JD 1-3, JD 2-1 and JD 4-4) and the hybrids obtained between them were used. They were mapped to the knobs in specific positions on chromosomes 3, 5, 7 and 9. All of this material was characterized in terms of male and female flowering time and genome size. The data were analysed using an adaptation of the Genome-Wide Association Study (GWAS), where knobs were used in place of molecular markers of single nucleotide polymorphism (SNPs). These analyses showed a significant association between the knob on the short arm of chromosome 9 and the flowering time, in which the knob presence in homozygosity or heterozygosis on chromosome 9 can lead to a reduction in the maize flowering time, making it earlier. The second chapter aimed to evaluate the effects of natural seed aging and its impacts on germination, behaviour of chromosomes and maize chromatin. In this study, genotypes of the JD 4-1 inbred line family were used, which were kept stored for 24 years in a cold chamber. Aged and non-aged seeds were analysed comparatively for the parameters of mitotic index, chromosomal changes, germination percentage, meiotic analysis and DNA methylation. There were significant differences between treatments for all parameters analysed. The most expressive result was observed in the methylation level, in which the aged seeds presented higher DNA methylation values than the non-aged seeds. Among the aged seeds, there were also differences in methylation, showing that there is a genotype-specific response to aging. The combined action between the genetic background and epigenetic indexing contributed to maintaining the aged seeds viability after long years of storage.

Keywords: *Zea mays*, Heterochromatic knobs, Flowering time, Genome size, Seed aging, Chromosomal changes, Epigenetics, Aging tolerance

1. INTRODUCTION

One of the most economically important cultivated species, *Zea mays* L. is also one of the best studied, considering its genomic complexity. The maize crop has a wide environmental adaptation since there are varieties adapted to different climatic conditions, altitudes, and latitudes (Poggio et al. 1998a; Bilinski et al. 2017; Fourastié et al. 2018). Due to its relevance, maize has become a model for cytogenetic and genetic studies that have significantly contributed to the comprehension of fundamental processes that include reproduction, photosynthesis, biosynthesis of primary metabolites, and chromosomal structure and function relationships (Zhou et al. 2009).

The first contributions made about the maize karyotype are dated to the beginning of the 20th century, with emphasis on the researcher Barbara McClintock, who was responsible for the initial study on the characterization of chromosomes from pollen grain mother cells (Mcclintock 1929). In this study, the author observed a variation in chromosome morphology due to the presence of knobs. Knobs are regions of heterochromatin, which in turn are made up of repetitive sequences, which occur in certain positions of the maize chromosomes. In further researchers, it was evidenced for the first time that the knobs had characters indicative maize varieties origin (Longley 1938; Longley 1941). The emergence of chromosomal banding techniques developed in the 1970s allowed the expansion of studies on maize chromosomes. In this context, the C-banding was used in studies showing the position and knob frequencies (Aguiar-Perecin and Vosat 1985; Aguiar Perecin 1985; Rayburn et al. 1985a).

Peacock et al. (1981) isolated repetitive sequences arranged in tandem into these structures, studying stocks of maize varying in knob numbers and B chromosomes. According to these authors, the sequence consisting of 180 bp would be the largest component of knobs found in chromosomes of maize complement A. Besides, in situ hybridization analyses have not shown the presence of these sequences in other types of heterochromatin. Opposite to that reported by Peacock et al. (1981), a new repetitive sequence was isolated from the knob of the maize chromosome 9. It was observed this new sequence was interrupting by insertions of the K180bp arrays, and when it described were found this repetitive sequences was composed primarily by motifs of approximately 350 bp in length. This new repetitive motif was disposed in tandem in the knob region and received the name Tandemly Repeated DNA Sequence 1 (TR-1) (Ananiev et al. 1998). As a result, the knobs may comprise tandem sequences of 180 bp, TR-1, or both (Albert et al. 2010; Ghaffari et al. 2013).

Currently, aided by fluorescent in situ hybridization (FISH), many studies have allowed to characterize the karyotype variability of the maize genome and to show the knob polymorphisms in inbred lines such as B73, KYS, and Mo17, which are commonly used in cytogenetic and genetic (Albert et al. 2010; Figueroa and Bass 2012; Mondin et al. 2014).

The maize genome is large, complex, and is estimated at 2.5 - 2.7 gigabases (Gb) (Springer et al. 2009). In addition, its genome is punctuated by complex motifs of repetitive sequences. About 85% of the genome consists of transposable elements, including numerous families of retrotransposons (SanMiguel et al. 1996). Studies about variability in genome size are performed across species and have been associated with evolutionary processes (Yang et al. 2011; Soltis et al. 2014). In a special case, investigations into the genome of *Zea mays* ssp. has shown a natural and complex variability in the intraspecific genome size (Rayburn et al. 1985b; Díez et al. 2013; Realini et al. 2015a).

This can be extended to the karyotype diversity described by some authors in landraces and varieties of maize due to the presence or absence of knobs (McClintock et al. 1981; Kato et al. 2004; Mondin et al. 2014). The knobs variation confers a characteristic of chromosomal variability between maize karyotypes and has been attributed as responsible for differences in genome size (Tito et al. 1991a; Biradar et al. 1994; Poggio et al. 1998b; Jian et al. 2017).

On this topic, with the development of DNA measurement techniques, much research has enlarged knowledge about the maize genome size (Rosado et al. 2009; Realini et al. 2015b; Tenaillon et al. 2016; Jian et al. 2017). Rayburn et al. (1985) determined the DNA content, heterochromatin percentage, and number of C-bands of 22 inbred and open-pollinated inbred lines and observed positive correlations between these parameters. These authors also divided the accessions according to their geographic distribution and found negative correlations between this characteristic and the heterochromatin percentage and genome size. The authors observed that inbred lines with the smallest genome have a short germination interval from flowering, while those with larger genomes have longer intervals, assuming that the selection by man for early maturation and plant size may be related to the lower maize genome size adapted to the higher latitudes.

A similar study was carried out with maize varieties and other *Zea* species (Tito et al. 1991a). Intra and interspecific variations have also been reported and positive correlations have been found between genome size and flowering time. According to these authors, the species of *Zea* are the result of the product of natural and artificial selection, in which it acts on different genotypic and nucleotypic constitutions at the diploid and/or tetraploid level. Other studies reported that the variation of maize genome size could be attributed to differences in the heterochromatin content, pointing to the correlations between the variability of the C-value with the nucleotypic effect and phenotypic and ecological factors (Poggio et al. 1998c; Rosato et al. 1998). Recently, studies using sequencing methodology have indicated relationships between genome size, the number of knobs, and flowering time, showing that knobs can play an important role in the maize genome (Jian et al. 2017; Kreiner and Wright 2018; Bilinski et al. 2018).

Additionally, the issue of aged seeds behaviour has also been reported in studies with maize (Peto 1933; Gutierrez et al. 1993; Bilia et al. 1994; Fluminhan and Kameya 1997; Revilla et al. 2006), an agricultural crop especially significant for food production. Aging directly affects seed viability. The seed is an organ of a plant that is susceptible to aging, but the understanding of this process is not the same observed for animals and even for the plants aging. Plants are modular organisms, which means that anatomically each part has its aging rate and therefore can be disposable at some point in its life cycle, without compromising the whole plant (Thomas 2002; Brutovská et al. 2013). Regarding the seed, it is not interesting that this happens, because the seed harbour the embryo, which is responsible for giving rise to the next generation.

Several factors contribute to seed aging. Storage conditions and storage time, moisture content, and temperature are the main factors that affect seed longevity (Waterworth et al. 2019). Consequently, in the embryo, there is a gradual increase in cellular damage, which affects macromolecules such as lipids, proteins, RNA, and DNA. This damage accumulation can lead to seed infeasibility, compromising its ability to germinate (Fu et al. 2015; Waterworth et al. 2015).

The compromise of the seed viability can lead to delay in the radicle emergence, in the seedling development, in the development of weak seedlings, inability to get along with environmental stress, in a low growth, and, consequently, in low yield. This is a reflection of the genomic stress caused by aging. In the embryo,

there are cellular mechanisms that trigger a reaction cascade, which in an organized manner, will promote actions that relieve all this stress such as activation of checkpoints, activation factors to DNA, programmed cell death, and endoreduplication. All of these reactions take place in an attempt to promote seed germination (Waterworth et al. 2019).

The understanding of the consequences of seed aging is the research object in the areas of genetic resources conservation and agriculture. In the first, particularly because it is of global interest to maintain germplasms that represent part of the genetic variability existing in different plant species. In the second, depending on the quality of the seed, which is imperative to guarantee food security and high yields in different crops (Fu et al. 2015; Waterworth et al. 2015; Kameswara Rao et al. 2017; Waterworth et al. 2019).

In the idealization of the maize inbred lines studied here and described in more detail in the first chapter, four inbred lines families gave rise to the set of materials used in the subsequent chapters, being: JD 1-3, JD 2-1, JD 4-4, and JD 4-1 (Decico 1991). The first three had a history of good development according to the study objectives that were developed in the laboratory at that time, being used extensively in several studies in later years (Fluminhan and De Aguiar-Perecin 1998; Bertão and Aguiar-Perecin 2002; Aguiar and Mendonça 2003; Mondin et al. 2014; Santos-serejo and Aguiar-perecin 2016). The fourth family of inbred line JD 4-1, for not meeting the predetermined objectives, was stored for 24 years in the cold chamber of the Department of Genetics, ESALQ/USP. It is in this context that the two studies were developed, and the thesis was structured. Due to the knowledge generated by previous studies, the first chapter was performed with the inbred lines families JD 1-3, JD 2-1, JD 4-4, and the hybrids obtained between them, which were used in the flowering time assays, FISH, and genome size quantification. The second chapter was designed from the multiplication of aged seeds stored of the JD 4-1 family. The recovered genotypes provided the possibility for carrying out the experiments by comparing aged and non-aged seeds at cytological, physiological, and epigenetic levels.

1.1. Objectives

The objectives of each study are described separately below.

“Gene-like effect of heterochromatic knob influences maize flowering time”

Objective: To analyse the effects of the knob condition (homozygous for presence, heterozygous or homozygous for absence), the variability in the knob numbers, and genome size in the flowering time of hybrids and maize inbred lines.

“Seed aging tolerance is associated to epigenetic program and genetic background”

Objective: To evaluate the effects of natural seed aging and its impacts on germination and behaviour of maize chromosomes and chromatin, comparing them with non-aged seeds.

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2. GENE-LIKE EFFECT OF HETEROCHROMATIC KNOB INFLUENCES MAIZE FLOWERING TIME

ABSTRACT

Maize flowering time is an important agronomic trait, which is associated with variations in the genome size and heterochromatic knobs content. We integrate three steps to show this association. Firstly, we selected inbred lines varying for heterochromatic knob composition at specific sites in the homozygous state. Then, we produced heterozygous hybrids seeking the establishment of a knob variability panel that would allow us to carry out genetic mapping. Second, we measured the genome size and flowering time for all the materials. Finally, we developed an association study and identified a knob marker on chromosome 9 that showed the strongest association with flowering time. Indeed, modeling allele substitution and dominance effects could offer only a heterochromatic knob locus and not the set of knobs in the maize genome could affect flowering time, making it earlier.

One Sentence Summary: A specific heterochromatic knob position is highly associated with decreased flowering time in maize tropical inbred lines and hybrids.

2.1. Introduction

The relationship between heterochromatic knobs composition and flowering time (FT) in maize is a long debate. Knobs have been intriguing geneticists for more than 100 years, and since they were discovered, their functions remain undercovered. These maize genomic regions are heterochromatin with late replication during the cell cycle, extensively composed of two highly repetitive satellite DNA families, 180 bp and TR-1 (*1*, *2*). Due to their monotonous DNA sequence organization, no direct function was attributed to the knobs. However, the first eminent observation was their wide variability among landraces, inbred lines and hybrids, and following a biogeographic distribution, suggesting a possible role affecting the expression of some phenotypic features, such as FT (*3*–*5*).

Moreover, other interesting aspects of the heterochromatic knobs in the maize genome have been elucidated by several studies. Knobs have been shown to affect local recombination (*6*, *7*) and genes adjacent to these regions reduce their level of gene expression (*8*). Buckler and colleagues (*9*) and Higgins and colleagues (*10*) pointed out that the meiotic drive mechanism influences the number of knobs' polymorphism in different species of *Zea*. Such a system would favor the transmission of knobs preferentially during female meiosis, which might have contributed to the maize genome's remodeling throughout its evolutionary history. These authors also showed that knobs would have a fitness cost that may be dependent on the environment. Response to abiotic stress was observed in maize plants from the transcriptional activation of the knobs' repetitive sequences. According to the authors, this activation was selective, temporary, and accompanied by epigenetic changes (*11*). Lastly, associations between heterochromatic knobs and agronomic characteristics of interest have also been reported (*12*).

In other species, possible functional roles of repetitive sequences have also been investigated. In *Arabidopsis thaliana*, a study with telomere sequences showed that, as in mammals, telomere length is not evenly

distributed in the root meristem cells. Also, it establishes a replication limit for a functional meristem and appears to be a critical mechanism that controls the growth and development of the roots (*13*). The mutant Like Heterochromatin Protein 1 of *A. thaliana*, which is homologous to HP1 of *Drosophila melanogaster*, affects developmental pathways, influencing the plant architecture and transition vegetative to reproductive phase (*14*). In humans, telomeric sequences' dysfunctions are associated with instabilities in the genome that can lead to tumorigenesis (*15*). Simultaneously, the overexpression of transcripts of satellite DNA sequences has also been linked to the development of cancer in mice and humans (*16*). Heterochromatic genes have also been described in *D. melanogaster*, *Saccharomyces cerevisiae*, *S. pombe*, *A. thaliana*, *Oryza sativa*, and in the human species (*17–21*). These genes are structurally different from euchromatic genes, and many of them have been shown to have functional roles within the species genome.

There is growing interest in the role of variation of genomic content in creating phenotypic modifications within a species. These changes are due to copy number variation (CNV), which has been used to describe duplications, deletions, and insertions between individuals of a species; and presence/absence variation (PAV) that describes the presence or not of sequences on the genome of different individuals of the same species (*22*). Based on the new sequencing technologies, it is now understood that within the species, a genome part is present in all individuals called the core genome and a part of collective genomic content that is present only in a subset of individuals called the dispensable genome. Together, they form the pan-genome of species (*23*, *24*). Maize is also a model species for studies of pan-genome. The first studies to show cytogenetic analyses performed all this variation present in the maize genome. Barbara McClintock and colleagues analyzed the content and size of heterochromatic knobs to characterize this variation in the genome (*25*). Studies of quantification of DNA content through flow cytometry have documented significant variability in the genome size (GS) between inbred lines and landraces (*26*, *27*). Currently, modern cytogenomic techniques have sampled the wide variation in the copy numbers resulting from repetitive sequences, which make up most of the maize genome (*28–30*).

New combinations of alleles arise from the variation of genomic content within species, contributing to the emergence of phenotypic variation. Therefore, this can have an influence on several important characteristics, including flowering time (*31*). Flowering in different plant species is marked by changes in the shoot apical meristem, leading the plant from the vegetative to the reproductive stage. This process is called floral transition and is highly regulated by different pathways in response to endogenous and environmental signals (*32*). Maize is a vegetatively determined plant, which means that the transition to flowering will only occur when the maize reaches a certain number of leaves (*33*). Besides, even though it is considered a short-day plant, maize is practically insensitive to the photoperiod at high latitudes. Thus, maize is more dependent on autonomous signs of development than on environmental changes to pass to flowering (*34*). Flowering time is a quantitative trait of extreme relevance for cultivated plants since it controls these plants' adaptation to the environment. Through this feature, in breeding programs, it is possible to outline strategies to make certain cultures earlier or later, allowing the expansion of crop to other regions and consequently increasing their yield (*35*).

There are some recent discussions about the triangle formed between genome size, knobs content, and flowering time in natural maize populations on the maize genome topic. These surveys have considered just the knob numbers or estimated the knob abundances by low coverage sequencing (*4*, *27*, *29*). However, few studies have evaluated the knob's effect depending on its homozygous or heterozygous condition (*36*, *37*). Different researchers have attributed variation in maize genome size to heterochromatin content differences, especially in

knobs (*3*, *38*, *39*). Moreover, it has been proposed that there is a relationship between decreased genome sizes in high altitudes with reduced flowering time (*29*, *39*). Besides, the same association is observed for knobs abundance occurring along altitudinal clines. However, no experimental design has been used to isolate the effects of homozygous or heterozygous knobs and test whether they are correlated genetically. In this case, the relationship between knobs and flowering time remains unclear.

2.1.1. Origin of the maize inbred lines and knob composition

For this research, the development process of inbred lines was idealized in the Department of Genetics at "Luiz de Queiroz" College of Agriculture – ESALQ, University of São Paulo – USP. The initial biological material was a commercial cateto variety Jac-Duro (JD - Flint type endosperm), donated by Agroceres Seeds, Brazil. Initially, the variety segregates to knob positions at K3L (where "K" refers to a knob, the number is the chromosome, L and S is for long and short arm, respectively), K5L, K7S, K9S, and it was homozygous for knobs positions at K6L, K7L, and K8L (Fig. 1 and 2, Data S1). Thus, over the self-fertilization cycles, the inbred lines were selected to have the presence or absence of knobs in those specific positions. Analyses of the C-bands frequency were performed on S3 (240-14-1 and 240-14-2) and S2 (240-14-4) progenies (Data S2) (*40*). The S3 inbred line family 240-14-1 segregated into two-knob positions: K3L and K9S; and those inbred line family 240-14-2 for one locus: K9S. The S2 inbred line family 240-14-4 segregated to the four loci: K3L, K5L, K7S, and K9S. All the inbred lines maintained the homozygous knobs for the positions K6L, K7L, and K8L. From these progenies, S4 and S5 inbred lines were obtained, derived from two self-fertilization cycles that became denominated in S5 = 14-1-3 and 14-2-1 and S4 = 14-4-1 and 14-4-4. This designation was abbreviated for lines JD 1-3, JD 2-1, JD 4-1, and JD 4-4 (Figure 1). The inbred lines used in this research were derived until S9 progenies (Data S2) (*4*), and the hybrids were obtained from crosses between JD 1-3 and JD 4-4. Therefore, we have a broad panel of heterochromatic knobs in different combinations, which can be homozygous for presence or absence or can be heterozygous, allowing to perform genetic mapping (Figures 2 and 3).

Here, we used these inbred lines and their hybrids to verify the association between genome size, knob constitution, and flowering time for male (MF) and female (FF) inflorescence. These near-isogenic inbred lines and hybrids, varying for knob positions at K3L, K5L, K7S, and K9S (Fig. 2 and Data S1), were used for flowering assay time under the well-controlled and stable environment and had their genome measured by flow cytometer. All data were used to carry out the genome association study.

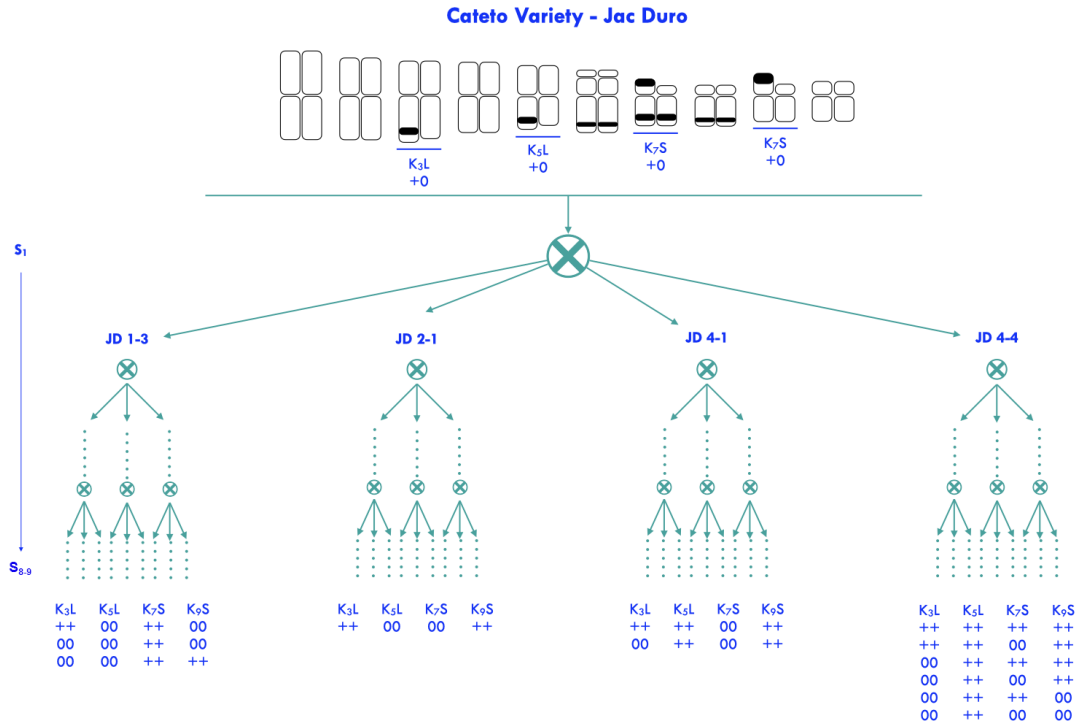

Figure 1. Maize inbred lines program for obtaining heterochromatic knobs in specific positions of chromosomes 3, 5, 7, and 9. Below each scheme is shown the classification for each condition 00 = knob absence and ++ = homozygous knob presence.

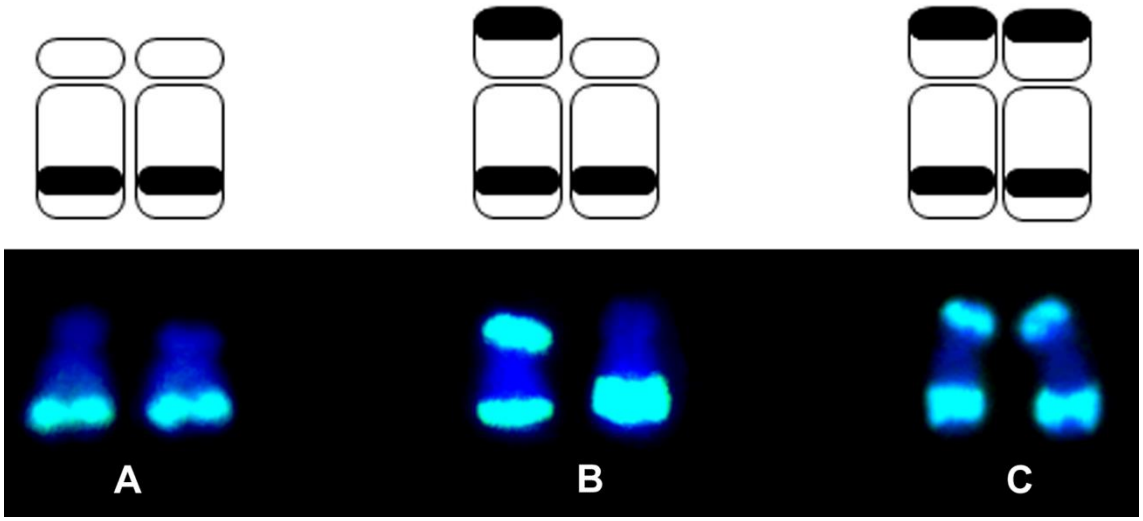

Figure 2. Representation of the knob conditions for chromosome 7 (K7S). (A) Absence knob; (B) heterozygous knob and (C) homozygous knob.

2.2. Material and Methods

2.2.1. Materials

The isogenic maize inbred lines were obtained by sibling crosses of S4 and S5 progenies that were established from seeds of two families of S3 and S2 progenies of the commercial variety Jac-Duro (JD - Flint type endosperm) (Agroceres Seeds, Brazil). As a result, were derivate four families of maize inbred lines, called JD 1-3, JD 2-1, JD 4-4 and JD 4-1. The hybrids were obtained from crosses between JD 1-3 and JD 4-4. Each inbred line family and hybrid has a specific knob composition.

2.2.2. Methods

2.2.2.1. Experimental design

For analysis of flowering time, two experiments were conducted in a greenhouse under 28ºC/25ºC day/light and 12h light/12h dark, in two years subsequent (2018/2019). The first assay counted with 199 plants, 24 inbred lines (parents) and 175 hybrids. The experiment was carried out in a completely randomized design, with 3 replicates for parents and 5 replicates for hybrids. The second assay was performed only with the inbred lines each one with 5 replicates, totalizing 100 plants. Both experiments were conducted from February to June and the maize plants were planted in 20L pots with 50 cm spacing between them. Flowering time was calculated as the number of days from planting until the first day of flowering. Inbred-lines and hybrids with different knob constitutions were evaluated individually for male flowering (MF) and female flowering (FF). Next, the whole data were analysed together.

2.2.2.1. FISH

The probe of 180 bp (*1*) were used to map the heterochromatic knobs in the inbred lines and hybrids. The steps of pre-treatment and in situ hybridization were based on Mondin and colleagues (*41*). Each cell preparation were conducted using 20 µl of a probe mixture containing the 180 bp probe. The probe mixture was denatured by heating at 96ºC for 10 minutes, cooled on ice and then dripped onto the slide preparations, which were covered with coverslip. The preparations were denatured in a thermocycler at 93ºC for 10 minutes. The hybridization were performed at 37ºC for 16h. After hybridization, the preparation were washed twice in 2x SSC at 37ºC and 42ºC for 5 minutes, twice in 20% formamide in 0.5x SSC at 42ºC (74% stringency) for 10 minutes and once in 0.5x SSC for 5 minutes at the same temperature. Probes of 180 bp were marked with FAM. The counterstain used was 0.2 µg/ml 4,6-diamidino-2-phenylindole (DAPI) and the slides were assembled in 5 µl of Vectashield H-1000.

2.2.2.1. Genome size measurements

Genome size of the inbred lines and hybrids were estimated following Praça-Fontes and colleagues (*42*). For these analyses, *Z. mays* ‘CE-777’ were used as internal standard (). Young leaves of the sample and standard were chopped into a Petri dish containing a solution of 0.5 ml of OTTO I nuclear extraction buffer (*43*). This solution was added with 2.0 mM of dithiothreitol and 50 µg/ml of RNase. Later was added the same volume of buffer solution. This homogenate was filtered, centrifuged and resuspended in OTTO I buffer for 10 minutes. The

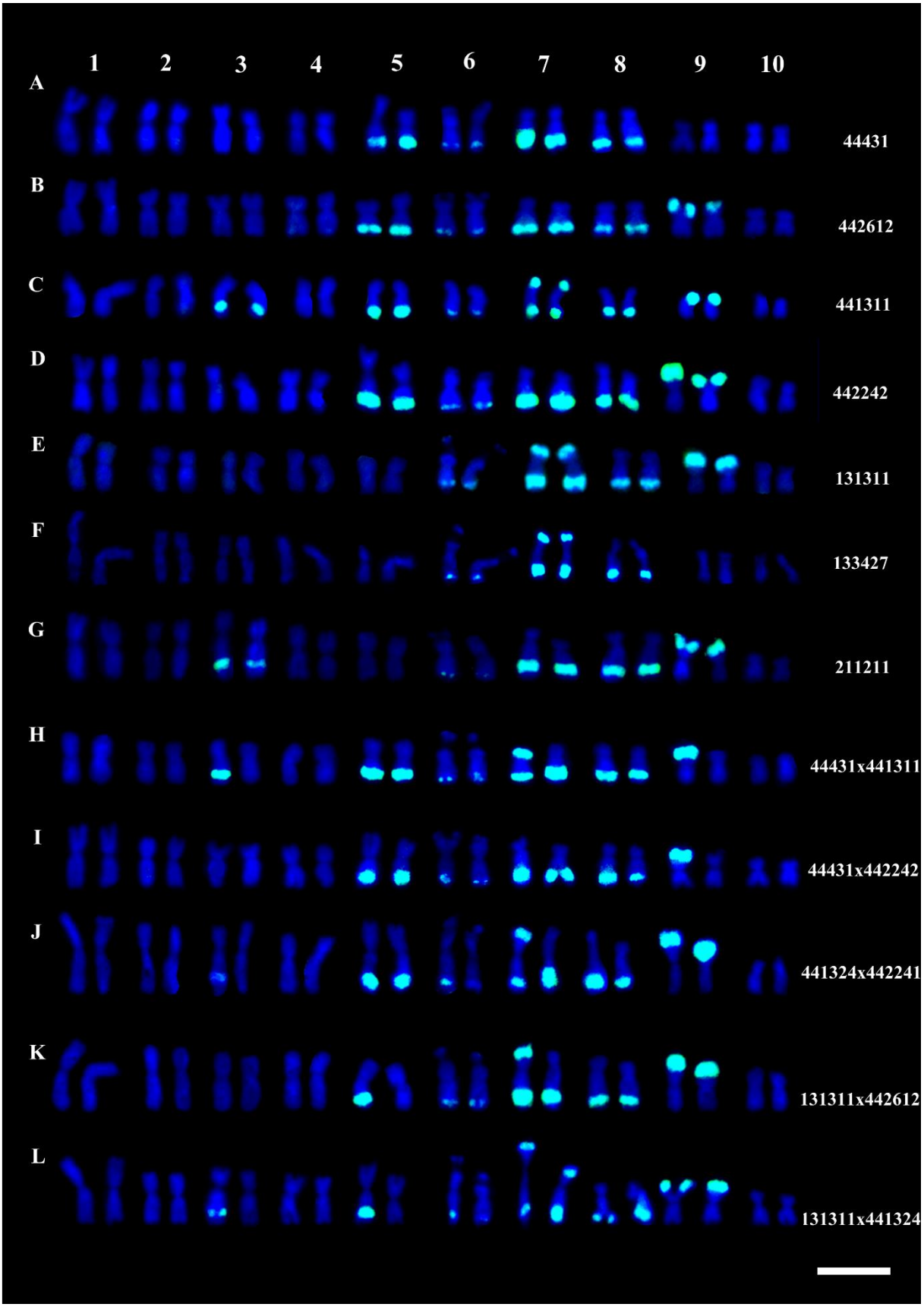

Figure 3. A sampling of genotypes used in the experiments on flowering time and genome size. (A) – (G) Somatic karyotypes of JD lines; and **(H) – (L)** hybrids, labeled by FISH with probes for the knob 180-bp repeat (green).

samples were stained in 1.5 ml of OTTO-I:OTTO-II (1: 2) staining buffer (*43*), supplemented with 50 mM dithiothreitol, 50 µl RNAse and 75 µM propidium iodide, for 20 minutes, to define the size of the nuclear genome (*44*). For each sample were used five replicates.

Nuclear suspensions were analyzed in a Partec PAS® flow cytometer (Partec® Gmbh, Munster, Germany), equipped with a laser source (488 nm) and a UV lamp (388 nm). The histograms were used to measure the nuclear genome by comparing the fluorescence peaks corresponding to the G0/G1 stages of the standard (*Z. mays* 'CE 777') and samples (inbred lines and hybrids). The genome size measurements were performed at the Laboratório de Pesquisa em Citogenética e Citometria, at the Universidade Federal de Viçosa.

2.2.2.2. Association study

A Mixed Linear Model (MLM) was run by the FarmCPU R package (*45*) to determine the SNP-trait associations. The MLM equation used in the analysis was as follows:

$$y = S\alpha + P\beta + K\upsilon + \varepsilon$$

where: y is the BLUP of the genotypes for FF or MF; α is the vector of fixed effects of the SNP; β is the vector of fixed effect of the population structure (first principal components used, depending on the trait); υ is the random effect of the relative kinship, where ~ N (0, Kσ_g^2).; ε is the error term, where ε ~ N (0, σ_e^2). S, P and K are incidence matrices that relate the independent vector effects from each matrix with the dependent *y* vector.

The additive and heterozygous (dis)advantage models were applied in GWAS analyses by using specifics encodings for the SNP matrix. Concerning the additive SNP effect with two alleles (A1 and A2), the SNP matrix was coded by 0 (A1A1), 1(A1A2), and 2 (A2 A2), considering the A2 as the minor allele. In this context, the additive GWAS model assumes there is a linear change in the phenotype regarding the minor allele number of copies. On the other hand, in the heterozygous (dis)advantage GWAS model, the homozygous genotypes (A1A1 or A2A2) were assumed to have the same effect while the heterozygous genotypes have a different one, implying an increase or decrease effect on the trait. Therefore, the SNP matrix was coded by 0 (A1A1), 1 (A1A2), and 0 (A2A2) (*46*).

To determine the p-value threshold, we used a resampling method. Therefore, first, the phenotypic values are shuffled, breaking their association with markers and then the random association between all markers to the phenotype is estimated and the corresponding best marker score (minimum p-value obtained among all markers) is recorded. This procedure was repeated 50 times for each trait, and the 95% quantile from the 50 best scores was defined as the threshold to declare a significant association.

2.3. Results and Discussion

2.3.1. Knob composition does not correlate with maize genome size

There is a wide variability of genome size in natural maize populations, and these differences often are correlated to heterochromatin percentage (*47*). Here, we measure the genome size of inbred lines and hybrids to test if the DNA content variation can be attributed to knob composition. Besides, in our analyses, we considered the genome size as a quantitative trait and estimated its heritability, which was 26%. The hybrids presented higher mean DNA values than inbred lines, showing 2C = 5.6 pg and 2C = 5.4 pg, respectively (Fig. 4). However, there were no significant differences between them. When considering only the knob condition, no differences between means were observed (Fig. S1).

Each inbred line and hybrid can have different knob arrangements at the chromosomes positions at K3L, K5L, K7S, and K9S. These combinations can be homozygous for presence or absence, or heterozygous. We used these combinations to create dosage classes and test their effects on GS and flowering time (Fig. 5). Our results demonstrated that only the dosage classes could not explain the increase or decrease in the inbred lines and hybrids' genome size. However, we observed that the hybrids presented the highest (441311/2 x 441324/1 – 2C = 6.31 pg) and the lowest DNA content (442213/1 x 441311/2 – 2C = 4.72 pg) showed the same knob constitution. For inbred lines, the largest genome size (131311/1-04 - 2C = 5.69 pg) had the knob absence for chromosomes K3L and K5L and knob presence for chromosomes K7S and K9S, while the shortest genome (442213/1 - 2C = 4.65 pg) showed knob homozygous presence for all the chromosomes. Therefore, no pattern was observed, which indicates that there is probably no linear relationship between the number of knobs present in the maize genome with the increase or decrease in the DNA content in our study.

Some studies have shown associations of the genome size with the heterochromatin percentage and knob numbers and with flowering time for the maize genome. Most of these studies showed significant positive correlations between genome size and knob content (*3*, *4*, *26*, *48*). Besides, a more recent study provided evidence that natural selection plays a substantial role in reducing the maize genome size at high altitudes (*29*). The authors also showed that the abundance of transposable elements (TEs) and heterochromatic knobs are significantly correlated with altitude. The knobs act as significant effect loci in the genome size. Kreiner and Wright (*49*) also pointed out that the genome expansion seems to be driven by the evolution of repetitive sequences since these sequences may be under other selective pressures and are additional sources for the variation of genome size in maize populations. From this perspective, variability results from the dynamic between the proliferation of repetitive sequences, diverseness in their costs and efficiency of selection against them, and exaptation of the genome size variation, which enabled the improvement of developmental traits.

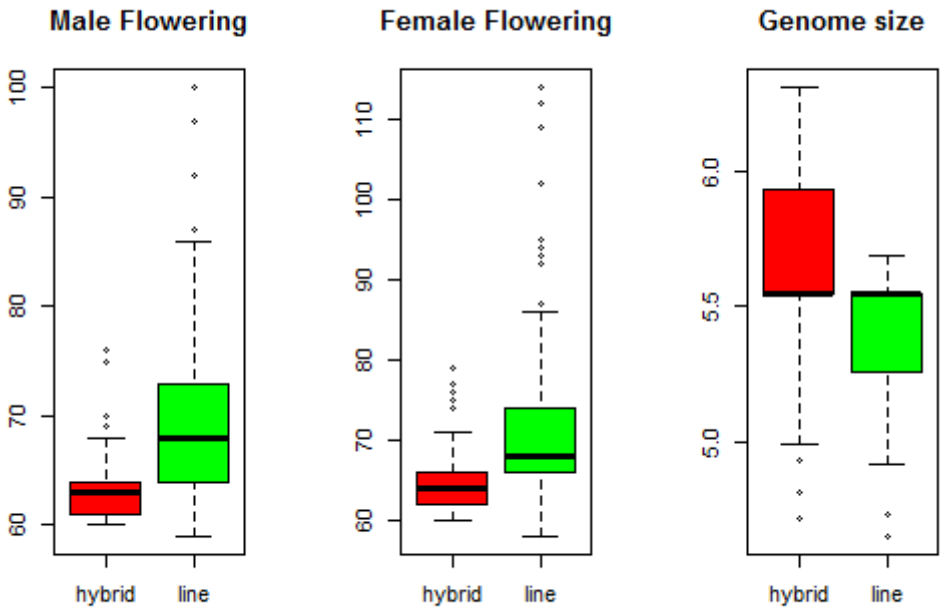

Figure 4. Flowering time and Genome Size of the hybrid and inbred lines. Boxplots compare mean values between male and female flowering time and genome size for inbred lines and hybrids. The boxes indicate the first quartile (lower line), the second quartile or mean (central line), and the third quartile (upper line). Additionally, the whiskers represent the standard deviation with the dots as the outliers.

Unlike what they found, our data does not show this same relationship. We performed a genome-wide association study (GWAS) with the heterochromatic knobs full panel to identify associations with genome size and flowering time (see above), where the knobs were used as our genetic markers (*46*, *50*). Our null hypothesis is that there are significant differences in genome size and days for male and female flowering due to the knob conditions in the K3L, K5L, K7S, and K9S positions. To test if this is true, we used the inbred lines and hybrids obtained from crossing them, to which all knobs were mapped (Fig. 3). As a result, we had many combinations of knobs presence in homozygous and heterozygous or absence of knobs to test our hypothesis. In this case, for genome size, only the allele substitution effect model was performed, and again none significant association was found (Fig. S2).

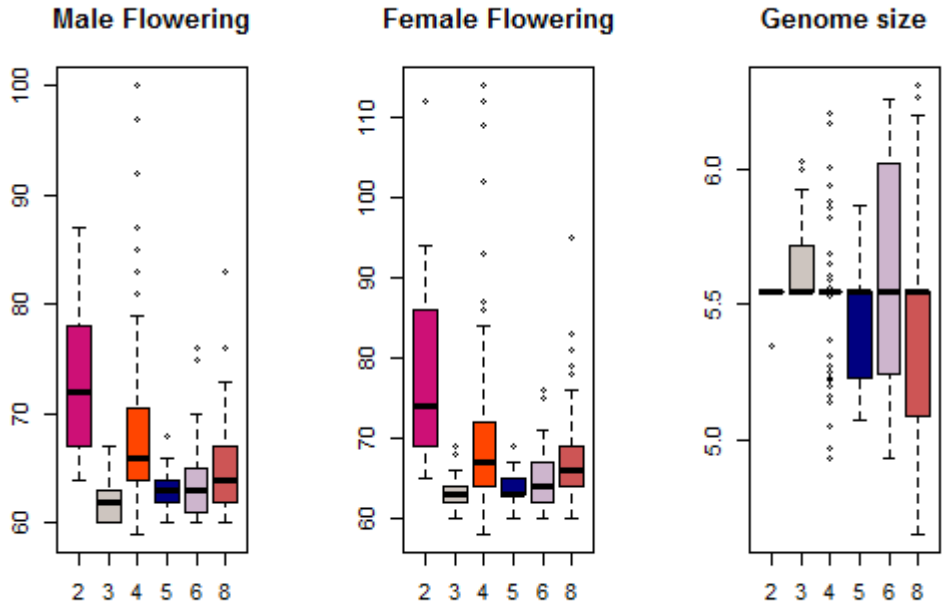

Figure 5. Comparison between dosage class of knobs for male and female flowering time, and genome size. The class means all possible combinations of presence and absence homozygous or heterozygous of the knobs considering only chromosomes 3, 5, 7, and 9. The boxes indicate the first quartile (lower line), the second quartile or mean (central line), and the third quartile (upper line). Additionally, the whiskers represent the standard deviation with the dots as the outliers.

Moreover, this information was supported by a null correlation between genome size and knob dosage classes (Table 1 and Fig. S3). Our results also corroborate a survey showing no significant correlation between maize populations' genome size and heterochromatin percentage. Even though larger genomes exhibited the highest heterochromatin content (*27*); hence, all these observations lead us to infer that maize genome size is independent of knob composition and might be controlled genetically.

2.3.2. Genome size does not affect maize flowering time

Compared to genome size by Pearson's correlation, both female and male flowering time showed negative values (Table 1). Based on our data, a negative correlation between genome size and a flowering time of maize inbred lines was found for the first time. Even so, the positive relationship between genome size and flowering time has also been noticed in some studies (*3*, *38*, *51*). Jian and colleagues (*4*) currently showed significant positive correlations between genome size, knobs abundance, and flowering time. However, when the authors introduced the kinship matrix, these correlations were lost. Once the authors performed an association study where genome size was also correlated with flowering time, three genomic regions associated with genome size were found, one of which was mapped close to the knob region on chromosome 8. Finally, these authors suggested that the selection for early materials could be aided by choice of germplasm with smaller genomes and the genome size determined based on the abundance of repetitive motifs of the knobs. In contrast, Realini and colleagues (*27*) analyzing maize landraces in northern Argentina found no correlation between genome size and flowering time. Nevertheless, a positive relationship between the vegetative cycle and heterochromatin percentage was observed.

Tabela 1. Pearson's correlation between traits.

	Male flowering	**Genome size**	**Dosage classes**
Female flowering	0.95	-0.14	-0.20
Male flowering		-0.17	-0.24
Genome size			-0.08

2.3.3. Relationship between flowering time and knob constitution

We evaluated days to MF and FF individually and estimated heritability to be 51% for MF and 41% for FF. The mean values for days to MF and FF hybrids were 63 and 64 days, respectively, while the flowering for inbred lines was 70 days to MF and 72 days to FF (Fig. 4). Heterochromatic knob configurations as heterozygotes exhibited shorter flowering time than those in homozygote states (Fig S1). The flowering time data also were plotted, showing its amplitude inside the inbred line families and hybrids (Fig. 6). We observed synchronicity for both traits within each group analyzed, and hybrids had shorter flowering time than inbred line families. Further, it is interesting to notice that the hybrids flowering time amplitude is much smaller than the other groups, ranging mainly between 60 and 70 days, in agreement with the observed mean values. It is not observed among lineage families, which show a wider variation in their amplitude (58 - 114 days), with distribution densities varying mostly between 60 and 80 days. Among families, the JD 4-4 family had a lower flowering time distribution for both FF and FM.

The knob dosage classes cannot explain the flowering behavior of all maize materials. It was possible to observe that the mean values of dosage classes were very similar for MF and FF (Fig. 5). It was also interesting to note that the inbred lines exhibited only two knobs (dosage class = 2) flowered later than those with other dosage classes (dosage class = 3-8). In their study, Bilinski and colleagues (*29*) argued that repetitive sequences would have indirect effects on flowering time due to their effect on genome size and might depend on the environment. However, this effect was not found in this study, since there was no relationship between genome size and knob composition (Table 1 and Fig S2).

The genetic architecture of flowering time in maize has been widely studied, given that this trait reflects the plant's adaptation to the environment. Since maize is distributed throughout America and is adapted to a wide range of environments, understanding how flowering time is regulated is of paramount importance and generates useful information for breeding programs (*52*). Besides, these studies, in the last two decades, dissected the maize flowering time using different approaches such as linkage and association mapping (*31, 53–55*), population genetics (*56, 57*), archaeological DNA studies (*58*), genome-wide association studies (*4, 59–61*), and gene analysis (*32, 62–64*). In inbred maize lines, the genetic architecture of flowering time has been attributed to the cumulative effect of numerous quantitative trait loci (QTL), each with a small impact on this trait (*52*). Most of the literature has mapped and identified several QTLs and genes related to flowering time. The first gene identified was *Indeterminate1* mutant (*id1*), which is responsible for regulating floral transition in maize (*33*). Considering all the

studies performed with different approaches, some regulatory network models proposed for flowering time in maize (*60*, *63*, *65*).

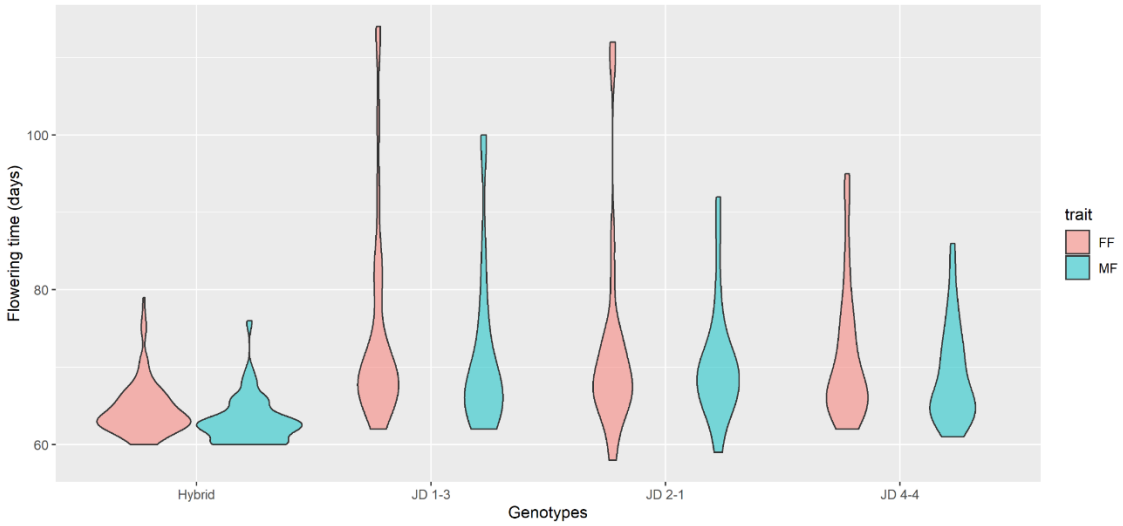

Figure 6. Flowering time among inbred line families and hybrids. The violin plot shows the FT distribution in days for all analyzed materials. The violin shape represents the estimated value density of the trait within each group. Hybrid, n = 175; JD 1-3, n = 36; JD 2-1, n = 25; JD 4-4, n = 63.

In the research described here, we used two GWAS models to illustrate the interactions between flowering time and knob conditions. The first model took into account only the allele substitution effect of the markers (Fig. 7A, 7B, and S4), and the second considered the dominance effect (Fig. 7C, 7D, and S5). Our analysis showed only one significant marker-trait association for both allele substitution and dominance effect models regarding flowering time. This significant association was observed only for the knob on the short arm of chromosome 9 (K9S) (Fig. 7). Regardless of the GWAS model, the knob marker on chromosome 9 displayed the same performance concerning the flowering time, showing the p-value highly significant (Table 2 and Table S1).

Regarding the allele substitution effect model, the presence of the K9S decreased the MF and FF of the inbred line and hybrids by -0.45 and -0.51 days, respectively. While on the dominance effect model, this effect was even more significant, decreasing the flowering time in one and a half days (MF = -1.40 and FF = -1.53) (Fig. 8). These results showed that the copy number of knobs was essential to model the effect of the genotype on the trait, but the knob condition, whether homozygous for the presence or heterozygous, contributed to the effect of decreasing flowering time. The heritability of the markers for both models varied from 0.02 to 0.15 (Table 2). The flowering time violin plot also supported the GWAS analyses (Fig. 6). Since all hybrids are homozygous or heterozygous for the K9S, corroborating the specific contribution of this knob to decrease maize flowering time.

Despite all genic action reported for flowering time, a continuing series of studies also showed the contribution of repetitive sequences for maize flowering time variation (*4*, *53*, *56*, *61*, *66*). The major maize FT-QTL *Vegetative to generative transition 1* (*Vgt1*) was characterized by a noncoding region and by insertion of a miniature inverted-repeat TE (MITE), which was strongly associated with maize flowering time (*66*). Romero Navarro and colleagues (*4*) found significant SNP associations for flowering time in pericentromeric and centromeric regions. TEs can also play a role in phenotypic variation by changing the gene expression pattern. For maize, some studies proposed the action cis-regulatory of TEs under two genes ZmCCT localized in two different

chromosomes, which are involved in photoperiod sensitivity (*56*, *61*). These authors argued that these sequences contributed to maize adaptation in higher latitudes.

Table 2. Significant marker-trait association for flowering time. Male flowering (MF), female flowering (FF), knob in the short arm of chromosome 9 (K9S), p-value Bonferroni test, minor allele frequency (MAF), allele substitution model (A), dominance model (D) and heritability (h^2).

Trait	Marker	Chr.	Position	*P-value*	MAF	Effect (A or D)	h^2
MF	K9S	9	2	1.32×10^{-2}	0.17	-0.51 (A)	0.05
	K9S	9	2	4.21×10^{-3}	0.46	-1.41 (D)	0.15
FF	K9S	9	2	2.12×10^{-2}	0.17	-0.45 (A)	0.05
	K9S	9	2	3.75×10^{-3}	0.46	-1.53 (D)	0.02

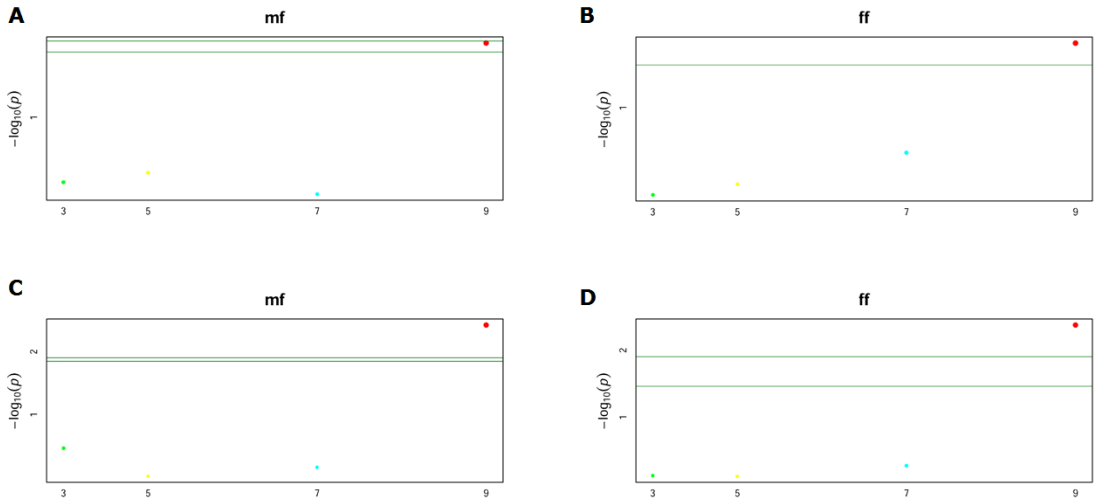

Figure 7. Manhattan plots of GWAS for male (mf) and female (ff) flowering time using the knob positions (K3L, K5L, K7S, and K9S). (A) and (B) are plots showing the allele substitution effect model, (C) and (D) the dominance effect model, both with p-values < 0.05. The green lines are the significance threshold, a Bonferroni-corrected significance threshold used to identify significant associations.

Especially in maize, the hypothesis that there is a putative contribution from heterochromatic knobs to the flowering time variation is described in the literature (*36*). Before that, studies already indicated that gene groups responsible for the plant's morphological and physiological traits would be correlated with the presence of knobs (*12*, *67*). According to the authors, once close to these knobs, recombination in adjacent regions was suppressed, influencing such characteristics. More recently, Ghafari and colleagues (*6*), using fluorescent *in situ* hybridization, demonstrated that knobs are located in dense regions of genes and that large knobs can reduce recombination locally. Comparisons between European maize genomes and US Corn Belt revealed variation in their repetitive and gene content. The germplasms were separated by the intensity and position of knob regions. However, additional positions with small sequences of knobs conserved in flint and dent lines were observed. This study also showed that the knob sequences could affect genes surrounded by them, decreasing their expression

level (*68*). Besides, the functional role of the knobs has also been traced by the relationship between their abundance, the genome size, and the flowering time, as shown above (*4*, *29*).

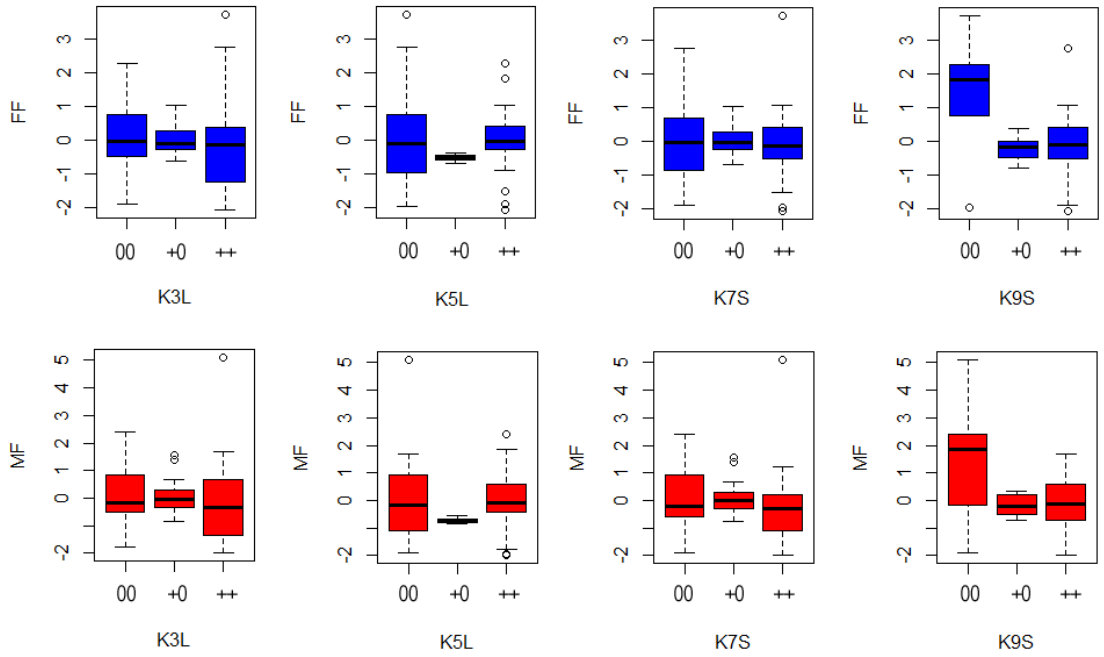

Figure 8. Boxplots show male (MF) and female (FF) flowering time for each knob condition in the K3L, K5L, K7S, and K9S chromosomes, considering both allele substitution and dominance effects. The x-axis shows the classification for each condition 00 = knob absence; +0 = heterozygous knob presence and ++ = homozygous knob presence.

Flowering time-related QTLs have been found across the maize genome (*52*, *55*, *58*). In chromosome 9, flowering time-related QTLs were found, and some genes were identified (*56*, *59*). The main gene located in chromosome 9 was ZmCCT9 (photoperiod sensitive) and is localized at the long arm, in an opposite location concerning the knob position. Other candidate genes were found along the chromosome, but the precise location was not defined yet (*53*, *69*). In our study, the knob significantly associated with flowering time was found at the short arm of chromosome 9. Knobs are regions of constitutive heterochromatin, but other constitutive heterochromatins have well-defined roles in species' genome, like centromeres or telomeres (*15*, *70*). That leads us to infer that heterochromatic knobs may also have a role in the maize genome, affecting certain phenotypic characteristics *per se*. However, further investigation in these regions will be needed to find possible candidate genes associated with knobs.

For maize, the flowering time is known to be governed for additive interactions mostly. However, other studies also demonstrated non-additive effects affording to the phenotypic variation in flowering time (*52*, *71*). Furthermore, an interesting study showed the proportion of phenotypic variance attributed to additive and dominance genetic variances for traits related to flowering time in maize and teosinte. This study clarified that, for those traits, the most phenotypic variation could be explained by additive genetic variance. Also, there is a small contribution of dominance effects on the trait, which remains virtually the same post domestication (*58*). Together, these data reinforce the results found here, where two models were used satisfactorily to detect the influence of heterochromatic knobs under flowering time.

2.3.4. Concluding remarks

The discussion on the role of knobs in the maize genome is old fashion, and it had shown that this region is an important fraction of the maize genome. Roessler and colleagues (*72*) led the elimination dynamics of components of the maize genome over successive self-fertilizations in their study. Among the five components analyzed in the genome (gene content, ribosomal DNA, B chromosomes, TEs, and knobs), the authors reported that TEs were the most significantly lost components. The results written by these authors provide insights into the constitutive role played by knobs in the maize genome. Since, like TEs, knobs are repetitive sequences, which make up about 10% of the maize genome and for unknown reasons, are not eliminated from it after generations of self-fertilization. That strengthens the hypothesis that heterochromatic knobs may have a functional role within the maize genome, even composed almost exclusively by repetitive sequences.

Throughout the maize genome evolution, it was proposed that flowering time was a trait influenced by changes in the genome size. At high altitudes, maize's flowering time was shorter than at low altitudes and followed by a smaller genome (*3*, *27*, *39*). At the same time, most studies have also indicated a positive correlation between the genome size and the knob abundance, and that the heterochromatin content decreases along the altitude clines. We found no significant association between knobs and genome size from our data, but we were able to identify one significant marker-trait association in chromosome 9 for flowering time. Moreover, opposing what was described in recent studies, we found that the knob presence in homozygosis or heterozygosis at chromosome 9 could lead to early maize flowering (Fig. 6).

Unravel the mystery behind the repetitive sequences have been hard work all over the years. It becomes even more difficult for maize since its genome comprises more than 85% of these sequences. For the first time, maize inbred lines were selected to have knobs in specific locations, and their hybrids developed to carry heterozygous knobs. After that, the analyses of adapted GWAS were carried out, showing the contribution of a single locus to the early maize flowering time. The maize flowering time is a complex trait, and several studies have provided insights into its genetic architecture. To this complexity, we add our data that suggest that gene-*like* components, such as specific heterochromatic knobs, are capable of affecting the maize flowering time, probably by interaction with particular genes playing a central role in the regulation of the feature's expression.

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Supplemental Material

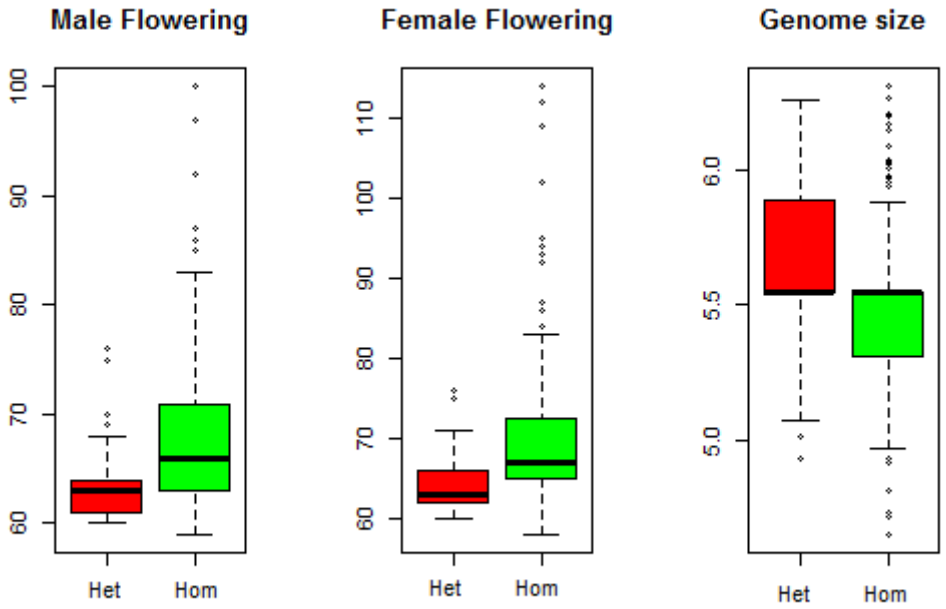


Figure S1. Flowering time and Genome Size versus knob condition. Boxplots shows the comparison between male flowering, female flowering and genome size regarding heterozygous knobs or homozygous knobs. The boxes indicate the first quartile (lower line), the second quartile or mean (central line), and the third quartile (upper line). Additionally, the whiskers represent the standard deviation with the dots as the outliers.

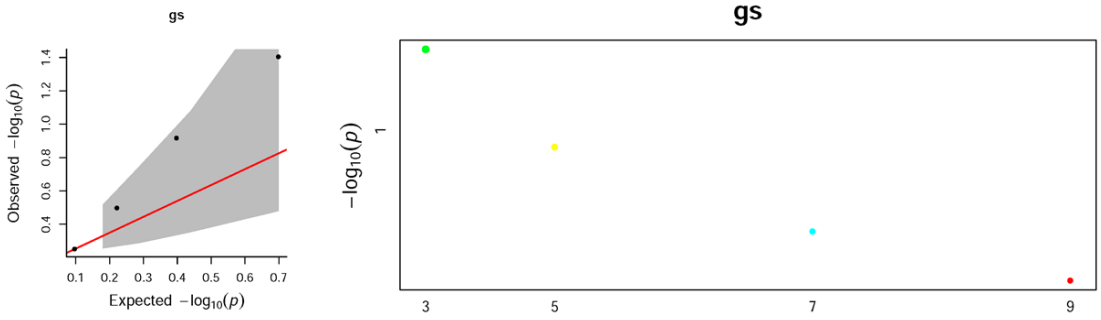


Figure S2. QQ-plot and Manhattan plot of GWAS for Genome size. Plots characterize the allele substitution effect. Significant associations are not find.

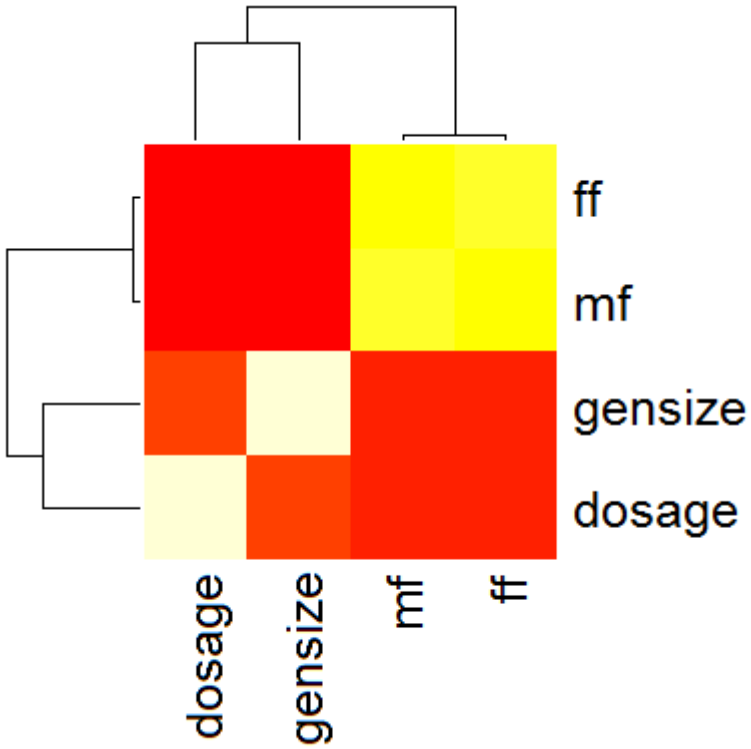


Figure S3. Heatmap. This heatmap shows the relationship between the traits: female flowering (ff), male flowering (mf), genome size (gensize) and knob dosage classes (dosage).

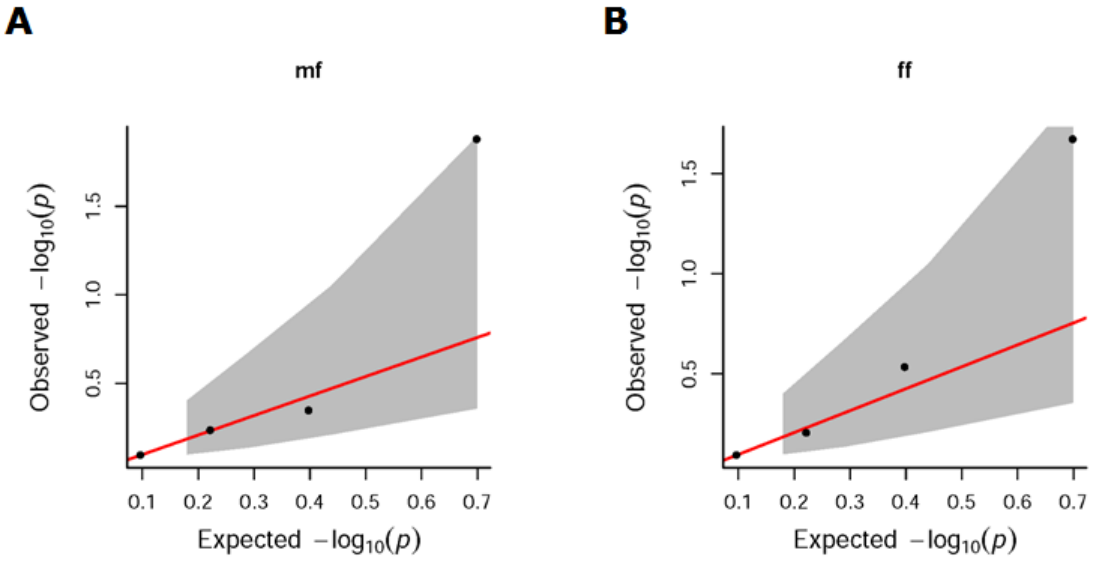


Figure S4. QQ-plot of GWAS of flowering time. Plots characterize the allele substitution effect for (A) male flowering (mf) and (B) female flowering (ff).

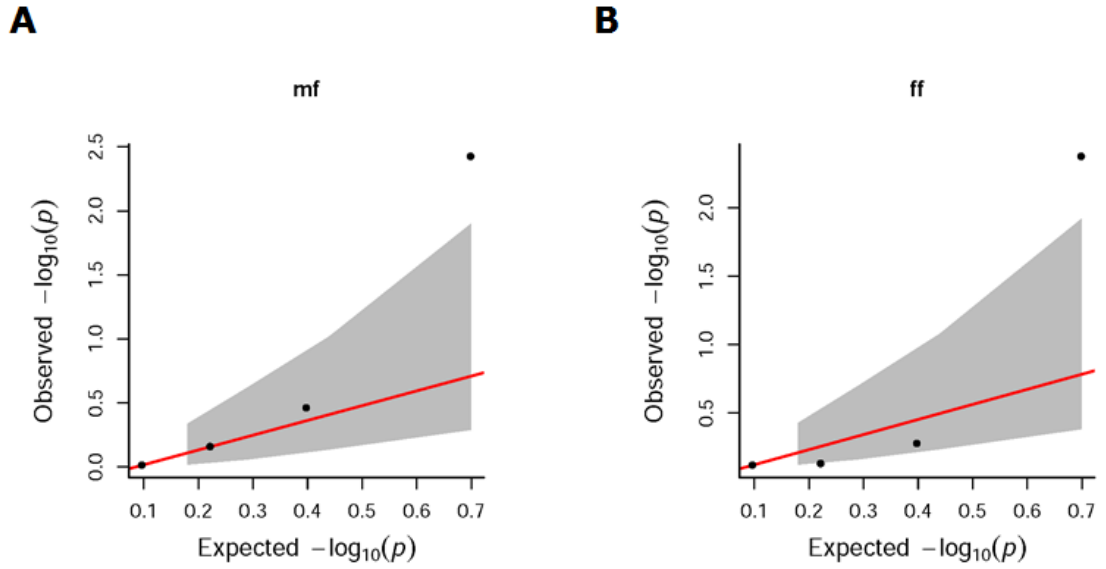


Figure S5. QQ-plot of GWAS of flowering time. Plots characterize the dominance effect for (A) male flowering (mf) and (B) female flowering (ff).

Table S1. List of additive and dominance effect for genome size and other knob loci. Male flowering (MF), female flowering (FF), Genome size (GS), Knob position, p-value via Bonferroni test, and minor allele frequency (MAF)

Trait	Marker	Chr.	Position	MAF	Additive Effect	*P value*	Dominance Effect	*P value*
MF	K3L	3	1	0.48	0.13	0.58	0.40	0.35
	K5L	5	1	0.26	-0.11	0.45	0.01	0.97
	K7S	7	2	0.44	0.06	0.81	0.17	0.70
FF	K3L	3	1	0.48	-0.05	0.81	0.74	0.13
	K5L	5	1	0.26	-0.07	0.62	0.76	0.10
	K7S	7	2	0.44	0.25	0.29	0.52	0.25
GS	K3L	3	1	0.48	0.01	0.04	-0.01	0.03
	K5L	5	1	0.26	0.004	0.12	-0.01	0.23
	K7S	7	2	0.44	0.005	0.32	-0.01	0.52

Data S1. The origin of the inbred lines from the Jac Duro variety. **Upper the table shows the original knob composition and the bottom shows the selected inbred lines and their current knob composition in S9 (separate file).**

JAC DURO ORIGINAL VARIETY						
K3L	**K5L**	**K6L1/K6L2**	**K7S**	**K7L**	**K8L1/K8L2**	**K9S**
segregating	segregating	homozygous knobs	Segregating	homozygous knobs	homozygous knobs	segregating
++/+0/00	++/+0/00	++	++/+0/00	++	++	++/+0/00

KNOBS					
Genotype	**K3L**	**K5L**	**K7S**	**K9S**	**Generation**
99	-	-	-	-	S1
200-14	-	-	-	-	S2
240-14-4	++/+0/00	++/+0/00	++/+0/00	++/+0/00	S2
240-14-4	++/+0/00	++/+0/00	++/+0/00	++/+0/00	S2
240-14-1	++/+0/00	00	++	++/+0/00	S3
240-14-2	++	00	00	++/+0/00	S3
4-1	++/+0/00	++/+0/00	++/+0/00	++/+0/00	S3
4-4	++/+0/00	++/+0/00	++/+0/00	++/+0/00	S3
1-1	-	-	-	-	S4
1-2	-	-	-	-	S4
1-3	-	-	-	-	S4
2-1	-	-	-	-	S4
2-2	-	-	-	-	S4
2-3	-	-	-	-	S4
41-1	+0/00	++	00	++/+0/00	S4
41-2	++/+0/00	++	00	++	S4
44-1	++/+0/00	++/00	++/+0/00	++/+0/00	S4
44-2	++/+0	++/+0/00	++/00	++/+0/00	S4
44-4	00	++	++/+0/00	+0/00	S4
13-1	00	00	++	++	S5
13-2	00	00	++	++	S5
13-3	++/+0/00	00	++	++?	S5
21-1	++	00	00	++	S5
21-3	++	00	00	++	S5
411-2	++	++	00	++/00	S5
412-3	++	++/+0	00	++/+0	S5
412-4	++/+0/00	++/+0	00	++/+0	S5
441-1	++	++/+0	++	++	S5
441-3	++	++	++/+0/00	++/+0	S5
442-2	+0	++/+0	++/+0/00	++	S5
444-3	00	++	00	00	S5
131-1	00	00	++	++	S6
131-5	00	00	++	++	S6
132-3	00	00	++	++	S6

		KNOBS			
Genotype	**K3L**	**K5L**	**K7S**	**K9S**	**Generation**
133-4	+0/00	00	++	00	S6
211-1	++	00	00	++	S6
211-2	++	00	00	++	S6
213-1	++	00	00	++	S6
213-3	++	00	00	++	S6
4112-1	++	++	00	++	S6
4112-2	++	++	00	++	S6
4123-3	++	++	00	++	S6
4123-4	++	++	00	++	S6
4124-2	00	++	00	++	S6
4411-2	++	++	++	++	S6
4411-3	++	++	++	++	S6
4413-1	++	++	++/00	++	S6
4413-2	++	++	++/+0/00	++	S6
4422-4	++/+0/00	++/+0	00	++	S6
4426-1	++	++	++	00	S6
4443-1	00	++	00	00	S6
1311-1	00	00	++	++	S7
1315-1	00	00	++	++	S7
1323-3	00	00	++	++	S7
1334-2	+0/00	00	++	00	S7
2111-2	++	00	00	++	S7
2111-3	++	00	00	++	S7
2112-1	++	00	00	++	S7
21311-2	++	00	00	++	S7
21331-1	++	00	00	++	S7
44433-2	00	++	00	00	S7
13131-1	00	00	++	++	S8
13151-1	00	00	++	++	S8
13233-1	00	00	++	++	S8
13342-1	++	00	++	00	S8
13342-2	++	00	++	00	S8
13342-7	00	00	++	00	S8
21112-1	++	00	00	++	S8
21113-1	++	00	00	++	S8
21121-1	++	00	00	++	S8
21311-2	++	00	00	++	S8
21331-1	++	00	00	++	S8
441123-3	++	++	++	++	S8
441132-2	++	++	++	++	S8
441311-2	++	++	++/+0/00	++	S8
441324-1	++	++	++	++	S8

		KNOBS			
Genotype	**K3L**	**K5L**	**K7S**	**K9S**	**Generation**
442213-1	++	++	++	++	S8
442213-2	00	++	++	++	S8
442242-1	00	++	+0/00	++	S8
442612-1	00	++	++	00	S8
444332	00	++	00	00	S8
131311-1	00	00	++	++	S9
131511	00	00	++	++	S9
132331-1	00	00	++	++	S9
133421	++	00	++	00	S9
133422	++	00	++	00	S9
133425	00	00	++	00	S9
133427	00	00	++	00	S9
211121	++	00	00	++	S9
211131	++	00	00	++	S9
211211	++	00	00	++	S9
213112	++	00	00	++	S9
213311	++	00	00	++	S9
441123-3	++	++	++	++	S9
441132-2	++	++	++	++	S9
441311-2	++	++	++	++	S9
441324-1	++	++	++	++	S9
442213-1	++	++	++	++	S9
442213-2	00	++	++	++	S9
442242-1	00	++	00	++	S9
442612-1	00	++	++	00	S9

3. SEED AGING TOLERANCE IS ASSOCIATED TO EPIGENETIC PROGRAM AND GENETIC BACKGROUND

ABSTRACT

A major factor in seed aging is the progressive DNA damage. These damages occur by altering the chromatin structure, through chromosomal changes or epigenetic regulation. In the present study, changes in the behaviour of the seeds viability, chromosomes, and chromatin of naturally aged maize seeds were investigated. The analyses were performed comparatively with non-aged seeds, evaluating parameters such as germination, mitotic index, chromosomal changes, and global DNA methylation level by immunolocalization. Aged maize seeds showed significant differences for all analysed parameters when compared to non-aged seeds. Changes in the DNA methylation levels were observed between aged and non-aged seeds, showing a significant increase in DNA methylation in aged seeds. A genotype-specific response was observed due to genotypes, demonstrating a certain pattern of aging tolerance. The genetic background affected viability maintenance of aged seeds even after long-term storage.

3.1. Introduction

Aging is a well-established process described in mammals, mainly. This process is characterized at the cellular level by the reorganization of the chromatin structure and it is associated with cellular senescence, which is initiated by replicative exhaustion, DNA damage, oncogene activation, or oxidative stress (Cristofari 2017). Consequently, this leads to numerous disorders in humans. Plants also have cell senescence, but the aging process is not the same (Brutovská et al. 2013).

In plants, the aging process is not yet well established, and some studies suggest that senescence is analogous to the aging process in mammals. Brutovská et al. (2013) discussed different aging theories, which are mainly based on animals, applying them to plants. These authors described the damage accumulation theory, which may be associated with oxidative stress or DNA damage directly. They also commented on the theory of somatic mutations and accumulation of mutations, which takes into account unrepaired DNA damage that may culminate in mutations or chromosomal breaks, or poor functionality of genes that are involved in plant aging. In addition, the authors have addressed other aging theories that relate to organism size, cell division processes, and adaptive processes that lead to the evolutionary compromise of plant aging.

On the other hand, unlike plant aging, seeds are structures passive of aging, once they can survive without germinating whether favourable conditions are not found. Seeds are plant organs responsible for carrying genetic information through the embryo and liable for establishing the next generation (Waterworth et al. 2015). Therefore, aged seeds are an important source for understanding the aging process of plants (Robbins 1957; Walters 1998).

Seed aging is characterized by vigour loss and seed viability (Walters 1998). In an attempt to explain the causes that lead to seed aging, several studies have been conducted considering cytological, physiological and biochemical parameters (Villiers 1974; Gutierrez et al. 1993; Elder and Osborne 1993; Shaban 2013; Waterworth et al. 2015). Seed quality is a key constituent for both the preservation of genetic

resources and agriculture (Waterworth et al. 2015; Waterworth et al. 2019). Therefore, it is imperative to understand the mechanisms controlling seed aging as a way of ensuring seed viability for long-term storage to germplasms maintenance and their use increasing crop yields.

Aging promotes several changes since the structural level, modifying the membrane permeability, to the genetic level, through chromosomal alterations. Particularly, DNA damage accumulates in the quiescent embryo progressively leading to increased base loss, generation abasic sites, base modification, single and double-strand DNA breaks (Waterworth et al. 2019). DNA cytosine methylation is a base modification that does not alter the DNA sequence but is associated with the regulation of gene expression, especially if it occurs in the promoter region, blocking its transcription (Liu et al. 2015). In the maize genome, it is estimated that about 30% of cytosines are found in their modified form, occurring in these three contexts CG, CHG, and CHH (Yu et al. 2020). Also, some studies have indicated that global DNA methylation plays an important role in seed development and viability (Michalak et al. 2015; Ogneva et al. 2016).

Aged seeds are one of the causes that lead to changes in germination patterns, poor seedling establishment, modifications in the chromatin structure and increased frequency of chromosomal abnormalities observed in different crops, including maize (Peto 1933; Murata et al. 1982; Murata 1991; Gutierrez et al. 1993; Bilia et al. 1994; Fluminhan and Kameya 1997; Yu et al. 2020). Although, most of these studies take into account such effects only in artificially aged seed experiments. Therefore, the main aim of the present study was to evaluate the effects of the natural process of seed aging and its impacts on germination and on the behaviour of maize chromosomes and chromatin, compared to non-aged seeds.

3.2. Material and Methods

3.2.1. Plant Material

Naturally aged seeds from the maize inbred line family JD 4-1 were used in the analysis (Aguiar-Perecin and Decico 1988; Mondin et al. 2014). This study was divided into two parts: the first comparing naturally aged seeds with non-aged seeds of the same genotype, and the second comparing aged seeds that had three different behaviour during their development: germination and plant normal growth, growth until the seedling stage followed of death; and seeds that did not germinate. The genotypes JD 41122, JD 41242, and JD 412331 were used in the first part for analyses of cell cycle and mitotic instability, also observing the germination rate. The JD 41122, JD 412331, JD 411232, JD 412321 and JD 411211 were used in the second part for analyses of germination, meiotic analysis, and DNA methylation by immunolocalization.

3.2.2. Cell cycle and chromosomal abnormalities

For this analysis, seeds from the JD 41122, JD 41242, and JD 412331 were divided into two treatments: seeds stored for 24 years (since 1992) in a cold chamber at 10ºC and 40% moisture

content and seeds recently multiplied (2017) and used immediately in the experiments not extending more than 12 months of storage under the same conditions.

For instability evaluation during the mitotic cycle, roots tips were stained by Feulgen method (Mello and Vidal 1978; Mondin et al. 2007). The roots tips were fixed in Carnoy 3:1 (ethanol: acetic acid), hydrolysed in HCl 1N at 60ºC for 8 minutes and exposed to Schiff Reactive for 45 minutes at room temperature in the dark. In the preparation of the slides, a drop of 1% acetic carmine was added over the meristem. The slides prepared by the squashing method were analysed under a light microscope Nikon Elipse E200.

At least three roots tips were analysed per treatment. The evaluated parameters were Mitotic Index (MI%), calculated by the number of cells in division divided by the total cells observed cells; and chromosomal alterations, obtained by dividing the number of alterations by the total cells analysed (%). The chromosomal alterations considered in the present study were anaphase bridge, telophase bridge, C-metaphase, stickiness, micronucleus and chromosome loss.

3.2.3. Germination

Previous analyses showed that genotypes of the JD 4-1 inbred line family had three different behaviours during their development: (i) JD 41122 and JD 412331 were genotypes that developed normally and generated offspring; (ii) JD 411232 and JD 412321 grew to the seedling stage and then died; and (iii) JD 411211 did not germinate. For this reason, these materials were chosen for this experiment and the subsequent experiments using seeds from KYS inbred line as controls. The germination data were also recorded from the first experiment, calculating the germination percentage.

The seeds were placed in petri dishes with water and were kept in a chamber at 28 C for 8 hours. After this step, they were germinated in Sphagnum in BOD at the same temperature for 7 days. Counts were performed on the fourth and seventh day after the installation of the experiment, following the criteria established in Brazil (2009). From the germination test were determined: (1) Germination percentage; (2) Germination speed index (GSI); (3) root length (fourth and seventh day); (4) seedling length; and (5) vigor seed.

The germination percentage was calculated by the number of seeds germinate at the end of the seventh day divided by the number of initial seeds (%). The GSI was calculated based on the formula: GSI = (N1/T1) + (N2/T2)+ (Nn/Tn), where where Nn corresponds to the total number of seeds germinated in a Tn interval; and Tn corresponds to the interval time (Gonzales de Souza et al. 2018). Seed vigor was calculated by seedling length (cm) x germination percentage (Khodarahmpour 2011).

3.2.4. Meiotic Analyses

For meiotic chromosome preparations, immature tassels were fixed in Carnoy 3:1 (ethanol: acetic acid) and kept at −20°C. Anthers from the genotypes JD 41122, JD 412331 were dissected in 1% propionic carmine (prepared in 45% propionic acid) and the microsporocytes were squashed (Mondin et al.

2014). Three meiotic phases during meiosis I – diakinesis, metaphase I and anaphase I were analysed. To estimate the chiasmata frequency, 20 diakinesis cells were counted per plant. Besides, 50 cells of metaphase I and anaphase I was analysed considering normal and abnormal cells (chromosome loss, misdivision, lagging chromosome, typical bridge and chromosomal bridge).

3.2.5. Immunostaining procedures

For this experiment, two contrasting genotypes were chosen based on their behaviour listed above (JD 41122 and JD 411211). Besides, non-aged seeds from JD 41122 genotype was used as controls. All seeds were vacuum fixed in 4% paraformaldehyde, diluted in 1xPBS. Then, the embryos manually removed from the seed were dehydrated in an increasing alcoholic series and infiltrated in L. R. White resin. The blocks were sectioned in a rotation microtome to obtain 5 μm thick sections for slide assembly and subsequent immunodetection of 5-methylcytosine (5mC).

The slides were incubated with 100 μl of primary antibody mouse anti-5-methylcytosine diluted in 1xPBS and kept in a humid chamber, overnight at room temperature. Subsequently, the slides were washed twice in 1xPBST for five minutes and incubated with secondary antibody rabbit anti-mouse TRITC diluted in 1xPBS, for 1h at 37°C. Then, washed twice in 1xPBST for five minutes and air-dried. The slides were counterstained with 4′,6-diamidino-2-phenylindole – DAPI (2 μg) and mounted with Vectashield.

3.2.6. Image Analyses

The mitotic cycle slides were photographed using the Zeiss Axiophot 2 microscope, acquired by the CCD camera and analysed with the IKAROS software. The immunostaining images were observed under the microscope with appropriate filters and analysed with the ISIS software. The images obtained to quantify the level of global methylation were analysed using the ImageJ 1.53c. The segmentation of nuclei was performed individually. The analysis was accomplished assuming parameters of total fluorescence intensity, based on the values of integrated density for DAPI and 5mC. The levels of methylation modifications were measured as a mean value from total TRITC fluorescence intensities. All images were processed with Adobe Photoshop CS5.

3.2.7. Statistical analysis

The cytogenetic experiment was carried out in a completely randomized factorial design 3x2, using a scheme with three genotypes (JD 41122, JD 41242 and JD 412331) and two treatments (seed aging and non-aged seeds). For each treatment, five repetitions were used (considering the seed as repetition). The collected data were organized in means and standard deviation. To verify the normality of the means, the Shapiro-Wilks test was used, followed by Duncan test at the 5% probability level. Data did not follow a normal distribution were subjected to nonparametric statistical analysis using the chi-square

test (X^2) at the 5% probability level. These analyses were performed using the Infostat program (Di Rienzo J.A., Casanoves F., Balzarini M.G., Gonzalez L., Tablada M. 2018).

The second part, corresponding to the germination experiment, was carried out a randomized block design with four blocks, five treatments (JD 41122, JD 412331, JD 411232, JD 412321 and JD 411211) and a control (KYS) and 10 replicates for each treatment within the block. No parametric analysis was used to analyse the data because there was a lot of missing data. Therefore, the data were shown using descriptive statistics (Table S1).

The DNA methylation experiment was carried out in a completely randomized factorial design 3x4 with three genotypes (JD 41122, JD 411211, and JD 41122 non-aged seeds as control) and four regions (coleoptile, coleorhiza, plumule, and radicle). For each genotype, three replicates were used. All data followed the normal distribution and were subjected to ANOVA, followed by Tukey test at the level of 5% probability. These analyses were performed using the R program (R Core Team 2017).

3.3. Results

3.3.1. Cell cycle and chromosomal abnormalities

The mean frequencies and standard deviations of the cell cycle phases are shown in Table 1. Considering the different cell cycle phases, no significant differences were found between treatments. However, it was possible to observe significant differences in mitotic indexes, both between treatments (genotype x seed age) and within the seed age factor (Figures 1 and 2). In this context, the non-aged seeds showed a higher mitotic index than the aged seeds.

Regarding chromosomal alterations, three types of abnormalities were commonly observed in all treatments: chromosome loss, anaphase bridge and micronucleus (Figure 3). Telophase bridges were observed at low frequency and C-metaphases and stickiness were not observed. Table 2 shows the average values observed for each type of abnormalities. Chi-square test showed an association between chromosomal alterations and seed age only for genotype JD 41122. Nonetheless, no significant differences were observed for the other two genotypes.

In this experiment, the three genotypes responded differently to germination. Germination percentage was lower in aged seed treatments, being genotype 41122 more responsive, which presented a germination rate of 40%, while the other two genotypes presented only 20%. All non-aged seeds of genotype 41122 also germinated, a response that was not observed for genotypes 41242 and 412331, which presented germination rate of 60% and 80%, respectively.

3.3.2. Germination test

As aforementioned, the raw data for the germination test experiment is presented. Thus, the data are described in table S1 (Supplemental material). In general, the table shows that the inbred lines JD 41122 and JD 412331 are the genotypes more responsive to germination in comparison to other genotypes, which

agrees with the results observed in the experiment that evaluated the cell cycle of non-aged and aged seeds. The other germination parameters analysed showed that the JD 412331 genotype performed better than JD 41122. Besides, two seeds of the JD 412321 genotype germinated, but the seeds followed the same trend observed in previous analyses, dying after reaching the seedling stage. It was also interesting to note that the mean seedling length of the JD 412321 genotype was smaller than the other two genotypes. KYS inbred line, which was used as a control showed average values, mostly, higher than the aged seeds.

3.3.3. Meiotic analysis

The descriptive results for these analyses are presented on the tables S2 and S3. Two plants for each genotype (JD 41122 and JD 412331) and the control (KYS) were analysed regarding the chiasmata frequencies and meiotic irregularities. The chiasmata frequencies ranged from 16 to 21 for inbred lines and 18 to 22 for KYS (Figure 4). The occurrence of univalent and tetravalent was observed in lower frequency to inbred lines, ranging to 1-4 (Figure 5). The inbred lines and the control did not show differences in the chiasmata frequencies and diakinesis alterations. The meiotic irregularities observed were chromosome loss, misdivision, lagging chromosome, typical bridge and chromosomal bridge (observed only for KYS) (Figure 6). Most of the meiotic irregularities were observed in metaphase I, being characterized by chromosome loss (Figure 6a-e-i). At the anaphase I, the most frequent occurrence was lagging chromosome (Figure 6c-g). Interestingly, it was possible to observe that the cells of the aged seeds showed mean values of irregularities superior to those of the control, although not statistically significant.

Table 1. Mean values standard deviations of frequencies of the cell cycle and mitotic index of meristematic cells of the JD 4-1 family genotypes in both treatments (aged seeds vs. non-aged seeds).

Genotype	**41122**	**41222**	**41242**	**41242**	**412331**	**412331**
Treatment	**Aged Seeds**	**Non-Aged Seeds**	**Aged Seeds**	**Non-Aged Seeds**	**Aged Seeds**	**Non-Aged Seeds**
Interphase	85.99±3.38a	84.25±3.54ab	84.04±1.90ab	80.83±2.62b	84.23±4.37ab	82.77±3.01ab
Prophase	4.91±1.29b	6.38±1.91b	5.71±0.80b	8.55±1.31a	6.18±2.19b	6.65±1.75b
Metaphase	3.37±1.48a	3.39±1.16a	3.20±1.10a	3.99±1.41a	4.08±1.58a	4.01±1.30a
Anaphase	3.66±1.27a	4.07±1.16a	4.01±1.07a	4.59±0.56a	4.25±1.51a	4.51±1.09a
Telophase	1.86±0.70ab	1.87±0.78ab	2.25±1a	2.05±0.63ab	1.25±0.96b	2.02±0.65ab
Mitotic Index	13.80±0.31b	15.71±0.36ab	15.16±0.23b	19.18±0.26a	15.77±0.44ab	17.18±0.30ab

Mean values followed by the same letter do not differ statistically at the 5% probability level.

Table 2. Mean values and standard deviations of frequencies of chromosomal abnormalities found in meristematic cells of the JD 4-1 family genotypes in both treatments (aged seeds vs. non-aged seeds). The data were analysed using the chi-square test (X^2) at the 5% probability level.

Genotype	**41122**	**41222**	**41242**	**41242**	**412331**	**412331**
Treatment	**Age Seeds**	**Non-Aged Seeds**	**Age Seeds**	**Non-Aged Seeds**	**Age Seeds**	**Non-Aged Seeds**
Micronucleus	0.78±1.01*	0.56±0.36*	0.81±0.43	0.50±0.30	0.30±0.47	0.27±0.31
Anaphase Bridge	0.47±0.24*	0.49±0.24*	0.24±0.15	0.39±0.22	0.50±0.23	0.41±0.27
Telophase Bridge	0.02±0.07*	0.01±0.00*	0.07±0.00	0.01±0.00	0.05±0.00	0.01±0.00
Chromosome Loss	0.65±0.51*	1.05±0.51*	0.92±0.69	1.06±0.73	0.85±0.23	0.78±0.40

*Mean values significant at the 5% probability level

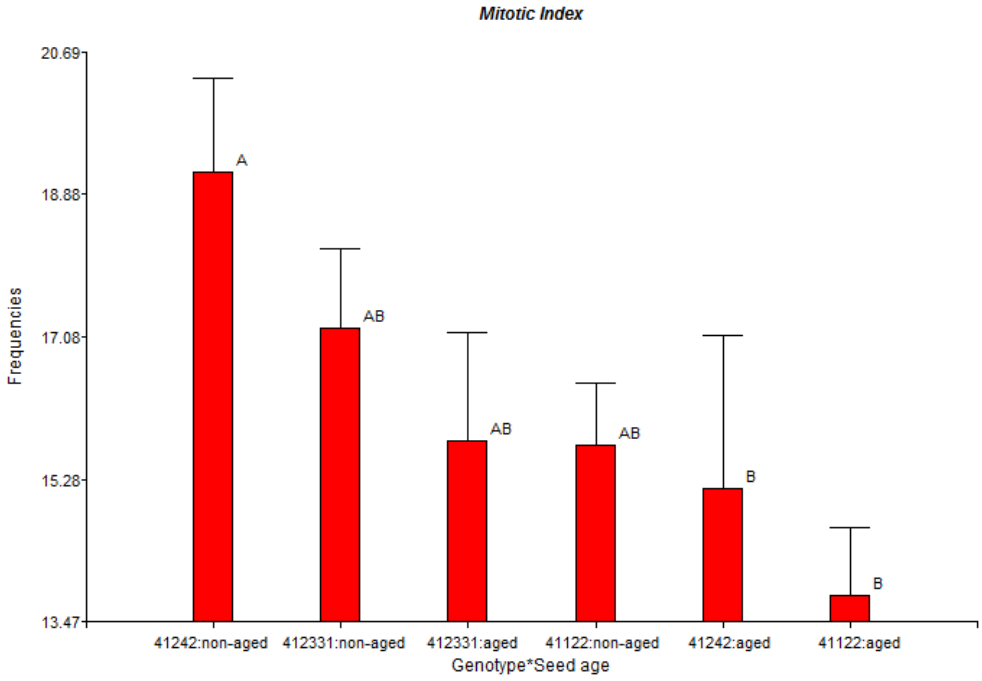


Figure 1. Bar-plot showing the interaction between the factors Genotype x Seed age for cytological parameter of Mitotic Index. Means followed by the same letter do not differ statistically at the 5% probability level. Non-aged seeds of genotype 41242 were statistically different from aged seeds of the same genotype. All genotypes with non-aged seeds showed higher MI than genotypes with aged seeds.

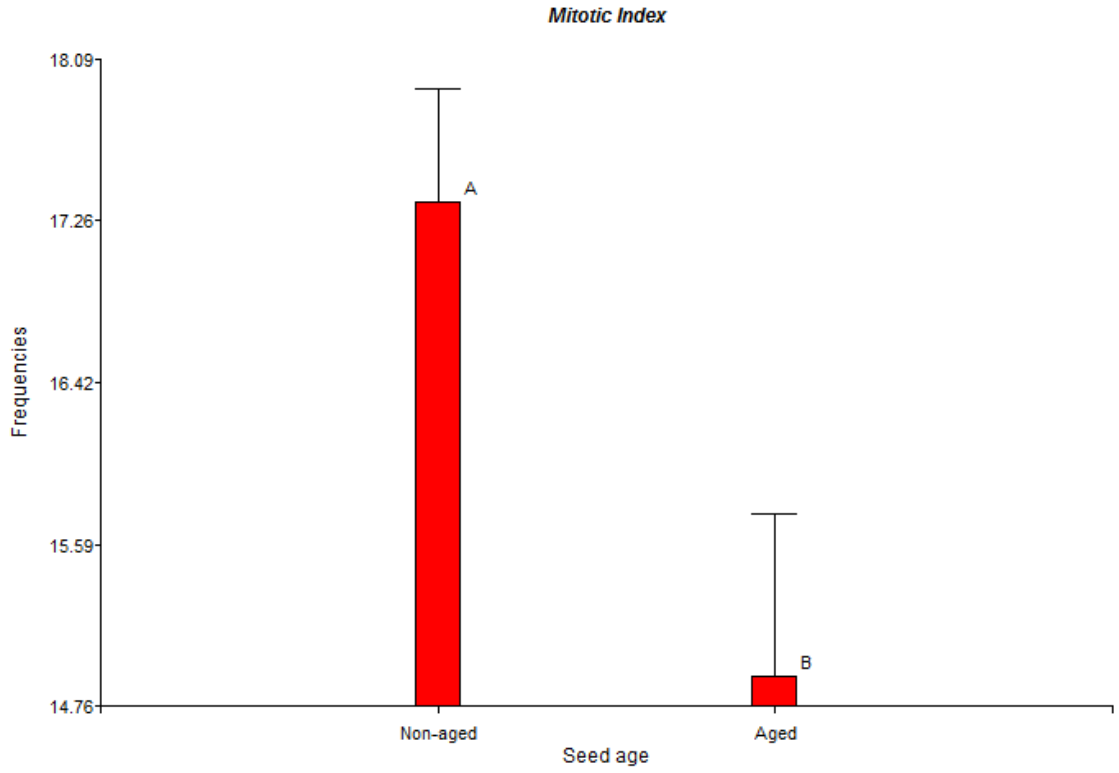


Figure 2. Bar-plot comparing treatments (non-aged seed and aged seed) within the seed age factor for the cytological parameter of Mitotic Index. Means followed by the same letter do not differ statistically at the 5% probability level. Non-aged seeds have higher MI than aged seeds, being statistically different.

3.3.4. Global DNA methylation

The levels of 5mC were measured for 4,348 nuclei, which comprised coleoptile, coleorhiza, plumule and radicle tissues (from now called regions) (Figure 7 and Table 3). ANOVA analyses revealed that there are significant differences in the total fluorescence intensities of 5mC and DAPI between the genotypes, regions, and in the genotype: region interaction (Tables 4 and 5). The comparison between the average values of the total fluorescence intensity of 5mC between the genotypes showed that the nuclei of the aged embryos were more methylated than the nuclei of the non-aged embryos, which in turn came from non-aged seeds. Furthermore, among the aged embryos, the JD 41122 genotype, which was the genotype with a better germination rate, exhibited higher DNA methylation patterns than the JD 411211 genotype (Figure 8 and Figure 9a). Total fluorescence intensity of DAPI did not show significant differences between the aged genotypes. However, aged seeds differed from the non-aged treatment, showing higher DAPI mean intensity values (Figure 9b).

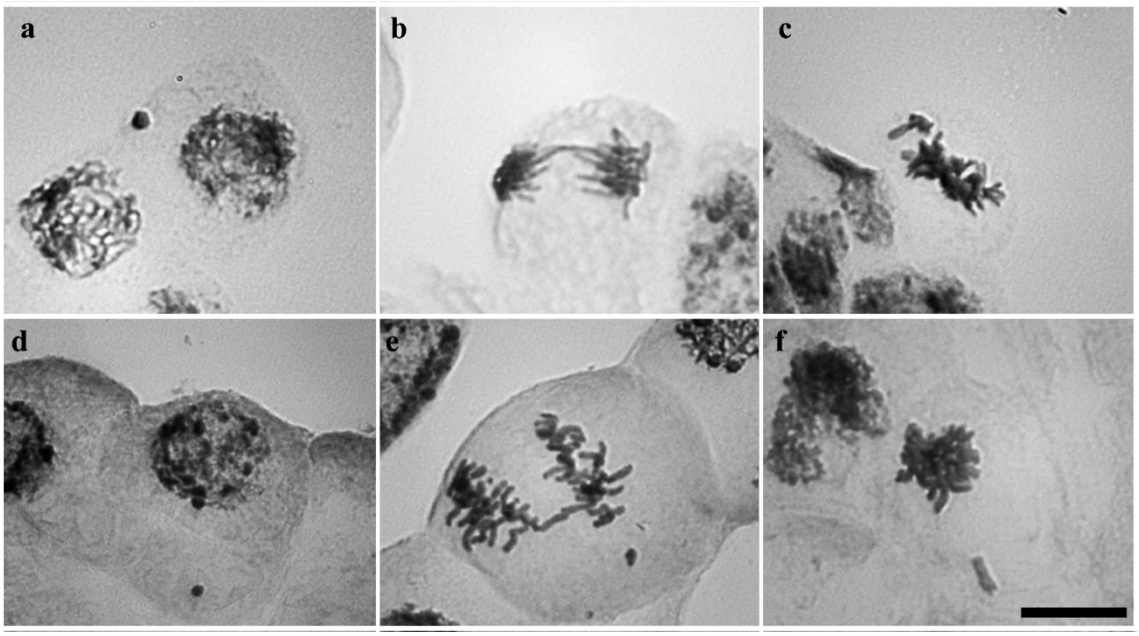


Figure 3. Chromosomal alterations observed in both treatments of the JD 41122 genotype (p-value <0.0001): aged (a-c) and non-aged seeds (d-f). a-d - micronuclei; b-e - anaphase bridge; c-f - chromosome loss. Scale bar = 10 μm.

Among regions, significant differences were also observed, with coleorhiza being the most methylated region followed by coleoptile, radicle, and plumule (Figure S1). In the genotype:region interaction, the levels of global DNA methylation between the aged nuclei of the coleorhiza region were the same. However, the methylation level of the JD 411211 genotype differed significantly from the non-aged (Figure 10a). It was possible to observe that the nuclei of the aged embryos exhibited higher methylation than the non-aged when comparing the coleoptile region between the genotypes. Again, the JD 41122 genotype showed higher methylation levels than the JD 411211 genotype. In the radicle region, JD 411211 and the non-aged showed the same DNA methylation level, which was lower than the JD 41122 genotype. The same observation is made when comparing plumule regions between genotypes.

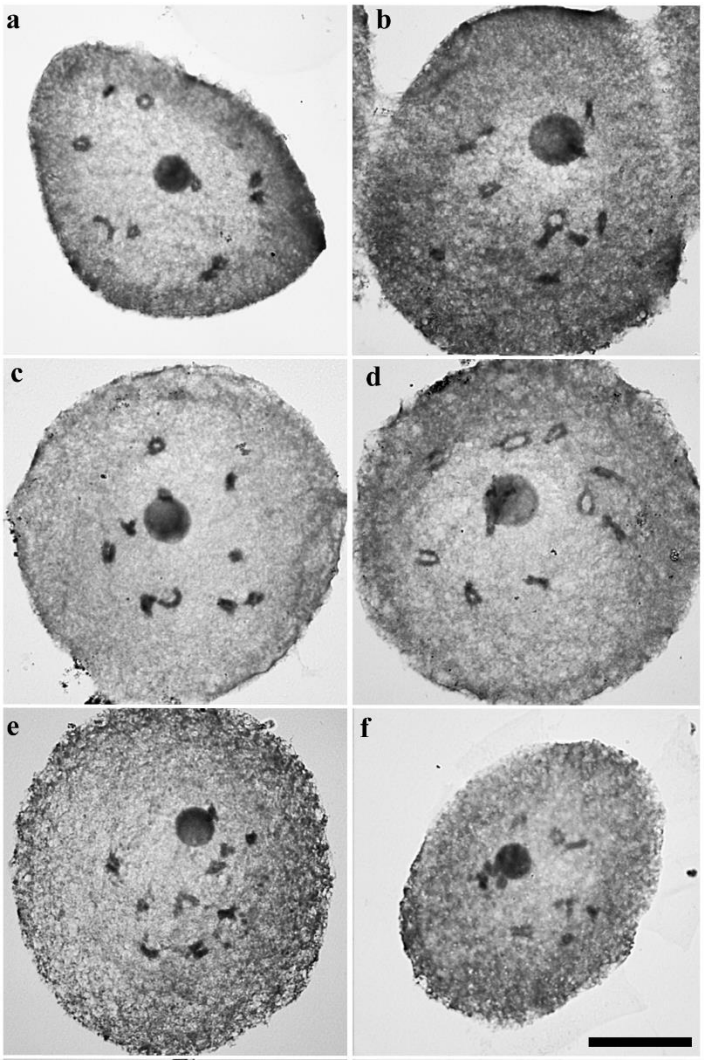


Figure 4. Propionic carmine-stained diakinesis chromosomes of the JD 41122 (a-b), JD 412331 (c-d) and KYS control (e-f). (a) 19 chiasmata; (b) 21 chiasmata; (c)19 chiasmata; (d) 20 chiasmata; (e) 19 chiasmata; (f) 20 chiasmata. Scale bar = 10 μm.

Moreover, in the genotype:region interaction, looking at each genotype and its regions individually, it was also possible to observe that there is a certain pattern of methylation levels shared by aged genotypes. These, showed-decreasing levels of global DNA methylation in the coleorhiza region, followed by coleoptile, radicle, and plumule. Interestingly, this same pattern is not seen in the non-aged genotype, which shows similar levels of DNA methylation between regions, except for coleorhiza. Regarding the total fluorescence intensities of DAPI in the genotype:region interaction, the same pattern described above is observed (Figure 10b), which reinforces the idea that the nuclei of the control embryos are metabolically more active than the nuclei of the aged embryos.

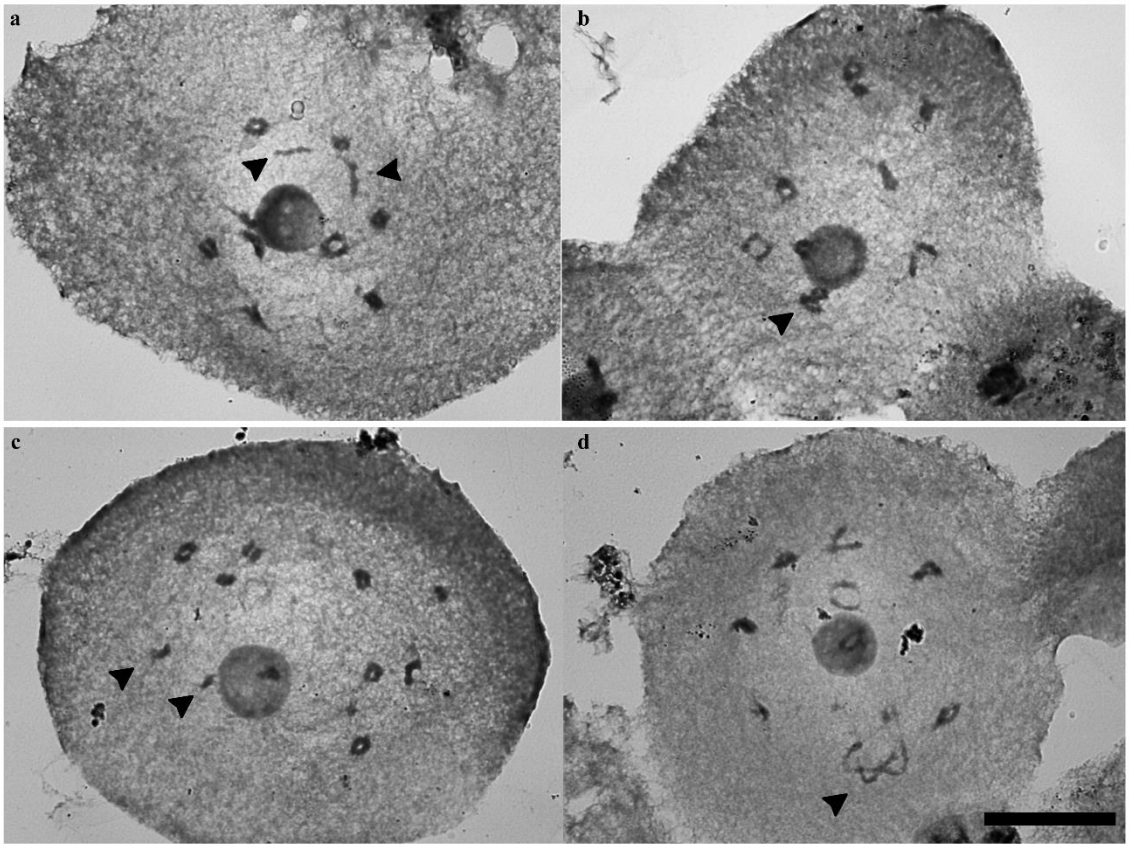


Figure 5. Diakinesis alterations of the JD 41122 (a-b); JD 412331 (c-d). a-c - a univalent chromosome (arrowhead); b-d - a tetravalent chromosome (arrowhead). Scale bar = 10 μm.

3.4. Discussion

In the present study, non-aged seeds had higher MI than aged seeds, being statistically different (Figure 2). Mitotic index analyses have been used as indicators of cell cytotoxicity when exposed to different types of destructive agents (Leme and Marin-Morales 2009; Pizzaia et al. 2019). In addition, MI can also be used to indicate seed deterioration during the aging process (Akhter et al. 1992; Menezes et al. 2014; Dantas et al. 2019). In this context, aged seeds in different studies exhibited a decrease in MI when compared to non-aged seeds, short-term stored seeds, or control seeds in artificial aging experiments (Murata et al. 1980; Akhter et al. 1992; Menezes et al. 2014).

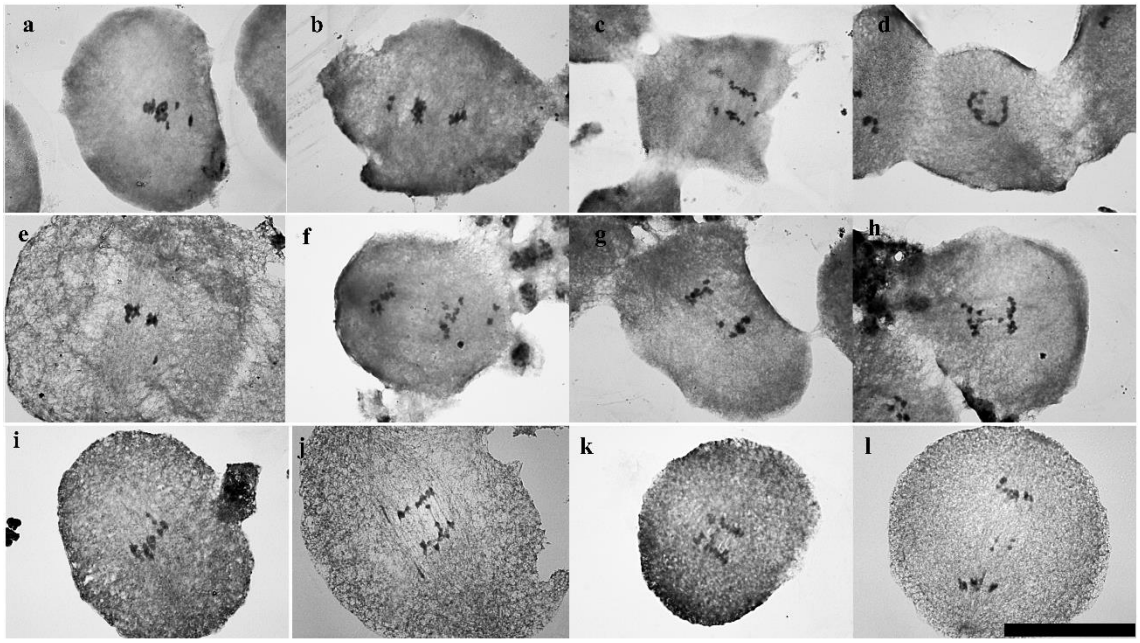


Figure 6. Meiotic irregularities observed in JD 41122 (a-d), JD 412331(e-h) and KYS control (i-l). a-e-i metaphase I - chromosome loss; b-f-l - misdivision; c-g - lagging chromosome; d-h-j - one typical bridge plus mis-division; k - chromosomal bridge. Scale bar = 10 µm.

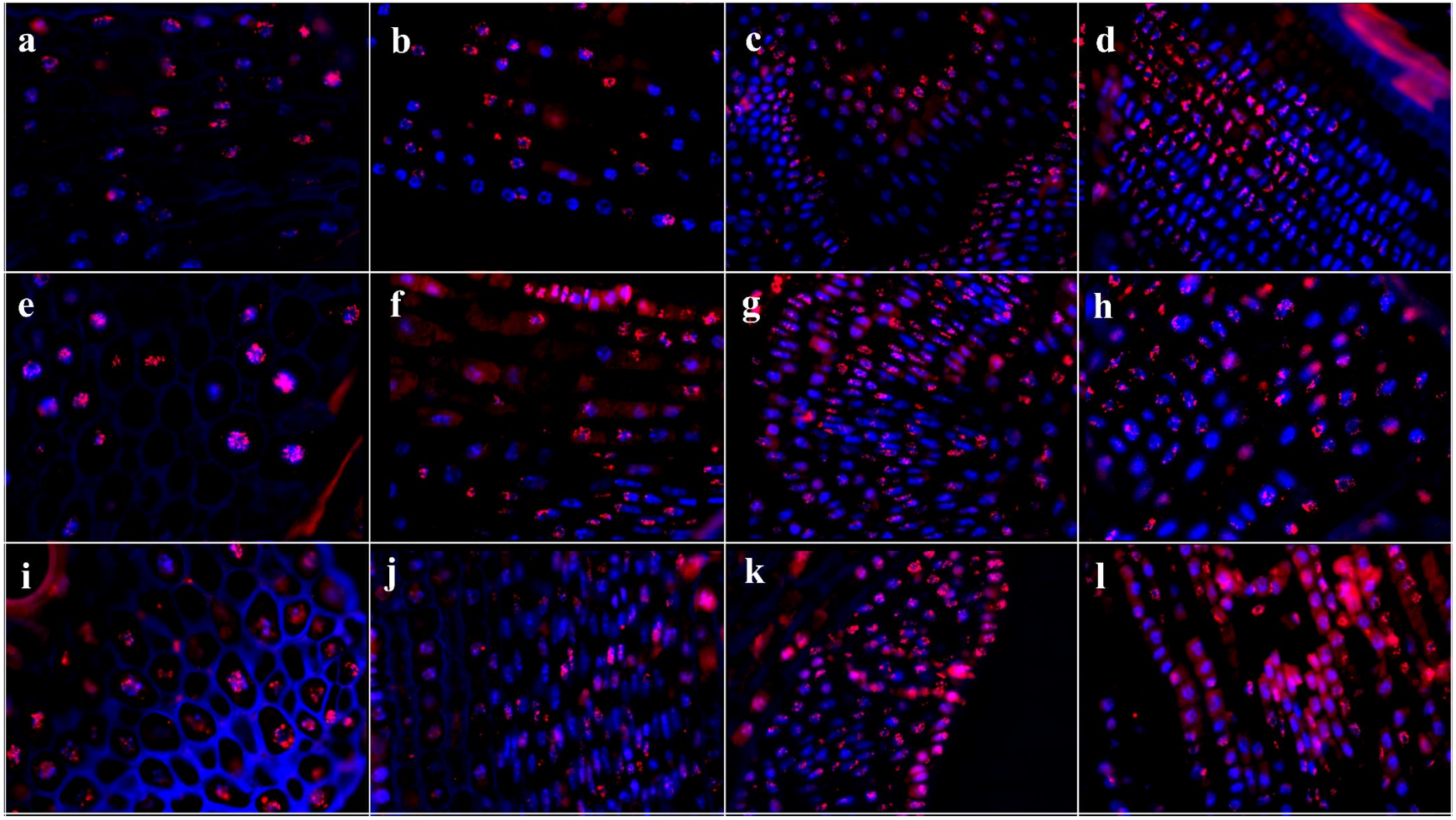
Table 3. Total number of nuclei evaluated by genotype and region.

	411211	**41122**	**41122 (Control)**	**Total**
Coleoptile	359	430	275	1064
Coleorhiza	265	140	256	661
Plumule	450	450	451	1351
Radicle	440	405	427	1272
Total	1514	1425	1409	4348

Table 4. ANOVA table for integrated density of 5mC.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Genotype	3	4.96E+08	1.65E+08	4803.614	< 2.20E-16 ***
Region	3	54474571	18158190	527.159	< 2.20E-16 ***
Region:Rep	8	3792102	474013	13.761	< 2.20E-16 ***
Genotype:Region	6	3156547	526091	15.273	< 2.20E-16 ***
Residuals	4325	1.49E+08	34445		

*** Mean values significant at the 5% probability level


a
b
c
d
e
f
g
h
i
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k
l

Figure 7. Immunodetection of 5mC (red) in maize embryos of JD 41122 (a-d), JD 411211 (e-h), and control (i-l). The columns show embryo regions. a-e-i - coleorhiza; b-f-j - coleoptile; c-g-k - plumule; d-h-l - radicle. Counterstain DAPI. 5 μm Longitudinal sections. Scale bar 10 μm

Menezes et al. (2014) analysed physiological and cytological parameters of wheat seeds. According to the authors, the artificial seed aging affected both plant vigor and mitotic divisions, showing a decrease in all physiological parameters analysed and in the MI. Similar experiments with artificially aged seeds of rice and common beans also showed a decrease in MI with increasing aging time (Dantas et al. 2019). The 41242 genotype was the only one to show a significant difference between treatments (seed age vs. non-aged seed), which suggests that there may be a genotype-specific response to seed aging.

Table 5. ANOVA table for integrated density DAPI.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Genotype	3	6.81E+08	2.27E+08	3397.292	< 2.20E-16 ***
Region	3	68169140	22723047	340.044	< 2.20E-16 ***
Region:Rep	8	4603338	575417	8.611	1.03E-11 ***
Genotype:Region	6	4183901	697317	10.435	1.61E-11 ***
Residuals	4325	2.89E+08	66824		

*** Mean values significant at the 5% probability level

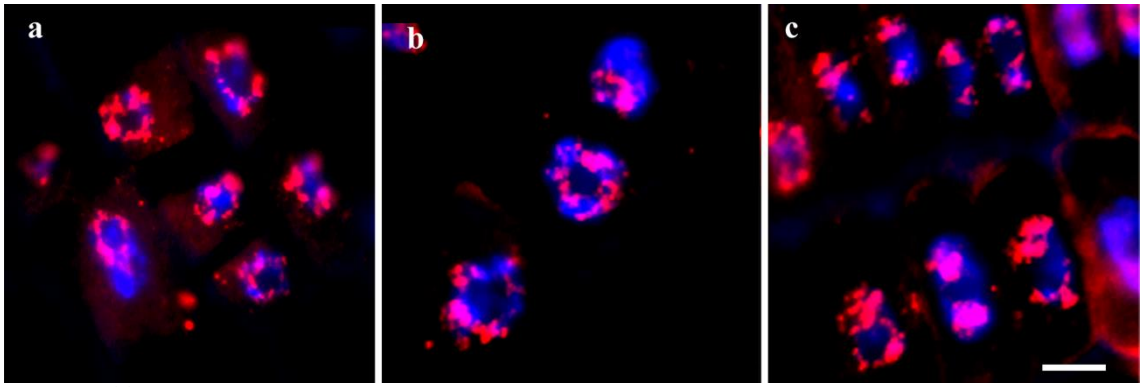

Figure 8. The radicle region nuclei. a - JD 41122 genotype; b - genotype JD 411211; control genotype. Scale bar 10 μm

Loss of seed viability during storage and aging is known to be associated with increased chromosomal changes in surviving seeds (Khan et al. 2003; Waterworth et al. 2015). Here, three types of chromosomal abnormalities were observed in both treatments: anaphase bridge, micronucleus and chromosome loss. Chromosomal abnormalities are changes in the structure or number of chromosomes that may arise spontaneously or as a result of exposure to chemical and physical agents (Albertini et al. 2000; Leme and Marin-Morales 2009). The action of abnormalities on cells can lead to malfunction of cytoplasmic structures, such as the mitotic spindle, leading to abnormal chromosome segregation and consequently aneuploid or polyploid cell formation. Additionally, structural changes are related to the

induction of chromosomal breaks during cell division, leading to loss of chromosomal fragments or whole chromosomes and micronucleus formation (Albertini et al. 2000; Leme and Marin-Morales 2009).

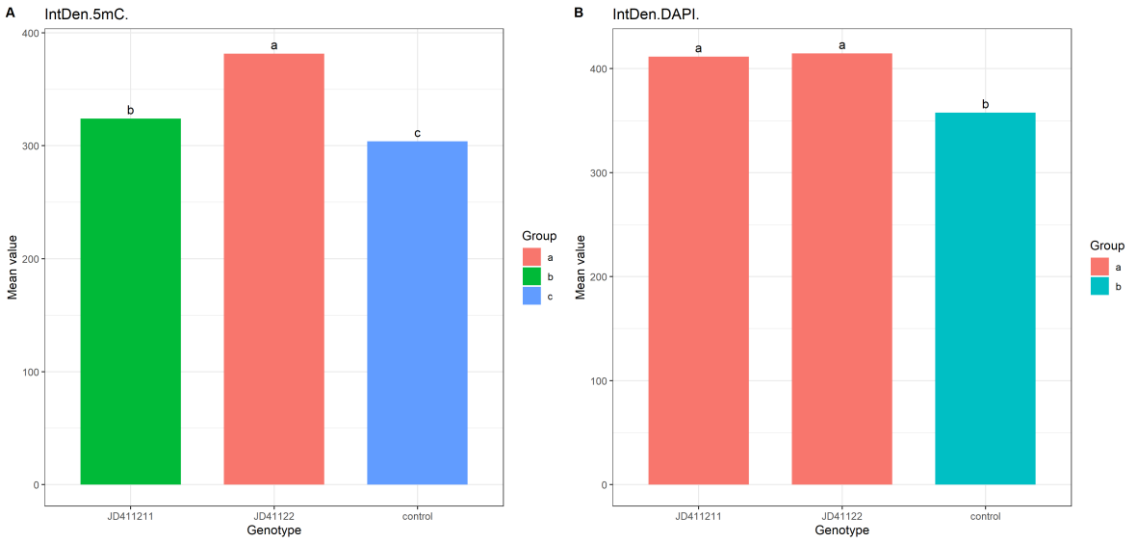

Figure 9. Plot showing the comparison of total fluorescence intensity between genotypes. A) Mean values of the integrated density (IntDen) for 5mC. B) Mean values of the integrated density for DAPI. Means followed by the same letter do not differ statistically at the 5% probability level.

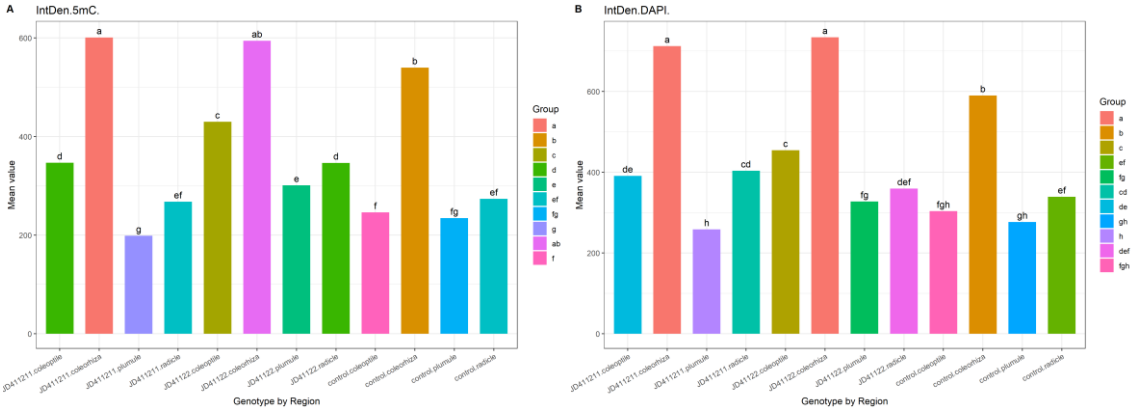

Figure 10. Plot showing the interaction between the Genotype: region. A) Mean values of the integrated density (IntDen) for 5mC. B) Mean values of the integrated density for DAPI. Means followed by the same letter do not differ statistically at the 5% probability level.

Anaphase bridges can arise from structural rearrangements, adhesion between chromosomes, or due to the influence of delayed knobs in the separation of chromatids, leading to terminal breaks of both chromatids and, consequently, the formation of lost fragments (Fiskesjö 1985; Fluminhan and Aguiar-Perecin 1998; Marcano et al. 2004). Chromosome loss arise from disturbances in mitotic spindle formation (Fernandes et al. 2009). Both types of chromosomal abnormalities may lead to the formation of micronuclei since they were not incorporated into the main nucleus during the cell cycle. Micronuclei are small in size and can occur throughout all phases of the cell cycle (Fenech 2000).

In a chronological order, investigations on the relationship between loss of seed viability and increased DNA damage throughout seed aging have been studied since the early twentieth century (Peto 1933; Innocenti and Avanzi 1971; Murata et al. 1981; Murata et al. 1982; Thant et al. 2017; Dantas et al. 2019). Peto (1933) studied the aging effect on maize and barley seeds. For maize, seeds aged naturally for six months, five and six years were tested, and for barley, the aging was artificially induced through temperature variation. For both species, the author observed a decrease in seed germination rate and appearance of chromosomal abnormalities along the seed aging process.

Similarly, Innocenti and Avanzi (1971) evaluated post-harvest wheat seeds (*Triticum durum*) for 4 years, analysing germination rate and chromosomal abnormalities. The authors observed a negative correlation between nuclear damage and germination rate during the storage time, indicating the same mechanism of action for both chromosomal abnormalities and decreased germination.

Murata et al. (1981) and (1982) tested the influence of temperature and moisture content on the germinability of artificially aged barley seeds. Chromosomal abnormality types were categorized the types of chromosomal abnormalities and verified whether there is a relationship between germination of these seeds and the presence of chromosomal abnormalities. High temperatures and humidity decreased seed germination rate, while chromosomal abnormalities increase. Both studies showed significant correlations between abnormalities and germination percentage. Dourado and Roberts (1984) analysed pea and barley seeds and showed that there was a relationship between loss of seed viability and the emergence of chromosomal abnormalities during seed aging. In addition, the authors discussed the numerous assumptions about the mechanisms that lead to the emergence of chromosomal abnormalities during seed aging, concluding that DNA damage occurs during seed storage as seeds age.

In the present results, the chi-square test analysis showed a significant association between seed age and chromosomal abnormalities for JD 41122 genotype, which supports the clue that there is a genotype-specific response to seed aging. Such association has also been reported for different species. Dimitrov (1994) evaluated chromosomal abnormalities in *Crepis capillares* stored at different ages (0, 2, 4, 6 and 8 years) and observed that chromosomal abnormalities increasing with increasing seed aging. A study of artificially aged pea seeds showed that there was a decrease in seed germination rate and vigour over time, while increasing electrical conductivity and DNA damage through chromosomal abnormalities in the same period (Khan et al. 2003).

In a more recent study, physiological, biochemical and cytological analyses were performed on soybean seeds that were collected at different harvest times to verify whether there is a relationship between these parameters and seed aging under field conditions (Thant et al. 2017). The authors showed a negative relationship between germination rate, seed aging in the field, and the percentage of chromosomal abnormalities. The longer the seed ages in the field, the lower is the germination rate, and the higher the percentage of chromosomal abnormalities.

For maize, seeds from two inbred-lines that varied in knob content were analysed in two aging treatments: seeds stored at room temperature (25ºC) and seeds stored in cold (5ºC) (Fluminhan and Kameya 1997). In this study, the authors observed that the percentage of chromosomal abnormalities was higher in the treatment with high temperature and related the types of abnormalities with the presence of knobs. For meiotic analyses, this association also were observed in plants grown from aged lettuce and barley seeds

(Murata et al. 1984; Rao and Roberts 1989). Both studies showed an increase in meiotic abnormalities with increasing aging time.

Aged seeds of different genotypes of the JD 4-1 inbred-line were stored in a cold chamber under the same conditions for 26 years. These seeds were bagged in sealed and identified kraft paper packaging (inbred-line number/year/crossing type). These packages were placed in hermetically sealed plastic boxes and stored in a cold chamber at 10ºC and 40% moisture content. Storage conditions are essential to ensure that plant seeds remain viable for long periods of time (Roberts 1972). In this case, the temperature and moisture content conditions to which the maize seeds were subjected guaranteed the longevity of the seeds after 26 years of storage.

Maize is classified as an orthodox seed and as a characteristic of this condition; its seeds are tolerant to desiccation during the ripening process. As a result, these seeds support long storage times as long as they are packaged correctly (Gimenes and Barbieri 2010). The longevity of orthodox seeds is closely related to moisture content and storage temperature (Roberts 1972; Nagel et al. 2015). Thus, the longevity of maize seeds can be extended by controlling these factors. As observed here, both moisture content and temperature were suitable in safeguarding maize seeds of the infeasibility, although not all genotypes showed the same germination performance.

Comparing the germination data from the first and second experiments (including all parameters), it was possible to notice that the most responsive aged genotypes were JD 41122 and JD 412331, once the other genotypes did not germinate. Several authors have studied the germination percentage of aged seeds for different species. Both naturally and artificially aged seeds have shown that there is a decrease in germination rate compared to the non-aged seeds (Kaul 1969; Villiers 1974; Murata et al. 1981; Akhter et al. 1992; Khan et al. 2003; Atici et al. 2007; Han et al. 2014; Menezes et al. 2014; Nagel et al. 2015).

For maize, as in the present study, some authors also exhibited these differences. Peto (1933) showed that naturally aged seeds showed a gradual decrease in germination rate when compared to new seeds. Similar results were also found for hybrid seeds during storage under different conditions of humidity and temperature (Bilia et al. 1994). Gutiérrez et al. (1993) studied the aging process in naturally and artificially aged maize seeds and showed that in both treatments the germination percentage was much lower in aged seeds. Moreover, the authors noted that the response of the germination percentage was genotype-responsive, since some genotypes, although aged, presented higher germination rate than others. Likewise, the same results were observed in the present study.

Differences in seed viability response have been shown between different maize inbred-lines stored for long periods. Revilla et al. (2006) showed that maize genotypes conserved in active collections showed variable responses regarding germination rate and vigour of plants after long storage period. In addition, a similar result was observed here for genotypes JD 41122 and JD 412331. Both aged and non-aged seeds of these genotypes exhibited a higher percentage of germination when compared to other genotypes.

Regarding the germination test parameters (GSI, root length, seedling length, and seed vigor), the seeds of the KYS genotype showed higher average values than the aged genotypes. Studies with different species have also shown a decrease in these parameters during seed aging (Basra et al. 2003;

Menezes et al. 2014; Dantas et al. 2019). In the present study, when considering the germination percentage, it was possible to observe that there are genotypes that tolerate the aging process more than other, thus showing a genotype-specific response. However, this was not linearly related to cytological parameters, since the genotype most responsive to MI did not perform as well in germination (Table 1).

The levels of global DNA methylation were studied in two aged genotypes, which showed different behaviours regarding their development (germinated or non-germinating), and thus were compared with a non-aged genotype. DNA methylation is an important epigenetic marker, which is directly related to the regulation of gene expression and, therefore can control seed response to aging (Yu et al. 2020). The most common modification found in plant and animal genomes is characterized by the addition of a methyl group at the C5 position of the cytosine, which confers changes in the chromatin structure, affecting the accessibility of the cell's machinery to the DNA strand (Law and Jacobsen 2010).

The data reported here showed that the seeds of the aged genotypes were more methylated than the seeds of the non-aged genotype. The relationship between seed aging and global DNA methylation levels has been the subject of studies described in the literature. Mira et al. (2020) verified changes in the methylation profiles of aged seeds of *Mentha aquatica* and seedlings from these seeds, showing that aging compromised the DNA integrity in these materials.

Michalak et al. (2013) and Mickalak et al. (2015) analysed the level of global DNA methylation and its relationship with the storage effects and, consequently aging in seeds of two species characterized by being orthodox and recalcitrant, respectively. Interestingly, the results of these studies were antagonistic. The orthodox seeds of *Pyrus communis* showed an increase in their methylation levels after dissection at very low levels (2-3%) and after one year of storage, these levels increased significantly. In contrast, in the experiment with recalcitrant seeds of *Quercus robur*, the authors observed demethylation in the genome with increasing aging, relating this to the viability of these seeds.

Aged maize seeds showed a higher level of global methylation than non-aged seeds. These results are in agreement with the data reported in the study with *Pirus communis*, strengthening the assumption that orthodox seeds can benefit from increased levels of 5mC methylation to maintain the DNA integrity and, consequently the viability of seeds over long-term storage (Michalak et al. 2015; Kurek et al. 2019).

Besides, comparatively among aged seeds, the genotype that is characterized by germinating seeds and generating offspring (JD 41122), showed higher methylation levels than the genotype that non-germinating (JD 411211). According to Kurek et al. (2019), there is a critical point of accumulation of changes in the genome, for example in the methylation levels, which then influence the viability of the seeds. However, this is a characteristic, which depends on many processes occurring in seeds. In this way, this would explain the results found here, where seeds that no longer germinate have reached critical levels of epigenomic changes, which prevent them from germinating. As well, methylation would have a protective effect on DNA, preventing the occurrence of damage that would lead to the accumulation of chromosomal changes and consequently the death of the seed. Thus, the more methylated the DNA, the greater the chances of the seeds surviving for longer periods.

Another possibility would be the action of DNA methylation in specific transcriptional regulatory pathways that lead to the synthesis of hormones that promote the balance between release seed

dormancy and its germination. Such processes are regulated by light signals and phytohormones, mainly gibberellic acid and abscisic acid (Yang et al. 2020). Failure to express these genes would compromise the seed's ability to release dormancy and subsequently germinate. Therefore, the global DNA methylation level observed in the JD 411211 genotype may have affected these genes, compromising the seed viability.

Among regions and in the genotype: region interaction, a certain pattern of the global DNA methylation level was observed, which was shared only between the aged genotypes. In a context of characterization of maize epigenomic landscape, it was reported that the genome of inbred lines B73 and Mo17 showed significant differences between the levels of DNA methylation, however between tissues of the same inbred line this was not observed (Yu et al. 2020). On the other hand, this same result was not found in the present study, since there were significant differences between regions of the same tissue, in this specific case, in the embryos of the inbred lines analysed.

In the case of embryos from non-aged seeds used as a control, these significant differences between regions did not exist, except for coleorhiza. The seeds deteriorate over time and several factors can influence this process (Waterworth et al. 2019). However as the seeds of the control genotype are new, the low levels of global DNA methylation observed in its different regions can be a suggestion of the viability of these seeds.

Regarding the total fluorescence of DAPI, an unexpected result was the observation of significant differences for all factors analysed (genotype, region, and genotype: region interaction). Interestingly, the aged genotypes did not differ statistically from each other and showed average values of the total intensity of DAPI greater than the control. In a mature and viable seed, the metabolism is maintained at very low levels, which conserves all the functional machinery of protein production, including mRNA produced before the embryo quiescence phase (Waterworth et al. 2019). As a result, two situations can be inferred in this case: (i) the nuclei of non-aged embryos were metabolically more active than the aged ones, being in different stages of the interphase, which in turn would influence the observed fluorescence intensity; and (ii) the nuclei of the aged embryos were in the same phase of the interphase, probably stationed in the G1 phase.

3.4.1. Concluding remarks

According to Fortanier and Jonkers (1976), in plant aging three types of aging should be considered: chronological, ontogenetic and physiological. The first is defined by the period from birth to death of any organism. In the case of plants, this is characterized from germination to plant death. Ontogenetic aging is distinguished by the passage of different phases of plant development, and these phase changes are genetically regulated. Already physiological aging can be understood as physiological modifications that occur in the plant from its aging. At this time, plants lose their ability to handle different types of stress, leading to loss of vigour and general deterioration (Fortanier and Jonkers 1976).

Taking these three types of aging into consideration and paralleling seed aging, the results found showed that aged seeds of different genotypes respond differently to the aging phenomenon. One possible explanation for this behaviour may be related to the later type of aging. If plants age physiologically, so can seeds. Seeds are organs that harbour embryos that will give rise to new offspring. It is in the

embryogenesis phase that root and stem meristems will be formed and all seed energy will be invested to ensure the full development of the embryo and the formation of a new plant (Barthélémy and Caraglio 2007).

As observed, after more than 24 years of storage the aged seeds did not show good performance regarding germination and cytological parameters. However, despite all this time, the seeds still germinating in some way, which may be the result of the genetic background and the established epigenetic indexing. The seeds that did not present this combination of responsive-genotype and epigenetic indexing failed to deal with genome changes occurred during the storage period, compromising the embryos response during the germination and cell division process.

Aging is still a poorly understood phenomenon in plants. In this research, under natural conditions, it was possible to observe the effects of aging on the germination process, on chromosome behaviour, and at the global DNA methylation level after long-term storage. The evaluated parameters did not show a linear relationship regarding their behaviour, thus showing that different genotypes belonging to the same inbred-line family respond differently to the aging process.

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Supplemental Material

Table S1. Descriptive data from the germination test.

Genotype	block	Germination Percentage	Germination Speed Index (GSI)	Mean Root length (cm/4° day)	Mean Root length (cm/7° day)	Mean Seedling length (cm)	Mean Seed vigor
41122	1	20	0.66	0.4	5	12.75	2.55
41122	2	10	0.33	1.7	9	13.7	1.37
41122	3	20	0.83	5.9	9	18.75	3.75
41122	4	20	0.66	5.55	17	34.5	6.9
412331	1	40	1.03	1.25	5.55	16.25	6.5
412331	2	60	1.33	0.67	3.86	7.23	4.34
412331	3	10	0.16	0.2	1	5	0.5
412331	4	0	0	0	0	0	0
412321	1	20	0.36	0.2	0.95	3.3	0.66
KYS (control)	1	60	1.99	3.98	15.23	26.41	15.85

Table S2. The mean values of the chiasmata frequency.

Genotype	16ch	17ch	18ch	19ch	20ch	21ch	22ch	Total
41122	1	3.5	2.5	2.5	6	4.5	0	20
412331	1	4	6	4.5	3.5	0	0	19
KYS (control)	0	0	3	8	7	1	1	20

Table S3. Mean values of normal meiosis cells and meiotic irregularities.

				Meiotic irregularities				
	Normal cells		CL	MD	LC	TB	CB	
Genotype	**Met I**	**Ana I**	**Met I**	**Ana I**	**Ana I**	**Ana I**	**Ana I**	**MI**
41122	32	44.5	18	3	2.5	0	0	4.7
412331	40.5	43.5	9.5	1	4	1	0	3.1
KYS (control)	46.5	43.5	3.5	0	2	1.5	3	2

CL = chromosome loss; MD = misdivision; LC = lagging chromosome; TB = typical bridge; CB = chromosomal bridge; and MI= mean irregularities

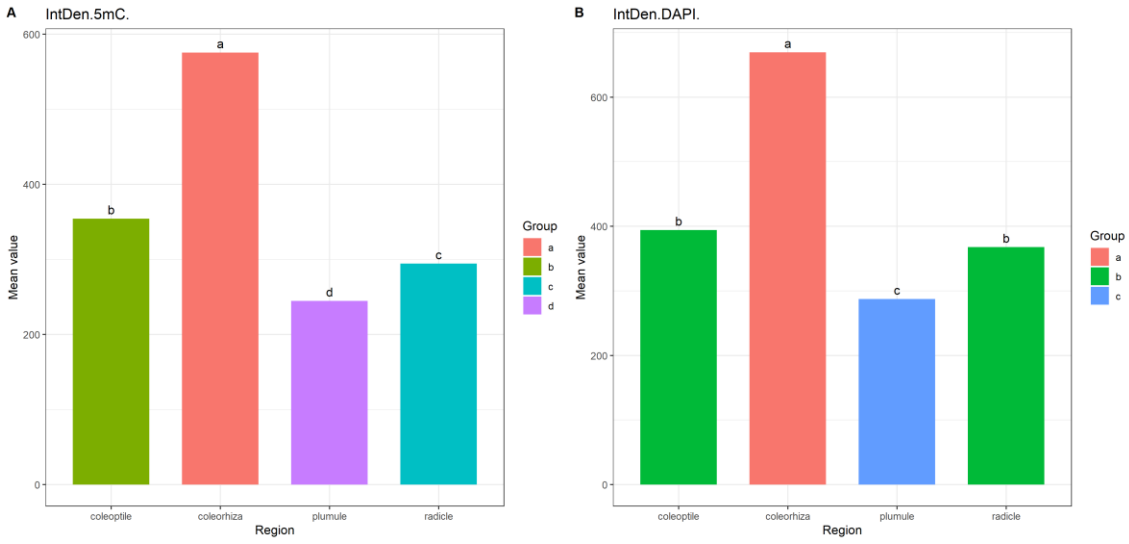

Figure S1. Plot showing the comparison of total fluorescence intensity between regions. A) Mean values of the integrated density (IntDen) for 5mC. B) Mean values of the integrated density for DAPI. Means followed by the same letter do not differ statistically at the 5% probability level.