Title: A heterochromatic knob reducing the flowering time in maize

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Abstract: Maize flowering time is an important agronomic trait, which is associated with variations in the genome size and heterochromatic knobs content. We integrated 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 for knobs, which allow us to carry out genetic mapping. Second, we measured the genome size and flowering time for all materials. Knob composition did not affect the genome size. Finally, we developed an association study and identified a knob marker on chromosome 9 showing the strongest association with flowering time. Indeed, modeling allele substitution and dominance effects could offer only one heterochromatic knob locus that could affect flowering time, making it earlier rather than the knob composition.

One Sentence Summary: Flowering time in maize is associated with specific knob position rather than genome size and knob composition.

Main Text: The relationship between heterochromatic knobs, genome size, and flowering time (FT) in maize is a long debate. Knobs have been intriguing geneticists for more than 100 years, and since their discovery, their functions remain under investigation. These maize genome regions are constitutive heterochromatins with late replication during the cell cycle, extensively composed of two highly repetitive satellite DNA families, K180 and TR-1 (1, 2). Due to their monotonous DNA sequence organization, no direct function has been attributed to the knobs. However, the first eminent observation was their wide variability among landraces, inbred lines, and hybrids, following a biogeographic distribution, suggesting a possible role affecting the expression of some phenotypic features FT (3–5). Moreover, other exciting 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 expression level (8). The meiotic drive mechanism influences the number of knobs in different species of *Zea* (9–11). Such a system would favour knob transmissions preferentially during female meiosis by a neocentromere activity, which might have contributed to maize genome remodelling throughout its evolutionary history. Response to abiotic stress was observed in maize plants from the transcriptional activation of the knob repetitive sequences, being this activation selective, temporary, and accompanied by epigenetic changes (12). Lastly, associations between heterochromatic knobs and agronomic traits have also been reported (13).

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. Together, they form the pan-genome of species (14). Maize is also a model species for studies of pan-genome. Differences in chromosomal structure between maize landraces were firstly identified through cytogenetic studies. Barbara McClintock and colleagues analysed the content and size of heterochromatic knobs to characterize this variation in the genome (4).

Moreover, quantification of DNA content through flow cytometry has documented significant variability in the genome size (GS) between landraces and inbred lines (15, 16). 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 (17–19). New combinations of alleles arise from the variation of genomic content within species, contributing to phenotypic variation (20). Therefore, this can have an influence on several important characteristics, including flowering time (17). Flowering time is a quantitative trait of extreme relevance for cultivated plants since it controls plant adaptation to the environment. Breeding programs through this feature outline strategies to make earlier or later varieties, allowing their expansion to other regions and consequently increasing yield (21).

Recent studies about maize genome present the triangle formed between genome size, knob content, and flowering time in the natural populations. These surveys have considered just the knob numbers or estimated the knob abundances by low coverage sequencing (16, 17, 22). However, few studies have evaluated the knob effect

depending on its homozygous or heterozygous condition (23). Different researchers have attributed variation in maize genome size to heterochromatin content differences, especially in knobs (16, 22–24). Moreover, it is proposed that there is a relationship between decreased genome sizes in high altitudes with reduced flowering time (17, 25). Besides, the same association is observed for knobs abundance occurring along altitudinal clines. However, no experimental design has isolated 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. Here, we derived a panel of inbred lines and their hybrids to verify the association between genome size, knob constitution, and flowering time for male (MF) and female (FF) inflorescence. Firstly, in chronological order, inbred lines with specific knob constitutions for chromosomes K3L, K5L, K7S, and K9S were derived until S9 generation from a segregating single seed of a flint variety (26). Then we created the hybrids between inbred lines, and finally, we started to estimate the flowering time. These near-isogenic inbred lines and their hybrids, varying for knob positions at K3L, K5L, K7S, and K9S (Fig. 1 and 2 and Data S2), were used to assay flowering time under controlled environmental conditions and their genome size measured by flow cytometry. All data were used to analyse the genome association study (for more information, see Supplementary Materials and Data S1).

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 associated with heterochromatin percentage (27). Here, we measured the genome size for the inbred lines and hybrids to test whether knob composition contributes to the DNA content variation. In our analyses, genome size was considered a quantitative trait, and its heritability was estimated at 26%. The hybrids presented a

higher mean genome size than inbred lines, showing 5477 Mbp ($2C = 5.6$ pg) and 5281 Mbp ($2C = 5.4$ pg), respectively (Fig. 3). However, no significant differences were found. Even when the presence and absence of the knob were considered, no differences were observed (Fig. S1). Each inbred line and hybrid can have different knob configurations at the chromosomes K3L, K5L, K7S, and K9S. These combinations can be homozygous for presence or absence in the lines or heterozygous in the hybrids (Fig. 1 and 2). These knob combinations were used to create dosage classes and test their effects on genome size and flowering time (Fig. 4). Our results show knob dosages are not enough to explain the genome size increase or decrease in the inbred lines and hybrids. However, we observed that genome size varies broadly despite the same knob composition. For instance, the hybrids presenting the highest (441311/2 x 441324/1 – $2C = 6.31$ pg) and the smallest genome size (442213/1 x 441311/2 – $2C = 4.72$ pg) share the same knob constitution (Data S1). For inbred lines, the largest genome size (131311/1-04 - $2C = 5.69$ pg) has a lesser number of knobs than the shortest genome (442213/1 - $2C = 4.65$ pg), where we have homozygous knob presence for all the chromosomes (Data S1). 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. The genome size associated with the heterochromatin percentage and knob numbers with flowering time for the maize genome is given in some studies. Most of these studies have shown positive correlations between genome size and knob content (5, 15, 22, 28). A recent study provided evidence that natural selection plays a substantial role in reducing the maize genome size at high altitudes (17). The authors also showed that the abundance of transposable elements (TEs) and heterochromatic knobs are significantly correlated with altitude, and the knobs act as significant effect loci in the genome size. Unlike what they

found, our data does not show this same relationship. We performed an adapted genome-wide association study (GWAS) with the heterochromatic knob full panel to identify associations with genome size and flowering time, where the knobs were used as our genetic markers (see Supplementary Materials). Our null hypothesis was that there were 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 our hypothesis, we used the inbred lines and hybrids panel, to which all knobs were mapped (Fig. 2, Data S1). The panel has a matrix-like structure with different knob combinations for presence (++ or 1) or absence (00 or -1) when homozygous and as heterozygous when just one of the homologous has a knob (+0 or 0). Firstly, only the allele substitution effect model was performed, and no significant association was found between knobs and FT (Fig. S2). 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 population genome size and heterochromatin percentage. Even though larger genomes exhibited the highest heterochromatin content (16); hence, all these observations lead us to infer that maize genome size is independent of knob composition and might be controlled genetically. Another hypothesis is that as our materials differ in the composition of only four knob positions, genome size measurements by flow cytometry would not detect slight differences in genome size due to knobs. Furthermore, we might infer differences in the genome size among our inbred lines and hybrids result from transposable element (TE) variability once the loss of TE during selfing has been demonstrated (29).

Genome size does not affect maize flowering time. Negative values for Pearson's correlation were found comparing genome size with female and male

flowering time (Table 1). Our results show a negative correlation between genome size and flowering time in maize for the first time. Previously, a positive relationship between genome size and flowering time has been reported (5, 28, 30). Also, significant positive correlations between genome size, knobs abundance, and flowering time were found in maize inbred lines (22). However, these correlations were lost when a kinship matrix was introduced in the analyses. In this study, the authors performed an association study where genome size was also correlated with flowering time. Three genomic regions associated with genome size were found, mapped close to the knob region on chromosome 8 (22). In contrast, another study analysing maize landraces in northern Argentina found no correlation between genome size and flowering time (16). Nevertheless, a positive relationship between the vegetative cycle and heterochromatin percentage was observed (16).

Relationship between flowering time and knob constitution. We evaluated days to MF and FF individually (Data S1) 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. 3). Heterochromatic knob configurations as heterozygotes exhibited shorter flowering times than those in homozygous states (Fig S1). The flowering time data also were plotted, showing its amplitude inside the inbred line families and hybrids (Fig. 4). We observed synchronicity for both traits within each group analysed, and hybrids had shorter flowering time than inbred line families.

Further, it is interesting to notice that the flowering time amplitude of hybrids is much smaller than the other groups, ranging mainly between 60 and 70 days, in agreement with the observed mean values. A broader variation in flowering time is observed for inbred line families (58 - 114 days), with distribution densities mainly

varying between 60 and 80 days. The JD 4-4 family had a smaller flowering time distribution for FF and FM among families. The knob dosage class's analyses cannot explain the flowering behaviour 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 with only two knobs (dosage class = 2) flowered later than those with other dosage classes (dosage class = 3-8). A previous study argued that repetitive sequences would have indirect effects on flowering time due to their impact on genome size and might depend on the environment (17). 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 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 valuable information for breeding programs (31). Besides, these studies, in the last two decades, dissected the maize flowering time using different approaches such as linkage and association mapping (32–35), population genetics (36, 37), archaeological DNA studies (38), genome-wide association studies (22, 39–41), and gene analysis (42–45). 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 (31). 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. 6A, 6B, and S4), and the second considered the dominance effect (Fig. 6C, 6D, 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 just for the knob on the short arm of chromosome 9 (K9S) (Fig. 6). 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 lines and hybrids by -0.45 and -0.51 days, respectively. While on the dominance effect model, this effect was even more significant, decreasing flowering time in one and a half days (MF = -1.40 and FF = -1.53) (Fig. 7). Only the knob on chromosome 9 significantly reduced the flowering time in the inbred lines and hybrids. Decreasing in flowering time is more affected by the presence of knobs in the homozygous or heterozygous configuration. Our analysis also showed slight effects for each of the knobs on flowering time, albeit these were not significant (Table S1). These results showed that the knob composition was not essential to model the genotype effects on the trait. 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. 5). Since all hybrids are homozygous or heterozygous for the K9S, corroborating the specific contribution of this knob to decrease maize flowering time.

Especially in maize, the literature describes the hypothesis of a putative contribution of the heterochromatic knobs to the flowering time variation (23). Previously, studies had already indicated that gene groups responsible for the plant morphological and physiological traits would be correlated with the presence of knobs (11, 13). According to the authors, once close to these knobs, recombination in adjacent regions was suppressed, influencing such traits. More recently, through fluorescent *in*

situ hybridization (FISH), it was demonstrated that knobs are located in areas dense in genes where large knobs can reduce recombination locally (6). Comparisons between European maize genomes and US Corn Belt revealed variation in their repetitive sequences and gene content. The germplasms were separated by the intensity and position of knob regions. However, additional sites with small arrangements 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 (8). 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 (17, 22).

Flowering time-related QTLs have been found across the maize genome (31, 35, 38). In chromosome 9, flowering time-related QTLs were found, and some genes were identified (40, 46). The main gene located in chromosome 9 was ZmCCT9 (photoperiod sensitive) and mapped at the long arm, opposite the knob position. Other candidate genes were found along the chromosome, but the precise location was not defined yet (33, 47). In our study, the heterochromatic knob mapped at the short arm of chromosome 9 is strongly associated with flowering time. Constitutive heterochromatins have well-defined roles in the genomes, such as centromeres and telomeres (48, 49). That leads us to infer that heterochromatic knobs may also have a role in the maize genome, affecting certain phenotypic traits. However, further investigation in these regions will be needed to find possible candidate genes associated with knobs due to the inhibition of crossing over on their neighbourhood.

Concluding remarks. Despite more than 100 years of studies on maize genetics, the discussion on the role of knobs remains a current issue and has shown that this region is an essential fraction of the maize genome. Elimination dynamics of

components of the maize genome over successive self-fertilizations were reported (29). Among the five components analysed in the genome (gene content, ribosomal DNA, B chromosomes, TEs, and knobs), TEs were the most significantly lost components. The results 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 flowering time was shorter than at low altitudes, followed by a smaller genome (5, 16, 25). 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. The knob present in the short arm of chromosome 9 is associated with reducing the flowering time when homozygous and heterozygous. Our results suggest a role of the knobs in the flowering time different from those previously described.

Unravel the mystery behind the repetitive sequences has 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 and homozygous 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 expression. Furthermore, as knobs suppress local recombination (6), some genes would be associated with them.

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478 **Supplementary Materials:**

479 Materials and Methods
480 Supplementary text
481 Figures S1-S5
482 Table S1
483 External Databases S1-S2
484 References

Fig. 1. Representation of the knob conditions for chromosome 9 (K9S). (A) Absence knob; (B) heterozygous knob and (C) homozygous knob.

Fig. 2. 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 using a probe for the knob 180-bp repeat (green).

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Fig. 3. Flowering time and Genome Size of the hybrid and inbred lines. Boxplots

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compare mean values between male and female flowering time and genome size for

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inbred lines and hybrids. The flowering time showed in days to flowering and the

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genome size in picograms. The boxes indicate the first quartile (lower line), the second

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quartile or mean (central line), and the third quartile (upper line). Additionally, the

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whiskers represent the standard deviation with the dots as the outliers.

500

501 **Fig. 4. Flowering time among inbred line families and hybrids.** The violin plot
502 shows the FT distribution in days for all analyzed materials. The violin shape represents
503 the estimated value density of the trait within each group. Hybrid, n = 175; JD 1-3, n =
504 36; JD 2-1, n = 25; JD 4-4, n = 63.

Fig. 5. Comparison between dosage class of knobs for male and female flowering time, and genome size. The flowering time is expressed in days to flowering and genome size in picograms. The class means all possible combinations of presence and absence homozygous or heterozygous of the knobs considering only chromosomes 3, 5, 7, and 9.

Fig. 6. 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.

Fig. 7. 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. The y-axis shows the flowering days of female and male plants.

Table 1. Pearson's correlation between traits

	Male flowering	Genome size	Knob dosage classes
Female flowering	0.95	-0.14	-0.20
Male flowering		-0.17	-0.24
Genome size			-0.08

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	<i>P</i> -value	MAF	Effect (A or D)	h^2
MF	K9S	9	Short Arm	1.32×10^{-2}	0.17	-0.51 (A)	0.05
	K9S	9	Short Arm	4.21×10^{-3}	0.46	-1.41 (D)	0.15
FF	K9S	9	Short Arm	2.12×10^{-2}	0.17	-0.45 (A)	0.05
	K9S	9	Short Arm	3.75×10^{-3}	0.46	-1.53 (D)	0.02