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

97 higher mean genome size than inbred lines, showing 5477 Mbp (2C = 5.6 pg) and 5281
98 Mbp (2C = 5.4 pg), respectively (Fig. 3). However, no significant differences were
99 found. Even when the presence and absence of the knob were considered, no differences
100 were observed (Fig. S1). Each inbred line and hybrid can have different knob
101 configurations at the chromosomes K3L, K5L, K7S, and K9S. These combinations can
102 be homozygous for presence or absence in the lines or heterozygous in the hybrids (Fig.
103 1 and 2). These knob combinations were used to create dosage classes and test their
104 effects on genome size and flowering time (Fig. 4). Our results show knob dosages are
105 not enough to explain the genome size increase or decrease in the inbred lines and
106 hybrids. However, we observed that genome size varies broadly despite the same knob
107 composition. For instance, the hybrids presenting the highest (441311/2 x 441324/1 –
108 2C = 6.31 pg) and the smallest genome size (442213/1 x 441311/2 – 2C = 4.72 pg)
109 share the same knob constitution (Data S1). For inbred lines, the largest genome size
110 (131311/1-04 - 2C = 5.69 pg) has a lesser number of knobs than the shortest genome
111 (442213/1 - 2C = 4.65 pg), where we have homozygous knob presence for all the
112 chromosomes (Data S1). Therefore, no pattern was observed, which indicates that there
113 is probably no linear relationship between the number of knobs present in the maize
114 genome with the increase or decrease in the DNA content in our study. The genome size
115 associated with the heterochromatin percentage and knob numbers with flowering time
116 for the maize genome is given in some studies. Most of these studies have shown
117 positive correlations between genome size and knob content (*5*, *15*, *22*, *28*). A recent
118 study provided evidence that natural selection plays a substantial role in reducing the
119 maize genome size at high altitudes (*17*). The authors also showed that the abundance of
120 transposable elements (TEs) and heterochromatic knobs are significantly correlated with
121 altitude, and the knobs act as significant effect loci in the genome size. Unlike what they

122 found, our data does not show this same relationship. We performed an adapted
123 genome-wide association study (GWAS) with the heterochromatic knob full panel to
124 identify associations with genome size and flowering time, where the knobs were used
125 as our genetic markers (see Supplementary Materials). Our null hypothesis was that
126 there were significant differences in genome size and days for male and female
127 flowering due to the knob conditions in the K3L, K5L, K7S, and K9S positions. To test
128 our hypothesis, we used the inbred lines and hybrids panel, to which all knobs were
129 mapped (Fig. 2, Data S1). The panel has a matrix-*like* structure with different knob
130 combinations for presence (++ or 1) or absence (00 or -1) when homozygous and as
131 heterozygous when just one of the homologous has a knob (+0 or 0). Firstly, only the
132 allele substitution effect model was performed, and no significant association was found
133 between knobs and FT (Fig. S2). Moreover, this information was supported by a null
134 correlation between genome size and knob dosage classes (Table 1 and Fig. S3). Our
135 results also corroborate a survey showing no significant correlation between maize
136 population genome size and heterochromatin percentage. Even though larger genomes
137 exhibited the highest heterochromatin content (*16*); hence, all these observations lead us
138 to infer that maize genome size is independent of knob composition and might be
139 controlled genetically. Another hypothesis is that as our materials differ in the
140 composition of only four knob positions, genome size measurements by flow cytometry
141 would not detect slight differences in genome size due to knobs. Furthermore, we might
142 infer differences in the genome size among our inbred lines and hybrids result from
143 transposable element (TE) variability once the loss of TE during selfing has been
144 demonstrated (*29*).

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

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

159 **Relationship between flowering time and knob constitution.** We evaluated
160 days to MF and FF individually (Data S1) and estimated heritability to be 51% for MF
161 and 41% for FF. The mean values for days to MF and FF hybrids were 63 and 64 days,
162 respectively, while the flowering for inbred lines was 70 days to MF and 72 days to FF
163 (Fig. 3). Heterochromatic knob configurations as heterozygotes exhibited shorter
164 flowering times than those in homozygous states (Fig S1). The flowering time data also
165 were plotted, showing its amplitude inside the inbred line families and hybrids (Fig. 4).
166 We observed synchronicity for both traits within each group analysed, and hybrids had
167 shorter flowering time than inbred line families.

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

172 varying between 60 and 80 days. The JD 4-4 family had a smaller flowering time
173 distribution for FF and FM among families. The knob dosage class's analyses cannot
174 explain the flowering behaviour of all maize materials. It was possible to observe that
175 the mean values of dosage classes were very similar for MF and FF (Fig. 5). It was also
176 interesting to note that the inbred lines with only two knobs (dosage class = 2) flowered
177 later than those with other dosage classes (dosage class = 3-8). A previous study argued
178 that repetitive sequences would have indirect effects on flowering time due to their
179 impact on genome size and might depend on the environment (*17*). However, this effect
180 was not found in this study since there was no relationship between genome size and
181 knob composition (Table 1 and Fig S2).

182 The genetic architecture of flowering time in maize has been widely studied,
183 given that this trait reflects the plant adaptation to the environment. Since maize is
184 distributed throughout America and is adapted to a wide range of environments,
185 understanding how flowering time is regulated is of paramount importance and
186 generates valuable information for breeding programs (*31*). Besides, these studies, in
187 the last two decades, dissected the maize flowering time using different approaches such
188 as linkage and association mapping (*32–35*), population genetics (*36*, *37*),
189 archaeological DNA studies (*38*), genome-wide association studies (*22*, *39–41*), and
190 gene analysis (*42–45*). In inbred maize lines, the genetic architecture of flowering time
191 has been attributed to the cumulative effect of numerous quantitative trait loci (QTL),
192 each with a small impact on this trait (*31*). In the research described here, we used two
193 GWAS models to illustrate the interactions between flowering time and knob
194 conditions. The first model took into account only the allele substitution effect of the
195 markers (Fig. 6A, 6B, and S4), and the second considered the dominance effect (Fig.
196 6C, 6D, and S5). Our analysis showed only one significant marker-trait association for

197 both allele substitution and dominance effect models regarding flowering time. This
198 significant association was observed just for the knob on the short arm of chromosome
199 9 (K9S) (Fig. 6). Regardless of the GWAS model, the knob marker on chromosome 9
200 displayed the same performance concerning the flowering time, showing the *p*-value
201 highly significant (Table 2 and Table S1).

202 Regarding the allele substitution effect model, the presence of the K9S
203 decreased the MF and FF of the inbred lines and hybrids by -0.45 and -0.51 days,
204 respectively. While on the dominance effect model, this effect was even more
205 significant, decreasing flowering time in one and a half days (MF = -1.40 and FF = -
206 1.53) (Fig. 7). Only the knob on chromosome 9 significantly reduced the flowering time
207 in the inbred lines and hybrids. Decreasing in flowering time is more affected by the
208 presence of knobs in the homozygous or heterozygous configuration. Our analysis also
209 showed slight effects for each of the knobs on flowering time, albeit these were not
210 significant (Table S1). These results showed that the knob composition was not
211 essential to model the genotype effects on the trait. The heritability of the markers for
212 both models varied from 0.02 to 0.15 (Table 2). The flowering time violin plot also
213 supported the GWAS analyses (Fig. 5). Since all hybrids are homozygous or
214 heterozygous for the K9S, corroborating the specific contribution of this knob to
215 decrease maize flowering time.

216 Especially in maize, the literature describes the hypothesis of a putative
217 contribution of the heterochromatic knobs to the flowering time variation (*23*).
218 Previously, studies had already indicated that gene groups responsible for the plant
219 morphological and physiological traits would be correlated with the presence of knobs
220 (*11*, *13*). According to the authors, once close to these knobs, recombination in adjacent
221 regions was suppressed, influencing such traits. More recently, through fluorescent *in*

222 *situ* hybridization (FISH), it was demonstrated that knobs are located in areas dense in
223 genes where large knobs can reduce recombination locally (*6*). Comparisons between
224 European maize genomes and US Corn Belt revealed variation in their repetitive
225 sequences and gene content. The germplasms were separated by the intensity and
226 position of knob regions. However, additional sites with small arrangements of knobs
227 conserved in flint and dent lines were observed. This study also showed that the knob
228 sequences could affect genes surrounded by them, decreasing their expression level (*8*).
229 Besides, the functional role of the knobs has also been traced by the relationship
230 between their abundance, the genome size, and the flowering time, as shown above (*17*,
231 *22*).

232 Flowering time-related QTLs have been found across the maize genome (*31*, *35*,
233 *38*). In chromosome 9, flowering time-related QTLs were found, and some genes were
234 identified (*40*, *46*). The main gene located in chromosome 9 was ZmCCT9 (photoperiod
235 sensitive) and mapped at the long arm, opposite the knob position. Other candidate
236 genes were found along the chromosome, but the precise location was not defined yet
237 (*33*, *47*). In our study, the heterochromatic knob mapped at the short arm of
238 chromosome 9 is strongly associated with flowering time. Constitutive
239 heterochromatins have well-defined roles in the genomes, such as centromeres and
240 telomeres (*48*, *49*). That leads us to infer that heterochromatic knobs may also have a
241 role in the maize genome, affecting certain phenotypic traits. However, further
242 investigation in these regions will be needed to find possible candidate genes associated
243 with knobs due to the inhibition of crossing over on their neighbourhood.

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

247 components of the maize genome over successive self-fertilizations were reported (*29*).
248 Among the five components analysed in the genome (gene content, ribosomal DNA, B
249 chromosomes, TEs, and knobs), TEs were the most significantly lost components. The
250 results provide insights into the constitutive role played by knobs in the maize genome.
251 Since, like TEs, knobs are repetitive sequences, which make up about 10% of the maize
252 genome and, for unknown reasons, are not eliminated from it after generations of self-
253 fertilization. That strengthens the hypothesis that heterochromatic knobs may have a
254 functional role within the maize genome, even composed almost exclusively by
255 repetitive sequences.

256 Throughout the maize genome evolution, it was proposed that flowering time
257 was a trait influenced by changes in the genome size. At high altitudes, maize flowering
258 time was shorter than at low altitudes, followed by a smaller genome (*5*, *16*, *25*). At the
259 same time, most studies have also indicated a positive correlation between the genome
260 size and the knob abundance and that the heterochromatin content decreases along the
261 altitude clines. We found no significant association between knobs and genome size
262 from our data. The knob present in the short arm of chromosome 9 is associated with
263 reducing the flowering time when homozygous and heterozygous. Our results suggest a
264 role of the knobs in the flowering time different from those previously described.

265 Unravel the mystery behind the repetitive sequences has been hard work all over
266 the years. It becomes even more difficult for maize since its genome comprises more
267 than 85% of these sequences. For the first time, maize inbred lines were selected to have
268 knobs in specific locations, and their hybrids developed to carry heterozygous and
269 homozygous knobs. After that, the analyses of adapted GWAS were carried out,
270 showing the contribution of a single locus to the early maize flowering time. The maize
271 flowering time is a complex trait, and several studies have provided insights into its

272 genetic architecture. To this complexity, we add our data that suggest that gene-*like*
273 components, such as specific heterochromatic knobs, are capable of affecting the maize
274 flowering time, probably by interaction with particular genes playing a central role in
275 the regulation of the feature expression. Furthermore, as knobs suppress local
276 recombination (*6*), some genes would be associated with them.

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Supplementary Materials:

Materials and Methods
Supplementary text
Figures S1-S5
Table S1
External Databases S1-S2
References

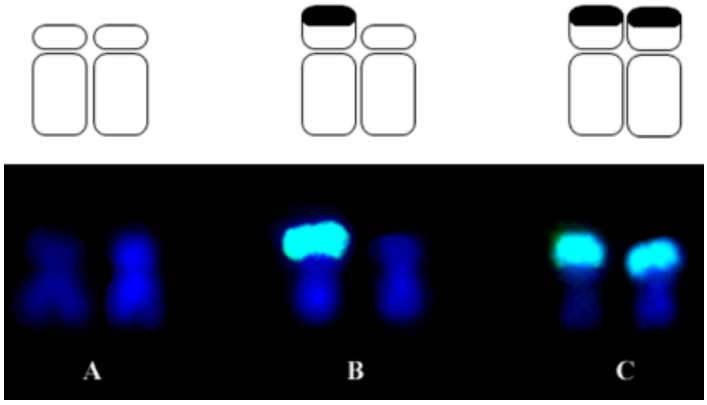


Fig. 1. Representation of the knob conditions for chromosome 9 (K9S). (A) Absence knob; **(B)** heterozygous knob and **(C)** homozygous knob.

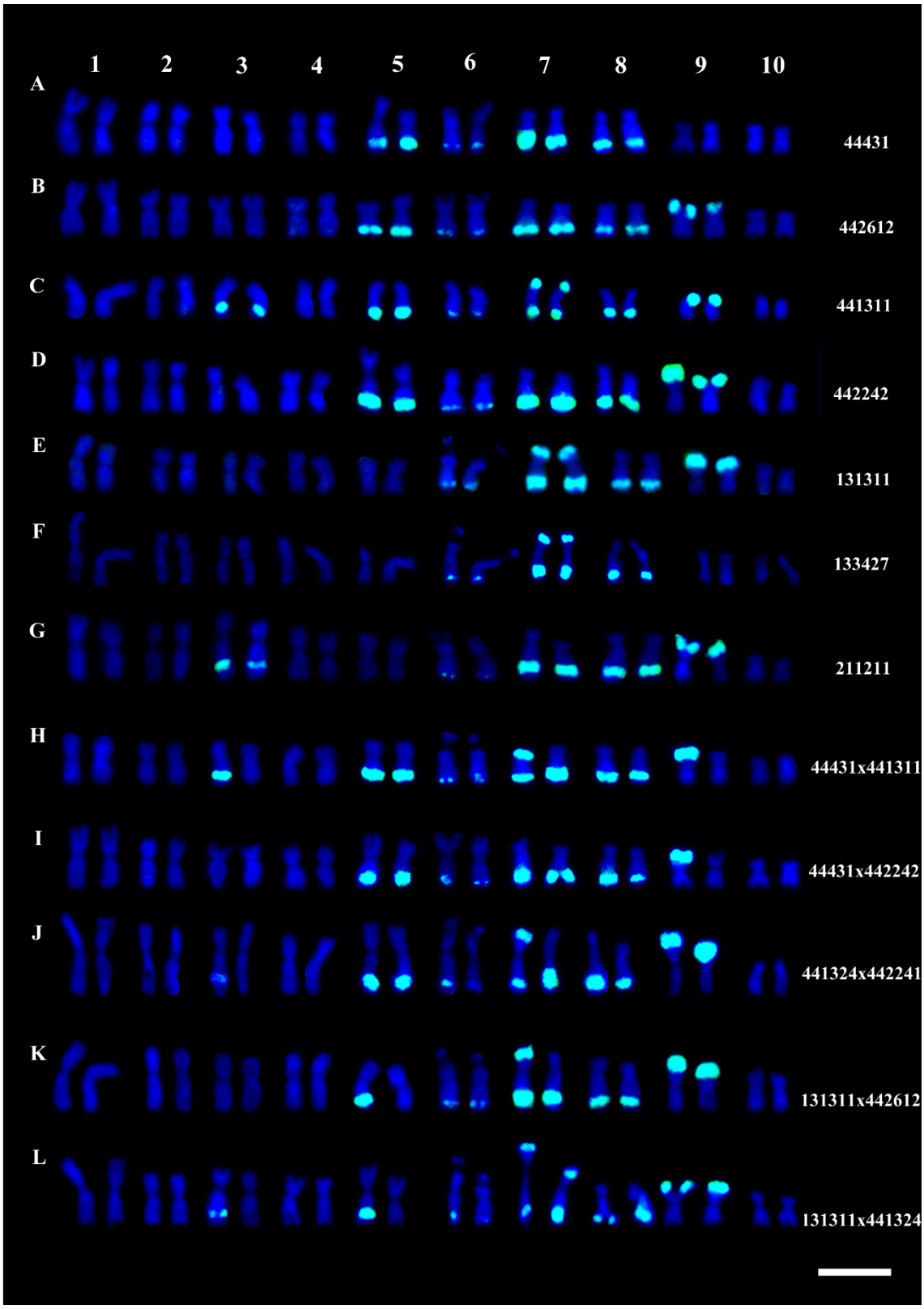


Fig. 2. A sampling of genotypes used in the experiments on flowering time and genome size. (A) – **(G)** Somatic karyotypes of JD lines; and **(H)** – **(L)** hybrids, labeled by FISH using a probe for the knob 180-bp repeat (green).

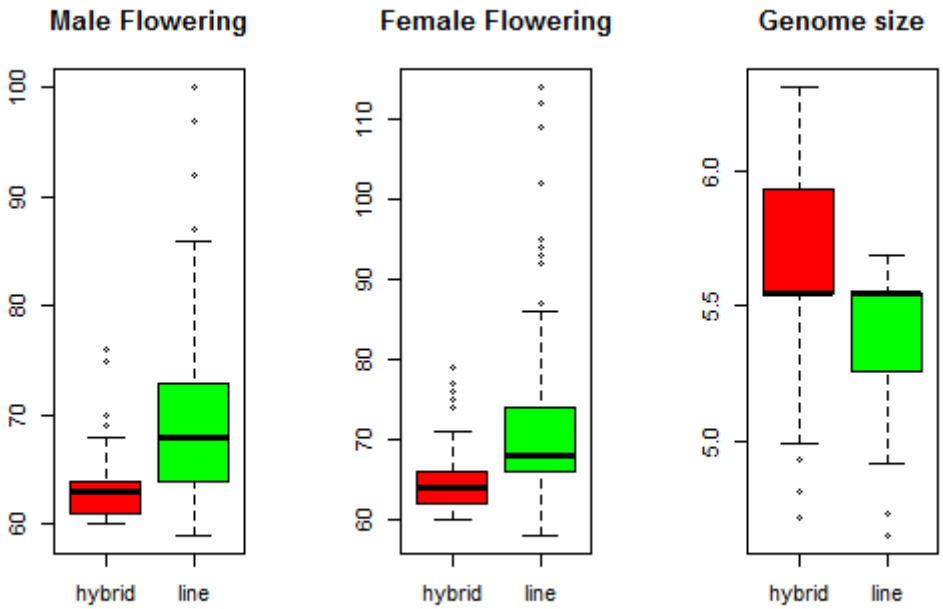


Fig. 3. Flowering time and Genome Size of the hybrid and inbred lines. Boxplots compare mean values between male and female flowering time and genome size for inbred lines and hybrids. The flowering time showed in days to flowering and the genome size in picograms. The boxes indicate the first quartile (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.

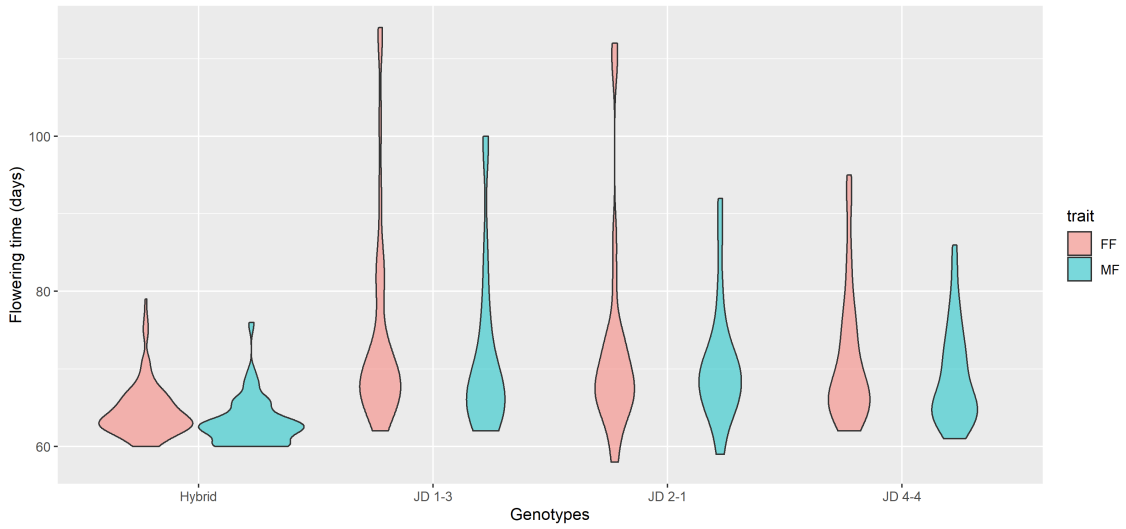
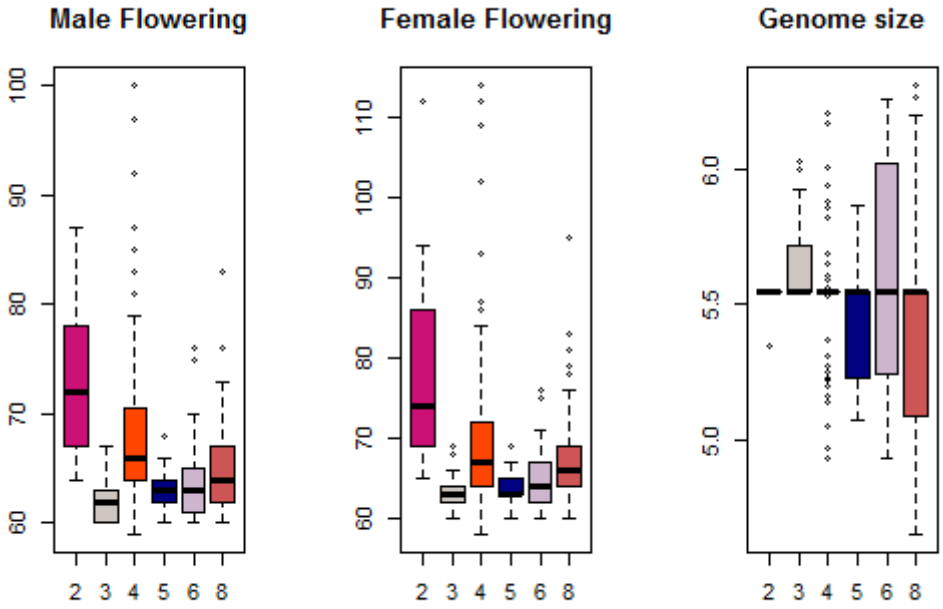


Fig. 4. Flowering time among inbred line families and hybrids. The violin plot shows the FT distribution in days for all analyzed materials. The violin shape represents the estimated value density of the trait within each group. Hybrid, n = 175; JD 1-3, n = 36; JD 2-1, n = 25; JD 4-4, n = 63.



505

506 **Fig. 5. Comparison between dosage class of knobs for male and female flowering**

507 **time, and genome size.** The flowering time is expressed in days to flowering and

508 genome size in picograms. The class means all possible combinations of presence and

509 absence homozygous or heterozygous of the knobs considering only chromosomes 3, 5,

510 7, and 9.

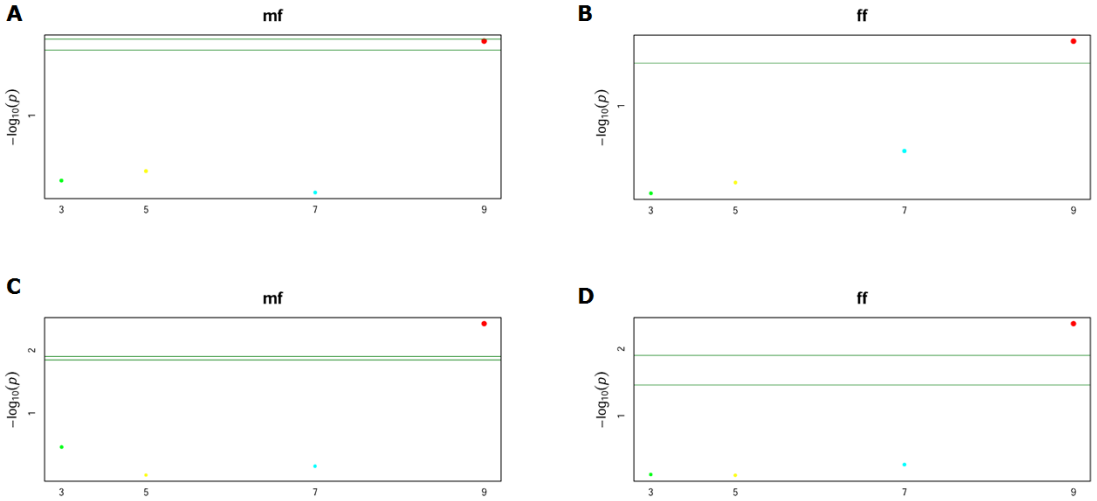


Fig. 6. Manhattan plots of GWAS for male (mf) and female (ff) flowering time using the knob positions (K3L, K5L, K7S, and K9S). (A) and **(B)** are plots showing the allele substitution effect model, **(C)** and **(D)** the dominance effect model, both with p-values < 0.05. The green lines are the significance threshold, a Bonferroni-corrected significance threshold used to identify significant associations.

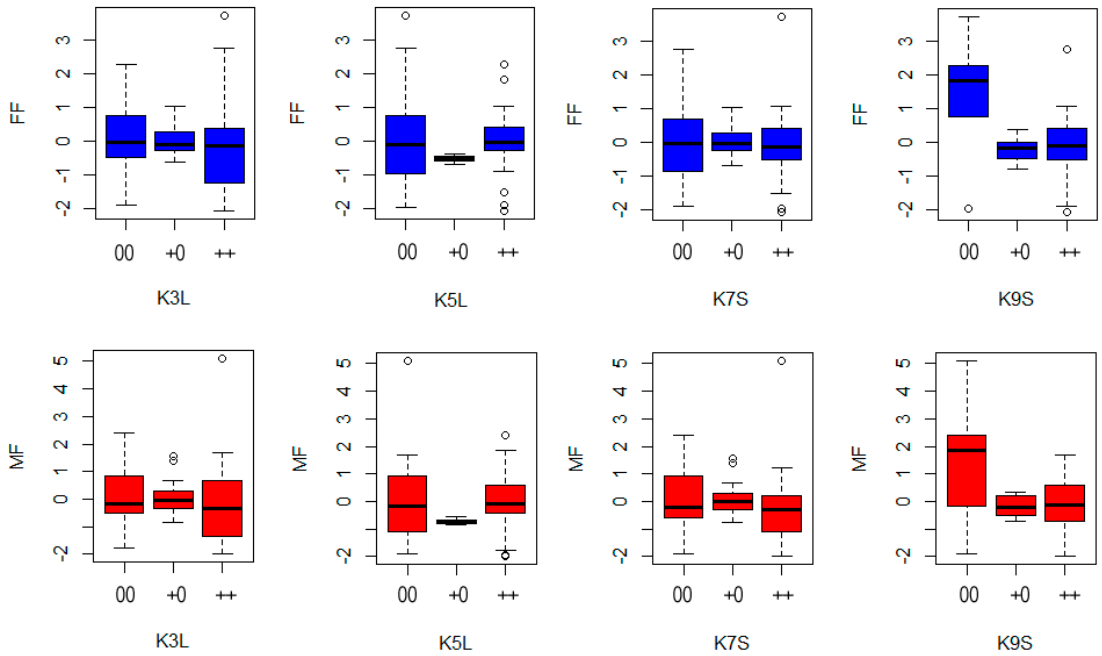


Fig. 7. Boxplots show male (MF) and female (FF) flowering time for each knob condition in the K3L, K5L, K7S, and K9S chromosomes, considering both allele substitution and dominance effects. The x-axis shows the classification for each condition 00 = knob absence; +0 = heterozygous knob presence and ++ = homozygous knob presence. The y-axis shows the flowering days of female and male plants.

523 **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

524

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

Trait	Marker	Chr.	Position	*P-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

529