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Chapter 5

Mediators of paramutation affect chromatin looping at the b1 locus

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To be submitted for publication
Abstract

B-I and B’ are tissue-specifically regulated epialleles involved in b1 paramutation in maize. B-I is high, and B’ low expressed. A hepta-repeat ~100kb upstream of the b1 transcription start site is required for high expression. Mutations in two Mediator of paramutation (mop) genes prevent paramutation. The B’ expression level in a mop2-1/Mop2 background is slightly down-regulated, while in mop3-1, it is up-regulated. 3C analyses on these mutants indicate that defined chromosome conformations are associated with a particular b1 expression level. In a mop2-1/Mop2 mutant background, the hepta-repeat interacts with the TSS region, giving rise to a single chromatin loop. This single loop is similar to the B’ conformation and is associated with low b1 expression. In a mop3-1 background, physical interactions occur between the TSS region, the hepta-repeat and regions ~15kb, ~47kb and ~107kb upstream. Such a B-I-like multi-loop structure mediates high b1 expression. FAIRE analysis on these mutants suggests that the occurrence of physical interactions appears not dependent on a particular chromatin state.

Introduction

Paramutation involves a heritable trans-interaction between two alleles, whereby one allele heritably changes the expression level of the other (Brink, 1956). This change in expression level is associated with a change in DNA methylation and/or chromatin structure, rather than with a change in DNA sequence, and is therefore epigenetic (reviewed in Chandler and Stam, 2004; Louwers et al., 2005; Stam and Mittelsten Scheid, 2005). One of the best studied paramutation phenomena is that at the b1 (booster1) locus. Several b1 alleles are known (Selinger and Chandler, 1999), but most of them do not participate in paramutation and are termed neutral alleles. At the b1 locus, paramutation occurs between B-I and B’ epialleles (Coe, 1966; Patterson et al., 1993; Stam et al., 2002a). B-I is expressed at a 10 to 20 times higher level than B’ (Patterson et al., 1993). When combined in one nucleus, the low expressing B’ epiallele changes (paramutates) the high expressing B-I epiallele into B’. This change in expression level is occurs at a 100% frequency and is heritable. The new B’ epiallele is fully capable of paramutating other B-I epialleles.

Multiple direct repeats of an 853bp sequence located ~100kb upstream of the b1 coding region are required for paramutation and for high expression. B’ and B-I contain seven copies of the 853 bp sequence (the hepta-repeat), while neutral b1 alleles contain only a single copy of this sequence (Stam et al., 2002b). Detailed
DNA methylation and Chromatin Immunoprecipitation (ChIP) analyses of the hepta-repeat indicated that the B’ epiallele is considered to be in an inactive state, carrying mostly silent chromatin marks, and B-I in an active state characterized by active histone marks (Haring et al., submitted). This epigenetic regulation is in agreement with the low and high expression levels of B’ and B-I, respectively. Furthermore, application of the Chromosome Conformation Capture technique (3C; Dekker et al., 2002; Tolhuis et al., 2002) indicated that the hepta-repeat physically interacts with the transcription start site (TSS) region in a tissue-specific manner (chapter 3). This interaction occurs in both B-I and B’, but the frequency of this interaction is higher in B-I than in B’. In high expressing B-I tissue, regions ~15kb, ~47kb and ~107 kb upstream interact with the TSS and hepta-repeat region as well: together, they form a multi-loop structure. This multi-loop structure has been postulated to mediate high b1 expression level (chapters 3 and 4). Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE; Giresi et al., 2007; Hogan et al., 2006) experiments suggested that nearly all regions involved in the multi-loop structure contain active regulatory sequences in B-I (chapter 3).

Paramutation is a complex epigenetic phenomenon. To unravel the underlying molecular mechanisms, mutants affecting paramutation are being studied. So far, three mediators of paramutation (mop) mutants in a B’ background have been isolated (Chandler, 2004). mop1-1 was the first mutant reported (Dorweiler et al., 2000). Map1 encodes an RNA-directed RNA polymerase (RDRP; Alleman et al., 2006) and is homologous to Arabidopsis RDR2 (Xie et al., 2004). Two other mutations have been isolated: the semidominant mop2-1 mutation (K. Kubo, J. Dorweiler and V. Chandler, unpublished results) and the recessive mop3-1 mutation (J. Dorweiler, D. Lisch and V. Chandler, unpublished results). Both mop mutations prevent paramutation at the b1 locus and alter the B’ expression level, but do not affect the epigenetic memory of the B’ allele. When the mop mutation is crossed out, the B’ expression level is restored and B’ can paramutate B-I again. The mop3-1 mutation prevents paramutation only as a homozygous mutation, mop2-1 already when heterozygous. Heterozygous mop2-1/Mop2 plants are weakly pigmented and B’ is expressed at an even lower level than in a wild-type background (Chandler, 2004; Fig. 1a). Homozygous mop3-1 plants are darkly pigmented and show a 6-8 times increase in b1 expression level (Chandler, 2004; Fig. 1a). Both mutants also affect the chromatin structure of the hepta-repeat: in mop2-1/Mop2 the B’ hepta-repeat carries silent, and in mop3-1 active histone marks (Haring et al., unpublished results).
Here we study the chromatin conformation of the $B'$ epiallele in the $mop2-1/Mop2$ and $mop3-1$ backgrounds to investigate the epigenetic regulation of $b1$ expression in these mutants. We investigate whether there is a correlation between the chromosome conformation of the $B'$ epiallele and its expression level in a mutant background, which is low in $mop2-1/Mop2$ and high in $mop3-1$. The 3C data in this chapter indicate that defined chromosome conformations are associated with a particular $b1$ expression level. FAIRE analysis suggests that the occurrence of physical interactions appears not dependent on a particular chromatin state.

![Figure 1. Features of the $b1$ and $Sam$ loci](image)

(a) RNA blot analyses of RNA isolated from husk tissue from $B'$, $B-I$, $B'mop2-1/Mop2$ and $B'mop3-1$ plants. The blots were hybridized with probes recognizing the coding region of $b1$ and $Sam$. The probe used for $b1$ recognized exons 7, 8 and 9 and the arrow left of the blot indicates the full-length $b1$ transcript. The probe used for $Sam$ is indicated in Fig. 1b by a black bar. The bar graph to the right of the blots shows the $b1$ transcript levels normalized to the $Sam$ transcript levels. The band-intensities of the full-length $b1$ and $Sam$ transcripts were quantified, the background signals subtracted and the $b1/Sam$ ratio calculated.

(b) Schematic representation of the $b1$ and $Sam$ loci. In the upper panel, the $b1$ transcription start site is indicated with a hooked arrow; the open box represents the $b1$ coding region, and the hepta-repeat is indicated with arrowheads. Distances are indicated in kb relative to the transcription start. The $BglII$ fragments analyzed by 3C are indicated with Roman numerals. Grey bars represent transposon and other repetitive sequences (Stam et al., 2002b). The $Sam$ locus in the lower panel was used to normalize the RNA, 3C and FAIRE data obtained for $b1$. The hooked arrow indicates the presumed $Sam$ transcription start site (in databases there is no consensus over the exact location). Keys: $B$ = $BglII$ sites; triangles = 3C primers.
Results

**Defined chromosome conformations associated with particular bI expression level**

Previous data indicated that the different epigenetic states of B-I and B’ gave rise to a multi- and single loop structure, respectively (chapter 3). We postulate that the multi-loop structure is required for high bI expression. To test this hypothesis, we applied 3C-qPCR technology (Hagege et al., 2007; Splinter et al., 2006) to low expressing B’mop2-1/Mop2 and high expressing B’mop3-1 husk tissue. The 3C experimental set up was the same as in chapters 2-4 of this thesis (for primers and TaqMan probes, see Table S2 in chapter 3). Figure 1b shows a schematic representation of the bI locus, indicating the TSS, the hepta-repeat located ~100kb upstream and the position of transposons and other repeated sequences. The 3C BglII fragments under study are represented by Roman numerals. The S-adenosyl methionine decarboxylase (Sam) locus (Fig. 1a) was used as an unrelated, internal control.

To investigate physical interactions between the transcription start site (TSS) region and the rest of the B’ epiallele in the mop mutants, we used fragment I, containing proximal bI promoter sequences and the transcription start (Fig. 1b), as a fixed fragment. In B’mop3-1 husk tissue, elevated interaction frequencies were observed between the TSS region and the hepta-repeat region (fragment X; Fig. 2a, turquoise line). High crosslinking frequencies were in addition observed between fragment I and fragments VII (~47kb upstream of the TSS) and XII (~107kb upstream) and possibly with fragment VII (~15kb upstream). Interestingly, the observed pattern and frequency of interactions in mop3-1 husk tissue is very similar to that observed in high expressing B-I husk tissue indicated in the same graph (Fig. 2a, purple line). In B’mop2-1/Mop2 husk tissue, elevated interaction frequencies were only detected between fragment I (TSS region) and fragment X, containing the hepta-repeat (Fig 2b; red line). This pattern of interaction frequencies is very similar to that in B’ husk tissue (Fig. 2b, green line) and different from that in B-I (purple line).
Figure 2. 3C-qPCR analyses demonstrate expression level-dependent chromatin looping of the $B'$ epiallele in mop3-1 and mop2-1/Mop2 mutant backgrounds.

Figure 2 includes data for $B$-I and $B'$ described in chapter 3 of this thesis. The $b$ locus is shown at the top of each graph (see also Fig. 1a). The X-axis shows the position in kb relative to the transcription start site (hooked arrow). The hepta-repeat is indicated with arrowheads. The position and size of the $BglII$ fragments analyzed is indicated by vertical gray shading and Roman numerals; black shading represents the fixed fragment. The Y-axis depicts relative crosslinking frequencies. Data were normalized against crosslinking frequencies measured for the $Sam$ locus. The highest, mean value of the normalized data for all experiments done with a specific fixed primer – TaqMan probe combination was set to 1. The data for $B'$ mop3-1 (panels a, c, e) and for $B'$ mop2-1/Mop2 (panels b, d, f) are indicated in turquoise and red, respectively. The data for $B$-I and $B'$ husk tissue are indicated in purple and green, respectively. Error bars indicate the standard error of mean of at least four different samples. Relative crosslinking frequencies are shown between (a,b) fixed fragment I (TSS region) and the rest of the $b$ locus; (c,d) fixed fragment X (containing hepta-repeat) and the rest of the $b$ locus; (e,f) fixed fragment VII (~47 kb upstream of the TSS) and the rest of the $b$ locus.
The 3C results on mop2-1/Mop2 and mop3-1 plants verify our hypothesis that the multi-loop structure mediates an elevated b1 expression level, and that the single chromatin loop is coupled to a low b1 expression level (chapter 3). Moreover, the 3C results on the mop2-1/Mop2 and mop3-1 mutants argue that the conformation of the b1 locus occurs independently of the epigenetic memory of the B’ allele, since that is not lost in a mop2-1/Mop2 and mop3-1 background. In addition, these results indicate that mediators of paramutation affect the conformation of the b1 locus.

Physical interactions are not dependent on particular chromatin state
Long-range interactions between DNA sequences are thought to be mediated by transcription factors binding to these sequences. FAIRE isolates nucleosome-depleted regions, which are enriched for active regulatory DNA sequences (Giresi et al., 2007; Hogan et al., 2006). At the b1 locus, enhancer activity has been assigned to the hepta-repeat (Stam et al., 2002b; Haring et al., submitted). 3C analysis indicated additional regions associated with high b1 expression and FAIRE analysis strongly suggested that these regions contained active chromatin in B-I (chapter 3).

Here we investigated the chromatin status of these and other sequences in high b1 expressing mop3-1 and low b1 expressing mop2-1/Mop2 mutants. The FAIRE primer sets used are indicated in Figure 3a (for primer sequences, see Table S3 in chapter 3). The Sam locus was used for validation of the FAIRE data (Fig. 3a).

FAIRE analysis on the B’ epiallele in both mutant mop backgrounds reveals enrichment of particular sites compared to wild-type B’, showing that mop2-1/Mop2 and mop3-1 also have an impact on nucleosome occupancy at selected sites of the b1 locus. For example, amplicons c (hepta-repeat), g and h (fragment VI) and i (fragment V) are enriched by FAIRE in B’ mop3-1 (Fig. 3b, turquoise bars) and B’ mop2-1/Mop2 (Fig. 3c, red bars), relative to B’ (green bars). Interestingly, these same sites are identified as potential regulatory DNA regions in B-I (purple bars).

In B’ mop2-1/Mop2, we did observe a discrepancy between the data for amplicon c obtained with FAIRE data and ChIP-qPCR using an antibody against histone H3. The ChIP data indicated higher nucleosome occupancy at the B’ hepta-repeat in B’ mop2-1/Mop2 than in B’ (M. Haring, unpublished results). FAIRE however suggests the opposite. A similar discrepancy for amplicon c existed between the FAIRE and ChIP data obtained for B’ mop1-1 and B’ (chapter 4). At present, it is unclear what could be the reason for this inconsistency.
To further investigate the spatial conformation of the B’ epiallele in a mop3-1 and mop2-1/Mop2 mutant background, experiments were carried out using other fixed fragments. Using the hepta-repeat fragment (X) on 3C templates derived from B’ mop3-1 husk tissue, elevated crosslinking frequencies were observed between fixed fragment X and fragments VII (~47kb upstream), IV (~15kb) and I (TSS region; Fig. 2c). As was seen with fixed fragment I (Fig. 2a), the interaction frequencies for B’ mop3-1 using X as fixed fragment are similar to the ones measured in high expressing B-I tissue (Fig. 2c). One difference is that the interaction with fragment IV is more pronounced in B’ mop3-1 tissue than in B-I tissue, when X is used as fixed fragment (Fig. 2c). Performing the same set of experiments on mop2-1/Mop2 husk tissue, a very different conformation of the b1 locus was observed. High interaction frequencies could only be observed between fragment X (fixed) and fragment I, the TSS region (Fig. 2d). In agreement with results obtained using fixed fragment I (Fig. 2b), the interaction pattern for B’ mop2-1/Mop2 is similar to that of B’ (indicated in the same graph, Fig. 2d). The results obtained with fragment X (Fig. 2c & d) confirm the 3C data acquired using fixed fragment I (Fig. 2a & b).

To examine the role of fragment VII in the interactions observed in B’ mop3-1 tissue, we used it as a fixed fragment on the 3C samples. In B’ mop3-1 husk tissue, high interaction frequencies were detected between fragment VII and fragments X and IV, and to a lesser extend with fragments I and XII (Fig. 2e). As perceived previously in Fig. 2a & c, the interaction pattern and frequencies for B’ mop3-1 husk are highly similar to the ones observed in B-I husk tissue (Fig. 2e). The interaction frequencies with fragment VII were not that elevated in B’ mop2-1/Mop2 compared to the ones in B’ mop3-1 (compare Fig. 2a & c with 2b & d, respectively). Therefore, as a negative control, we carried out the same experiment using VII as a fixed fragment in B’ mop2-1/Mop2 husk tissue. Interactions were observed with fragments IV, X and XII (Fig. 2f), but not as frequent as those observed for mop3-1 or B-I. The results for mop2-1/Mop2 with fixed fragment VII match the B’ results very well (Fig. 2f).

In conclusion, in high expressing B’ mop3-1 plants we observed a multi-loop structure involving the TSS, the hepta-repeat and regions ~15kb, ~47kb and ~107kb upstream of the TSS. A similar multi-loop structure has been observed in high expressing B-I husk tissue (chapter 3). In low expressing B’ mop2-1/Mop2 plants, the TSS region interacts with the hepta-repeat in a single chromatin loop. Such a single loop has also been observed in low expressing B’ tissue (chapter 3).
Figure 3. FAIRE enrichment at ~110kb chromatin domain in B’mop3-1 and B’mop2-1/Mop2 husk tissues.

Figure 3 includes data for B-I and B’ described in chapter 3 of this thesis.

(a) Schematic representation of the b1 and Sam locus, indicating the primer sets used for FAIRE with letters. See legend Fig. 1a for further details.

(b) Quantitative FAIRE analysis on B’mop3-1 husk tissue, and on B-I and B’ husk tissue for comparison. FAIRE values obtained for b1 were normalized against those measured for Sam using amplicon q. The highest value was set to 1. Error bars indicate the standard error of mean of four samples. Turquoise bars represent B’mop3-1 husk tissue, purple bars represent B-I husk tissue and green B’ husk tissue. Values that differ significantly between B’mop3-1 and B’ are indicated with one, two or three green asterisks, specifying a 90%, 95% and 99% confidence interval, respectively, in a two-tailed student’s t-test. In analogy, values that differ significantly between B’mop3-1 and B-I are indicated with one, two or three purple asterisks.

(c) Quantitative FAIRE analysis on B’mop2-1/Mop2 husk tissue, and on B-I and B’ husk tissue for comparison. Red bars represent B’mop2-1/Mop2 husk tissue. See (b) for further details.
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In B’mop3-1 husk tissue the b1 expression level is high and a B-I-like multi-loop structure involving fragments XII, X, VII and IV has been observed. In B-I husk, amplicons in these regions showed high FAIRE signals indicating the presence of active regulatory sequences. In high expressing B’mop3-1 however, these regions were not significantly FAIRE enriched compared to B’, except for amplicon c at the hepta-repeat region (X). The FAIRE data in this chapter indicate that MOP3 affects the b1 chromatin structure, but this mainly occurs at regions that are not involved in the formation of a multi-loop. Remarkably, the FAIRE enrichment pattern observed in B’mop2-1/Mop2 is very similar compared to that in B’mop3-1: in both mutants, amplicons in fragments X, VI and V are significantly enriched over B’. Despite this similarity, there is a large difference in expression level and chromosome conformation between B’mop2-1/Mop2 and B’mop3-1 plants. This suggests that the presence of active chromatin does not automatically result in DNA looping and elevated b1 expression or vice versa.

Discussion

Regulation of gene expression often involves physical interactions between promoters, enhancers and/or locus control elements (Carter et al., 2002; Spilianakis and Flavell, 2004; Tolhuis et al., 2002). At the maize b1 locus, the regulatory hepta-repeat has been shown to interact with the TSS region in low expressing B’ and high expressing B-I husk tissue. In B-I, besides the hepta-repeat, additional regions interact with the TSS region. Together they form a multi-loop structure and we hypothesized that the multi-loop structure is required to mediate high b1 expression (chapters 3 and 4). In this chapter, using the low b1 expressing mop2-1/Mop2 and the high b1 expressing mop3-1 mutant, we further tested and verified this hypothesis.

The 3C data on high b1 expressing B’mop3-1 husk tissue indicated multiple in cis interactions, involving the hepta-repeat, the TSS and regions ~15kb, ~47kb and ~107kb upstream of the TSS, resulting in the formation of a multi-loop structure (Fig. 4a). This structure is very similar to the chromosome conformation in high expressing B-I and intermediately expressing B’mop1-1 husk tissue, confirming our hypothesis that such a multi-loop structure is associated with elevated b1 expression.
Figure 4. Chromatin looping and expression of $B'$ in the mop3-1 and mop2-1/Mop2 mutants: a model. 
(a) The $B'$ epiallele in a mop3-1 mutant background forms a multi-loop structure as previously observed at the $B$-I epiallele. In mop3-1, the enhancer function of the hepta-repeat is activated by binding of a transcription factor and various enhancer proteins. The activated enhancer, as well as sequences ~107kb, ~47kb and ~15kb upstream of the TSS, interact with the TSS region, resulting in the formation of a $B$-I like multi-loop structure that mediates high $b1$ expression levels.
(b) In a mop2-1/Mop2 mutant background, the $B'$ hepta-repeat interacts with the TSS region, forming a single loop structure associated with low $b1$ expression. A similar single loop is also observed in a wild-type background.

(Chapters 3 and 4). In low expressing $B'mop2-1/Mop2$ husk tissue, the hepta-repeat region interacts with the TSS region forming a single chromatin loop, similar to the conformation of the $B'$ epiallele (Fig. 4b). This validates our hypothesis that the single chromatin loop mediates low $b1$ expression and is not sufficient to trigger high expression (Chapter 3). The 3C results in this chapter strengthen our previously proposed model for epigenetic regulation of $b1$ expression (Haring et al., submitted; Louwers et al., Chapter 3). In this model, a putative tissue-specific transcription factor mediates chromatin looping between the hepta-repeat and the TSS in husk tissue. Additional interactions with other regions throughout the ~110kb $b1$ chromatin domain stabilize the interaction between the hepta-repeat and the TSS region, resulting in a high expression level. This model is in line with previously described looping models in which upstream regulatory sequences physically
interact with a promoter (Liu and Garrard, 2005; Spilianakis and Flavell, 2004; Tolhuis et al., 2002; Vernimmen et al., 2007). In the Active Chromatin Hub (ACH) model proposed for the β-globin locus, several hypersensitive sites cluster together and interact with active globin genes (deLaat and Grosveld, 2003; Tolhuis et al., 2002).

The data obtained for the mop2-1/Mop2 and mop3-1 mutants show that expression level, paramutagenicity and epigenetic memory of the B’ allele are three different aspects that can be separated from each other (see also chapters 1 and 6). First, both the mop2-1/Mop2 and mop3-1 mutants have lost the ability to paramutate B-I alleles (Chandler, 2004). Second, each mutant affects the B’ expression level in a very different manner: in mop2-1/Mop2 the expression level is down-regulated, in mop3-1 up-regulated. This difference in expression level is associated with different chromosome conformations. Third, the B’ epigenetic memory is retained in both mutant backgrounds. When the mutation is crossed out, the B’ epiallele behave again like B’. The epigenetic memory has been proposed to be determined by DNA hypermethylation at the hepta-repeat (Haring et al., submitted; Louwers et al., chapter 4). DNA methylation data on mop2-1/Mop2 and mop3-1 are consistent with this (R. Bader and M. Stam, unpublished results); the B’ hepta-repeat in a mop2-1/Mop2 or mop3-1 background still carries DNA methylation. Together, this indicates that chromosome looping is tightly associated with the expression level of B’ and independent of the epigenetic mark determining the heritability of the B’ epiallele.

In high expressing B-I tissue, the regions involved in the multi-loop structure are indicated to contain active regulatory sequences (chapter 3), suggesting that regulatory proteins, such as transcription factors, enhancer complexes and histone acetyltransferases, interact with these regions. We hypothesized that the multi-loop structure results in the local concentration of regulatory proteins that trigger the high b1 expression level in B-I. FAIRE data on mop1 mutants was in line with this (chapter 4). In B’ mop1-1 tissue, in the regions involved in the multi-loop structure, more features of an active chromatin state were present than in B’Mop1 tissue, but less than in B-I tissue (chapter 4). This intermediately active chromatin state is associated with an intermediate b1 expression level in B’ mop1-1 husk tissue. The FAIRE data obtained for the B’ allele in mop2-1/Mop2 and mop3-1 mutants however do not correlate well with the 3C data obtained for these mutants. In mop3-1 plants 3C fragments ~107kb, ~47kb and ~15kb upstream physically interact with the TSS. Amplicons within these fragments showed however no significant FAIRE
enrichment in high $b1$ expressing $\textit{mop}3$-1 compared to low $b1$ expressing wild-type plants. Strikingly, $B'\text{mop}2$-1/$\text{Mop}2$ and $B'\text{mop}3$-1 husk tissue gave rise to comparable patterns of FAIRE enrichment (Fig. 3), whereas the effect of the $\textit{mop}2$-1/$\text{Mop}$2 and $\textit{mop}3$-1 mutations on the $b1$ chromatin structure, chromatin looping and resulting $B'$ expression level is very different (data in this chapter, and M. Haring, R. Bader and M. Stam, unpublished results). In $\textit{mop}2$-1/$\text{Mop}$2, the $B'$ epiallele displays a silent chromatin state at the hepta-repeat, forms a single chromatin loop and is expressed at a very low level, while in $\textit{mop}3$-1, the $B'$ epiallele shows an active chromatin state at the hepta-repeat, forms a multi-loop and is expressed at a high level. Our FAIRE data however suggest that at the amplicons tested, the FAIRE-selected chromatin feature is similarly affected in $\textit{mop}2$-1/$\text{Mop}$2 and $\textit{mop}3$-1 plants.

$\text{MOP2}$ and $\text{MOP3}$ both prevent paramutation, indicating they act in a similar pathway. We have observed a very different nucleosome density at the $B'$ hepta-repeat in $\textit{mop}2$-1/$\text{Mop}$2 and $\textit{mop}3$-1 plants, but cannot exclude that the nucleosome density of the other regions tested in 3C and FAIRE is similarly affected by the two mutations. Studies on the role of DNA methylation and histone modifications in the epigenetic regulation of numerous genomic loci have shown that the interplay between the various proteins involved varies on the sequence looked at (Lippman et al., 2003; Lister et al., 2008; Penterman et al., 2007; Tran et al., 2005). Possibly, the chromatin structure at the $B'$ hepta-repeat is regulated by similar, but also by different proteins than the chromatin structure at most of the other regions examined within the $b1$ locus.

In order to get a better understanding of the findings described in this chapter it will be necessary to characterize the various regions within the 110 kb $b1$ locus in more detail. DNA methylation, nuclease sensitivity and ChIP-qPCR assays will indicate the chromatin structure of these regions. 3C experiments using another restriction enzyme will enable finemapping of the regions involved in the multi-loop structure, while transgenic experiments will be helpful to examine the function of the various sequence regions. Furthermore, cloning and identification of the $\text{Mop}2$ and $\text{Mop}3$ gene products is required to put all data into perspective. The identification of proteins binding to the regions enriched by FAIRE will also provide more insight into the epigenetic regulation of the $B'$ epiallele.
Acknowledgements

We would like to thank Vicki Chandler and Luyda Sidorenko for providing seeds. Vicki Chandler is thanked for sharing unpublished data. We are grateful to Wouter de Laat for helpful discussions on the results and for critical reading. Ludek Tikovsky, Harold Lemereis and Thijs Hendrix are thanked for taking excellent care of the maize plants.

Materials and Methods

Plant stocks and tissues
The plant stocks containing the \(b_1\) alleles examined (\(B', B'mop2-1/Mop2, B'mop3-1\) and \(B-I\)) were obtained from V.L. Chandler (University of Arizona, Tucson, USA) and were grown in a greenhouse. All plants had dominant functional alleles for the anthocyanin biosynthetic genes required in vegetative plant tissues. To obtain homozygous \(mop3-1\) mutant plants, \(B'mop3-1/mop3-1\) plants were crossed to \(B'mop3-1/Mop3\) plants, resulting 50% homozygous and 50% heterozygous \(mop3-1\) plants. The homozygous plants were identified by their dark pigmentation. Heterozygous \(mop2/Mop2\) plants were the F1 progeny of a cross between wild-type and homozygous \(mop2-1\) mutant plants. For husk tissue we used the leaves surrounding the maize corncob, whereby the tough, outer leaves were discarded. Depending on the amount of daylight, husks were harvested when the plants were between two and three months old, before silks appeared; the actual corncobs were between three and six cm long.

RNA blot analysis
RNA isolation and blot analysis were performed according to the method described in chapter 3 of this thesis.

Chromosome Conformation Capture
3C analysis was basically performed according to the method described in (Hagege et al., 2007), but with plant-specific adjustments (Louwers et al., chapter 2 of this thesis). For implementation of 3C at the \(b_1\) locus, see chapter 3.

FAIRE analysis
FAIRE analysis was basically performed as described in (Hogan et al., 2006), but with plant-specific adjustments. For implementation of FAIRE at the \(b_1\) locus, see chapter 3 of this thesis.

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