CHROMATIN–LEVEL REGULATION OF THE MAIZE *PURPLE PLANT1* GENE.

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by
KYUNGJU SHIN

Dr. Karen Cone, Dissertation Supervisor
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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

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presented by Kyungju Shin

a candidate for the degree of Doctor of Philosophy

and hereby certify that in their opinion it is worthy of acceptance

Karen Cone

Emmanuel Liscum

James Birchler

Kathleen Newton

Michael McMullen

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CHROMATIN–LEVEL REGULATION OF THE MAIZE *PURPLE PLANT1* GENE

Kyungju Shin

Dr. Karen Cone, Dissertation Supervisor

ABSTRACT

In all eukaryotes, gene expression is regulated at chromatin level by mechanisms that are termed "epigenetic". Actively expressed genes tend to be associated with loosely packed chromatin, and inactive genes tend to be associated with tightly packed chromatin that prevents access by the transcriptional machinery. The basic unit of chromatin packaging is the nucleosome, composed of a core of positively charged histone molecules wrapped nearly twice by DNA. In order for a gene to be expressed, chromatin must be altered to weaken DNA and nucleosome interaction or to move nucleosomes away from the binding site for the transcriptional machinery so that transcription can occur. Changes in chromatin structure are commonly accompanied by covalent modifications to histones, which helps impose or reinforce transcriptional states, and by chromatin remodeling complexes, which use ATP energy to reposition nuclesomes. Exactly how these changes in chromatin structure lead to changes in gene expression is not well understood. The goal of my research was to address some of the unanswered questions about chromatin level regulation.

In maize, *Pl1-Blotched* is a stable epiallele of *purple plant1 (pl1)* that leads to a variegated pattern of anthocyanin pigmentation that contrasts markedly with the uniformly purple pigmentation seen in plants with the *Pl1-Rhoades* allele. However, the amount of pigmentation in plants with *Pl1-Blotched* is increased in the presence of a modifier called *Suppressor of plant blotching* (*Spb*). At the molecular level, the phenotypic effect of *Spb* is due to increased *Pl1-Blotched* mRNA levels and

correspondingly higher levels of mRNAs for the structural genes encoding the anthocyanin biosynthetic enzymes. In addition, *Spb* leads to changes in the pattern of DNA methylation at several methylation sensitive restriction sites in *Pl1-Blotched*. These observations led us to hypothesize that *Spb* increases *Pl1-Blotched* expression epigenetically by altering the chromatin structure of the gene.

To evaluate this hypothesis, DNase I sensitivity and chromatin immunoprecipitation (ChIP) assays were used for pair-wise comparisons of *Pl1-Rhoades vs*. *Pl1-Blotched,* and *Pl1-Blotched* in the presence *vs.* absence of *Spb*.

Results of the first comparison showed that the highly expressed *Pl1-Rhoades* allele was more sensitive to DNase I digestion and had more histone modifications typical of active genes, such as H3K4me3 and H4 tetra-ac, than in *Pl1-Blotched*. These observations are consistent with the interpretation that expression differences between these alleles are due to epigenetic influences on chromatin structure, including histone modifications.

Results of the second comparison showed that *Pl1-Blotched* in the presence of *Spb* was more sensitive to DNase I digestion than in the absence of *Spb*. Results of ChIP assays showed that H4 tetra-ac was more enriched at downstream regions of *pl1* gene in *Pl1-Blotched* in the presence than absence of *Spb*. Because histone acetylation is usually associated with loosely packed chromatin, this result indicates that chromatin structure of the more acetylated downstream region of *pl1* gene is more relaxed than the upstream region. This suggests that the downstream of *pl1* may be involved in gene regulation, although the mechanism for such regulation is not yet clear. Except for H4 tetra-ac modification, *Spb* did not have a significant effect on other modification of histones associated with *Pl1-Blotched* chromatin; instead, *Spb* led to a change in nucleosome occupancy, especially at the downstream region of *Pl1-Blotched*. These results suggest that *Spb* may increase *Pl1-Blotched* expression by chromatin

remodeling, leading to a more relaxed structure that is more accessible to the transcriptional machinery.

To discover the gene(s) responsible for the *Spb* effect on *Pl1-Blotched*, I conducted QTL mapping. This result confirmed previous genetic analysis indicating that *Spb* is not a single gene. Four main effect QTLs were detected with significant effects on *Pl1-Blotched* pigmentation and these loci also showed that the major effects were due to epistatic gene interactions.

In summary, the results of my research have led to important insights on the chromatin level control of a maize regulatory gene, and have provided a launching point for future studies aimed at evaluating candidate chromatin genes to discover the identity of the epigenetic modifier, *Spb*.

CHAPTER 1. INTRODUCTION

Epigenetic Gene Regulation and Chromatin Organization

In eukaryotes, including plants, chromatin-level regulation is responsible for many non-Mendelian types of inheritance, such as paramutation, transgene silencing, transposon activity, gametic imprinting and nucleolar dominance (reviewed in Henikoff and Matzke, 1997; Hollick et al., 1997). Chromatin configuration is also critically important for many processes during growth and development of the plant, including cellular differentiation, vegetative phase changes, flowering and development of embryo and endosperm (reviewed in He et al., 2003; Wagner, 2003; Ausin et al., 2004). Changes in chromatin can be mitotically, and sometimes meiotically, heritable, serving as a mechanism for the plant to remember environmental or cellular cues inducing gene expression changes (Klar, 1998; Murfett et al., 2001; Probst et al., 2004). In some case, the memories are passed–as altered chromatin states–to future generations. Such heritable changes in gene expression, which occur in the absence of changes in nucleotide sequence, are termed epigenetic (Wolffe and Matzke, 1999).

Chromatin Structure

Chromatin is a complex of DNA and proteins. The basic molecular repeating unit of chromatin is a nucleosome, in which 146-147 bp of DNA is wrapped nearly twice around an octamer containing two molecules of each histones H2A, H2B, H3 and H4. The linker histone H1 that separates adjacent core particles is required for folding to higher-order chromatin structure. The core histones, especially histone H3 and H4, are well conserved between plant and animal systems, whereas the linker histone H1 is less conserved (van Holde et al., 1995; Goodrich and Tweedie, 2002).

Chromatin packaging is dynamic. Tightly packed chromatin physically restricts access to DNA by the transcriptional machinery, leading to transcriptional inactivity

(Struhl, 1999). By contrast, loosely packed chromatin is more accessible to the transcriptional apparatus, leading to active gene expression (Farkas et al., 2000). Conversion between these alternate chromatin states is controlled by two basic processes. The first is chromatin remodeling, in which interactions between DNA and histones are altered by energy derived from ATP hydrolysis. Changes in chromatin structure by chromatin remodeling include nucleosome sliding to a new position on the genomic DNA, conformational changes in histone-DNA interactions, and dynamic loss and gain of histones through nucleosome disassembly and reassembly (Kwon and Wagner, 2007). The second mechanism influencing the packaging of chromatin is covalent modification of histone proteins and DNA. These mechanisms are reviewed briefly in the following sections.

Chromatin Remodeling

Chromatin remodeling is defined as any event that alters the nuclease sensitivity of a region of chromatin to digestion by small nucleases like DNase I or micrococcal nuclease (Aalfs and Kingston, 2000). Increased nuclease sensitivity can occur from absence of nucleosomes, rearrangement of nucleosome position, or creation of an otherwise more accessible conformation of nucleosome. Mechanisms driving these changes can vary in the extent and duration of their effect. For example, some remodeling mechanisms act locally on a single nucleosome and others act more broadly (Eberharter and Becker, 2002). Temporally, some mechanisms have transient effects, whereas others have heritable effects (Turner, 2002; Sims III et al., 2003).

Chromatin remodeling complexes are ATP-dependent and functionally very conserved from yeast to human (Gendrel et al., 2002; Goodrich and Tweedie, 2002; Narlikar et al., 2002; Wagner, 2003; Kwon and Wagner, 2007). These complexes can be divided into several classes based on their core ATPase subunits (Clapier et al., 2001). The yeast SWI/SNF (Switch/Sucrose Non-Fermenting) and RSC (Remodels the

structure of chromatin) proteins contain SNF2-SWI2 type ATPases. The CHD ATPase (chromodomain-Helicase-ATPase-DNA-Binding) group includes the mouse CHD1 protein and the Mi-2 protein found in the NuRD (Nucleosome remodeling and deacetylation) complex. The ISWI (Imitation switch) ATPase is present in another group of complexes, including *Drosophila* NURF (Nucleosome remodeling factor), ACF (the ATP utilizing chromatin assembly and remodeling factor), and CHRAC (the chromatin accessibility complex). In *Arabidopsis,* DDM1 (Decreased in DNA Methylation1) represents another group, which lacks the COOH-terminal SANT (SWI3, ADA2, N-CoR, TFIIIB) domain found in ISWI, and the AT hook domain (AT rich DNA binding domain) found in SWI2/SNF2. The function of all of these complexes is the same; all are able to induce nucleosome sliding on DNA (Clapier et al., 2001).

Chromatin modification

Both the core histones and DNA can serve as substrates for covalent modifications. Lysines and arginines in the unstructured amino-terminal tails of histones are targets for various types of modification, such as acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation, sumoylation, deimination, and proline isomerization (Grunstein, 1997; Jenuwein and Allis, 2001; Eberharter and Becker, 2002). In plants, DNA is modified by cytosine methylation in multiple sequence contexts: symmetric (on both strands) in CG and CNG contests, or asymmetric in the CNN context (Lindroth et al., 2001; Jackson et al., 2002; Tariq et al., 2003). Histone modifications can change chromatin structure at two basic levels. First, specific modifications have been shown to serve as binding sites for proteins involved in silencing or activation (Asifa et al., 2000; Jacobson et al., 2000; Hassan et al., 2002; Jacobs and Khorasanizadeh, 2002; Kanno et al., 2004; Chua et al., 2005). Second, histone modification can change the interaction between DNA and the histone octamer, influencing the sliding or

movement of nucleosomes and thereby affecting gene activity (Grunstein, 1997; Turner, 2000; Lusser et al., 2001; Eberharter and Becker, 2002).

On histones, combinational or sequential modifications have been proposed to constitute a histone code, which is read by distinct sets of proteins that bring about downstream responses that change gene expression (Strahl and Allis, 2000; Jenuwein and Allis, 2001). The most intensively studied histone modifications are methylation and acetylation on lysines in histones H3 and H4. Highlights of these studies are presented below.

Histone Methylation

Methylation of lysines in histones is catalyzed by a group of enzymes called histone methyl transferases (HMTases). Most of these proteins have a conserved structure composed of a SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain flanked by cysteine-rich (PRE-SET and POST-SET) domains (Goodrich and Tweedie, 2002). Methylation occurs at the ε-amino group of conserved lysine residues on the N-terminal tails of core histones, and up to three methyl groups can be added. The degree of methylation, such as mono-, di-, and tri- methylation, appears to have a different biological function, thus providing for complex layers of gene regulation (Rea et al., 2000; Rice and Allis, 2001).

Distinct patterns of histone lysine methylation are thought to mark chromatin for downstream events that regulate the activity of single genes or large chromosomal regions (Sims III et al., 2003). Maintenance of trancriptionally active chromatin depends on methylation of lysine 4 on histone H3 (Noma and Grewal, 2002). The nomenclature for histone modification includes: histone, residue and position of that residue in the protein, followed by type and number of modifications to that residue. So, mono-methylation of histone H3 on lysine 4 would be designated H3K4me1 (Turner, 2005).

The association of di-and tri-methylation of H3K4 (H3K4me2 and H3K4me3) with gene activation is universal from yeast to human, including plants (Santos-Rosa, 2002; Sakamoto et al., 2004; Alvarez-Venegas and Zoya, 2005; Barski et al., 2007). For example, in *Saccharomyces cerevisiae*, the Set1 protein can catalyze di- and tri-methylation of H3K4. Both H3K4me2 and H3K4me3 are found in euchromatin, but not in silent heterochromatic regions. Within euchromatin, H3K4me2 is found in both active and inactive genes, whereas H3K4me3 is present exclusively in active genes. Thus, the presence of H3K4me3 defines an active state of gene expression (Bernstein et al., 2002; Santos-Rosa, 2002).

Methylation of histone H3 K9 (H3K9me) is associated with transcriptionally inactive chromatin (Jenuwein and Allis, 2001), especially in heterochromatic areas, such as centromeric and pericentromeric regions that contain tandemly repeated sequences. In fission yeast (*Schizosaccharomyces pombe*), heterochromatin formation involves a complex set of steps that begins with removal of acetylation at histone H3K14 by the *cryptic loci regulator3* (*clr3*) gene product. Then a HMTase, encoded by *clr4,* preferentially methylates H3K9. This modification results in binding of *Swi6* protein, a homolog of *Drosophila* heterochromatin protein1 (HP1), which helps to propagate chromatin condensation (Nakayama et al., 2001).

Although histone H3K9 methylation is widely viewed as a mark of silent genes in heterochromatin (Lachner et al., 2001; Houben et al., 2003; Lindroth et al., 2004; Naumann et al., 2005), this modification can occur in active, euchromatic genes, as well. In *Drosophila*, the discs absent, small, or homeotic-1 (ASH1) protein methylates histone H3K9, in addition to H3K4 and H4K20, and the combination of three methylated histone residues activates gene expression (Beisel et al., 2002). In humans, the physical association between a HMTase, which methylates at both histone H3K4 and histone H3K9 (Lachner and Jenuwein, 2002), and the histone acetyltransferase p300/CBP,

which activates gene expression, suggests that H3K9 methylation might not be restricted solely to inactive genes (Vandel and Trouche, 2001). In maize, immunological staining of chromosomes with an antibody against H3K9me2 showed that this modification occurs in euchromatin, not in the heterochromatin associated with knobs or pericentromeres. In fact, H3K9me2 staining was seen in the same chromosome arm regions as H3K4me2 staining, although the signals did not precisely overlap (Shi and Dawe, 2006).

H3K27 methylation has also been associated with silent genes in multiple organisms. H3K27 methylation occurs in conjunction with polycomb-group silencing of *hox* genes in *Drosophila* (Cao et al., 2002) and with X-inactivation in humans (Plath et al., 2003). These modifications are catalyzed by related protein complexes: ESC-E(Z) in *Drosophila* and EED-EZH2 in humans. H3K27 also occurs in plants. In *Arabidopsis thaliana*, H3K27 methylation is associated with silencing in euchromatic genes and in heterochromatin (Lindroth et al., 2001). In maize, immunostaining of chromosomes revealed this modification is present in a few focused euchromatic domains, although whether it is associated with transcriptional silencing in maize is not yet known (Shi and Dawe, 2006).

H4K20 methylation has traditionally been associated with inactive chromatin in animal systems; however, recent studies show that the chromosomal distribution of modified H4K20 depends on the number of methyl groups added to amino acid. For example, in *Drosophila* polytene chromosomes, H3K20me3 is found in condensed regions (Ebert et al., 2006) In humans, this modification is also associated with silent heterochromatic regions, but H4K20me1 is found near transcription start sites of active genes (Barski et al., 2007). In *Arabidopsis*, H4K20me1 is heterochromatic mark, whereas H4K20me2 and H4K20me3 are euchromatic (Fischer et al., 2006) and in maize, H4K20 methylation is low or absent (Shi and Dawe, 2006).

Histone Acetylation

Acetylation of histones is one of the most abundant and dynamic modifications, with the actual level of histone acetylation dependent on regulated interplay between histone acetyltransferases (HATs) and histone deacetylases (HDACs; Turner, 2002). Proper acetylation and deacetylation of core histones is critically important for many cellular processes, including nucleosome assembly (Grunstein, 1997; Johnson et al., 1998), higher-order packaging of chromosomes (Turner, 2000), and transcriptional regulation (Chua et al., 2001; Lusser et al., 2001).

Acetylation occurs on the ε-amino group of conserved lysine residues in the N-terminal regions of each histone. This kind of modification has two possible effects on the transcriptional activity of a gene. First, histone acetylation reduces the affinity of the histone for DNA by abrogating the positive charge of lysine residues; this leads to relaxation of the nucleosomal organization and increased accessibility of DNA to the transcriptional machinery. Second, modifications on the lysine residues can serve as binding surfaces for the recruitment of other proteins that regulate gene activity (Kurdistani et al., 2004).

Acetylation of histones can occur at several sites in the same histone molecule, but not all acetylations have the same functional activity (Dion et al., 2005). For example, in yeast, H4 can be acetylated on K5, K8, K12 and K16, but only K16 acetylation has a specific regulatory function in formation of telomeric heterochromatin (Kimura et al., 2002; Suka et al., 2002). In human cells, K12 recruits a bromodomain factor, Bdf2 (Finnegan and Dennis, 1993; Ma et al., 1998; Cao et al., 2000; Bartee et al., 2001; Lindroth et al., 2001) and acetylated K8 recruits a SWI/SNF chromatin remodeling factor (Kanno et al., 2004).

In plants, acetylation of histones can modulate gene expression in response to environmental cues. In pea, the plastocyanin gene (*petE*) is induced by light (Chua et

al., 2001; Chua et al., 2003). Chromatin immunoprecipitation (ChIP) assays were used to show that transcriptional activation of *petE* was associated with acetylation of histone H3 and H4 in the promoter and enhancer regions. These modifications were not seen in the absence of light when the gene was not active. Another example of how environmental cues regulate chromatin changes is for the *FLC* flowering gene in *Arabidopsis thaliana*. In that system, vernalization leads to increases in histone deacetylation, followed by methylation of H3K27 and H3K9 (He and Amasino, 2004)

DNA methylation

In plants, like mammals, cytosine methylation is a major component of epigenetic, chromatin-mediated control. Cytosines, in CG and CNG or CNN contexts, are all potential substrates for methylation by DNA methyltransferases (Finnegan and Dennis, 1993; Cao et al., 2000; Bartee et al., 2001; Lindroth et al., 2001). Heavy cytosine methylation is found typically in heterochromatin, where the DNA is tightly compacted and transcriptionally silent. DNA methylation serves as a binding site for methylcytosine binding (MBD) proteins that can recruit or stably associate with HDACs, leading to a series of chromatin modifications that can condense and inactivate the DNA (Jones et al., 1998; Nan et al., 1998).

A number of studies implicate important mechanistic connections between DNA methylation and histone modification in plants. These include the discovery (coincident with studies in Neurospora) that methylation of histone H3K9 is required for cytosine methylation (Dobosy and Selker, 2001; Jackson et al., 2002). Similarly, other studies have demonstrated that DNA methylation is required for maintenance of H3K9 methylation (Gendrel et al., 2002; Soppe et al., 2002; Volpe et al., 2002; Tariq et al., 2003). Analysis of rDNA gene silencing accompanying nucleolar dominance led to the proposal of a concerted mechanism involving DNA methylation and histone modifications resulting in silencing (Lawrence et al., 2004). This model was supported

by additional experiments examining the global effects on transcription of mutating a histone deacetylase gene (Tian et al., 2005).

At the biochemical level, the tie between DNA methylation and histone modification occurs because the responsible enzymes are associated with one another in multi-protein complexes. For example, as mentioned above histone acetyltransferases, deactylases, DNA and histone methyltransferases and chromatin remodeling proteins in the SWI/SNF and/or ISWI ATPase families have all been shown to be parts of larger protein complexes (*e.g.*, Jones et al., 1998; Nan et al., 1998; Wade et al., 1998; Wade et al., 1998; Zhang et al., 1998; Kaya et al., 2001; Carrozza et al., 2003).

Anthocyanin Biosynthesis Pathway in Maize

Anthocyanins are red and purple flavonoid pigments found in plants. Because anthocyanins are conspicuous and non-essential for the growth, the anthocyanin biosynthetic pathway has been intensively studied at biochemical and genetic levels (Figure 1.1). Many genes involved in this pathway have been cloned and characterized, and the transcriptional regulation of the pathway is well understood (Dooner et al., 1991; Holton and Cornish, 1995).

Structural Genes

The anthocyanin structural genes encode enzymes in the biosynthetic pathway (Fig. 1.1). The first reaction of the synthetic pathway is the condensation of three molecules of malonyl CoA and one molecule of *p*-coumaroyl CoA to form chalcone by chalcone synthase (CHS) encoded by the maize *c2* gene. Then isomerization of chalcone by chalcone isomerase (encoded by *chi*) produces a colorless flavonone. This flavonone is hydroxylated by flavonoid 3-hydroxylase (*fht1*) to form dihyroflavonol. The next step is the reduction of dihydroflavonol by dihyroflavonol 4- reductase (*a1*) to produce a colorless leucoanthocyanidin. The conversion of a colorless

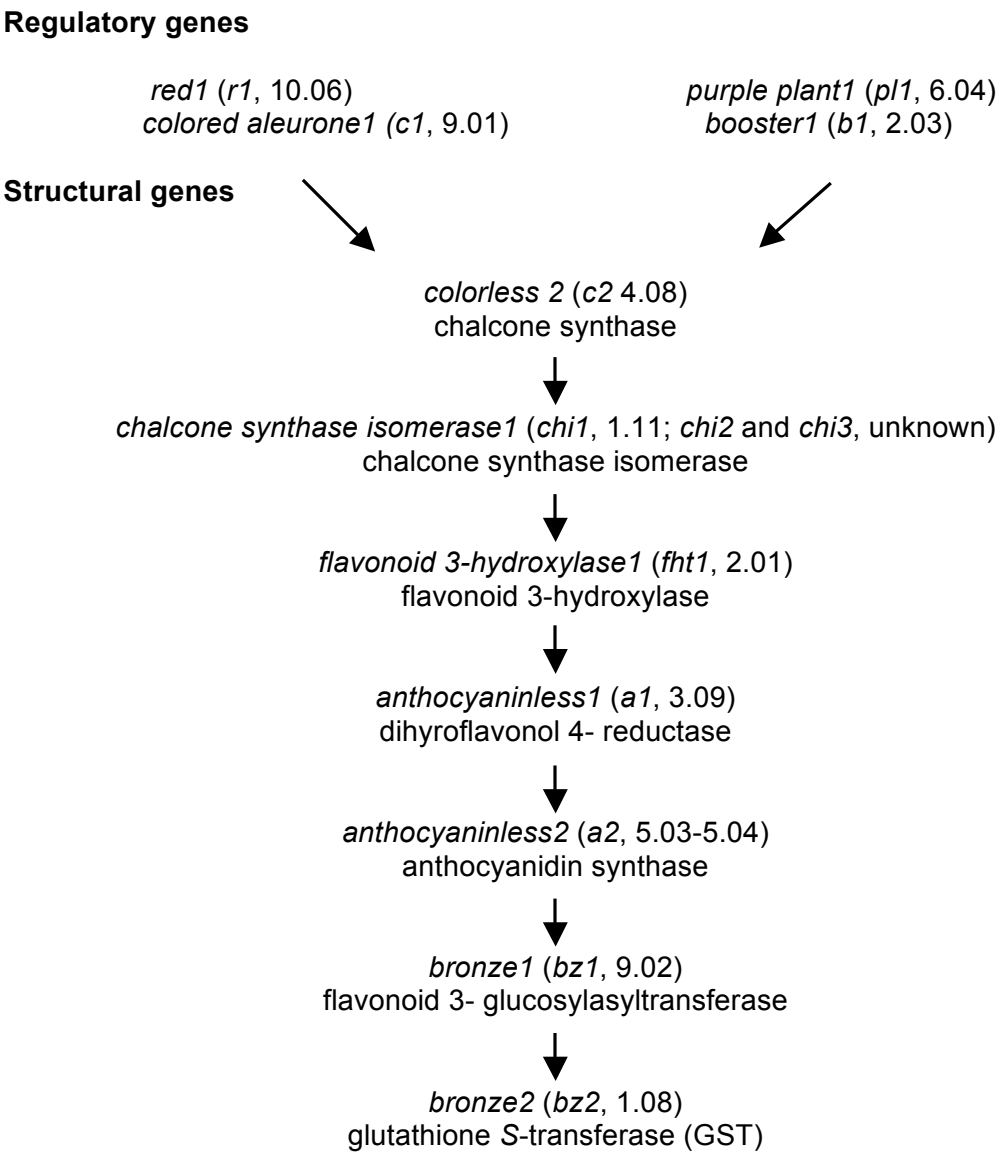


Figure 1.1. Schematic of maize anthocyanin biosynthetic pathway. Shown are regulatory and structural genes, their chromosomal locations (chromosome.bin number), and the enzymes encoded.

leucoanthocyanidin to a colored anthocyanidin involves the *a2* gene that encodes anthocyanidin synthase. The final step is the glycosylation of anthocyanidin to form an anthocyanin by flavonoid 3- glucosylasyltransferase (*Bz1*). The *bz2* gene encodes a glutathione *S*-transferase (GST), which is involved in transport of anthocyanins to vacuoles (Dooner et al., 1991; Holton and Cornish, 1995; Grotewold, 2006).

Regulatory Genes

In maize, two families of regulatory genes activate the coordinate expression of the anthocyanin structural genes. One family the *colored aleurone1* (*c1*) and *purple plant1* (*pl1*) genes, which encode *myb*-like transcription factors (Paz-Ares et al., 1987; Cone et al., 1993a). The second family, includes the *red1* (*r1*) and *booster1* (*b1*) genes, which encode related transcription factors that have a basic helix-loop-helix (b-HLH) motif at the C-terminus of the protein (Ludwig et al., 1989; Perrot and Cone, 1989; Robbins et al., 1991; Consonni et al., 1992; Petroni et al., 2000). Studies in maize showed that activation of structural gene transcription requires interaction of the b-HLH domain of the B1 protein with the N-terminal MYB domain of C1 protein (Grotewold et al., 2000). The regulatory genes control the tissue-specificity of anthocyanin biosynthesis. The R and C1 proteins mainly activate anthocyanin biosynthesis in the aleurone layer of the kernel, and the B and PL proteins mainly activate pigmentation in vegetative portions of the plant such as leaf sheath, husk and stem (Cone, 1994).

Pl1-Rhoades and Pl1-Blotched

Plants carrying *Pl1-Rhoades* and the strong *b1* allele, *B1-I*, show uniform and intensive purple pigmentation in the vegetative and floral tissues (Cone et al., 1993b). By contrast, plants carrying *Pl1-Blotched* allele and *B1-I* show a variegated pattern of pigmentation (Fig. 1.2). At the molecular level, this difference in pigmentation is correlated with differences in *pl* mRNA; *Pl1-Blotched* mRNA levels are much lower than in *Pl1-Rhoades* (Cocciolone and Cone, 1993).

The difference in expression of *Pl1-Rhoades* and *Pl1-Blotched* is due to epigenetic regulation. The DNA sequences of the two alleles are nearly identical, but the patterns of DNA methylation are altered (Cocciolone and Cone, 1993; Hoekenga et al., 2000). In both alleles, DNA methylation of many sites exhibit developmentally sensitive changes, but some of the changes are allele-specific. For example, methylation status at several *Msp*I/*Hpa*II sites downstream of the coding sequence changes from hypo- to hypermethylated in *Pl1-Blotched,* as plants mature from seedling to adults. Methylation of the same sites in *Pl1-Rhoades* is constant through development. The two alleles also differ in their chromatin structure (Hoekenga et al., 2000). *Pl1-Rhoades* is more sensitive to DNase I digestion than *Pl1-Blotched*, consistent with the higher level of expression in *Pl1-Rhoades*. In *Pl1-Blotched*, chromatin structure across the gene changes with development. In seedlings, the coding region of the gene is resistant to DNase I cleavage. With maturation, the closed chromatin structure expands, to include upstream and downstream flanking regions so that in husks (adult tissue), all regions of the gene are equally resistant to nuclease digestion (Hoekenga et al., 2000).

Suppressor of plant blotching (Spb): a modifier of Pl1-Blotched

To better understand how chromatin structure influences *Pl1-Blotched* expression, the Cone laboratory has adopted a genetic approach to identify modifiers that alter pigmentation in *Pl1-Blotched* plants. One such modifier, *Suppressor of plant blotching* (*Spb*), leads to increased pigmentation (Fig. 1.2). *Spb* was recovered in F_2 progeny from a cross of one of the Cone lab *Pl1-Blotched* stocks (*Skb, Pl1-Blotched*) with a stock (Coop 607 B*; *Pl1- Blotched*) obtained from the maize genetics stock center. To produce a true-breeding *Spb* line, intensively pigmented *Pl1-Blotched* plants were repeatedly self-pollinated, selecting each generation for the most pigmented plants.

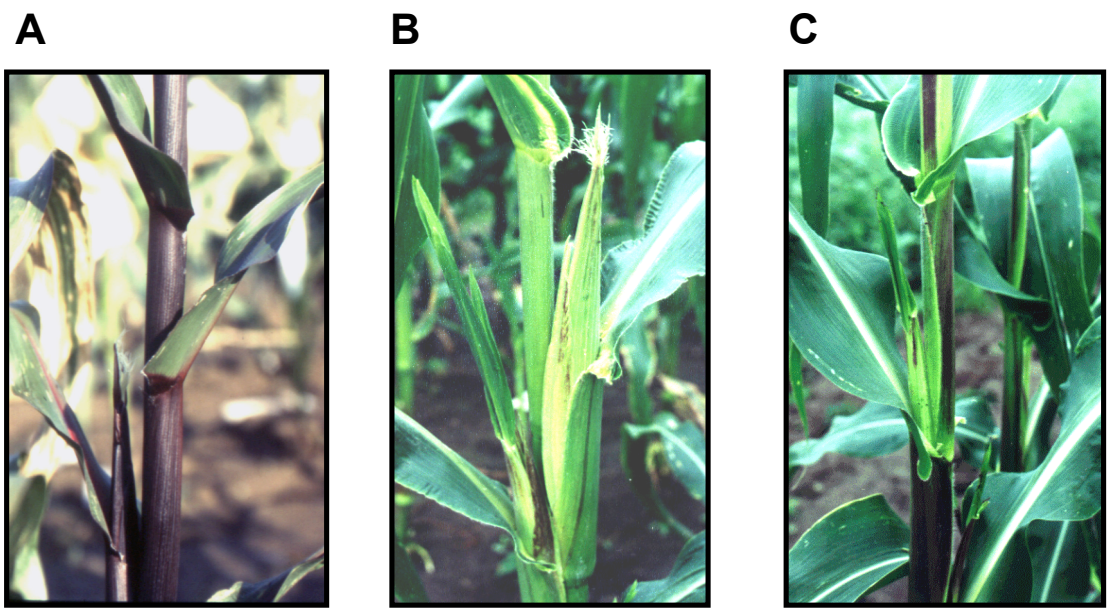


Figure 1.2. Plant phenotypes. A, *Pl1-Rhoades*; B, *Pl1-Blotched* without *Spb*; C, *Pl1-Blotched* with *Spb*.

At the molecular level, the increased pigmentation of *Pl1-Blotched* plants with *Spb* is due to increased *Pl1-Blotched* mRNA levels and corresponding increase in mRNA levels for the genes encoding the anthocyanin biosynthetic enzymes. In addition, *Spb* leads to changes in the pattern of DNA methylation at several methylation sensitive restriction sites in *Pl1-Blotched* (Courtney, 2001). These observations led to the proposal that the effect of *Spb* on *Pl1-Blotched* is epigenetic.

Surprisingly, the *Spb* effect on *Pl1-Blotched* does not seem to be due to a single gene. When *Spb* was crossed by a less pigmented *Pl1-Blotched* line, F_1 plants had pigmentation levels intermediate between the two parents, and the *Spb* phenotypes did not segregate in the simple 3:1 ratio expected for a single-gene effect on pigmentation. Instead, pigment levels in the F_2 plants showed a continuous, non-normal distribution. Thus, *Spb*-enhanced pigmentation is a quantitative trait (Courtney, 2001).

Goals of this study

The goal of this study was to address the hypothesis that *Spb* regulates *Pl1-Blotched* at the level of chromatin. I took two complementary approaches. First, I used molecular assays to describe the kinds of chromatin-level changes induced at *Pl1-Blotched* by *Spb*. I used DNase I sensitivity assays to analyze the chromatin configuration of *Pl1-Blotched* in the presence *vs*. absence of *Spb*. Based on previous comparisons of *Pl1-Blotched* to *Pl1-Rhoades*, I predicted that *Pl1-Blotched* chromatin would be more relaxed in the presence of *Spb*. Results of these experiments are presented in Chapter 2. I also used chromatin immunoprecipitation assays to study patterns of histone modification. In these experiments, I performed two pair-wise comparisons: *Pl1-Rhoades* to *Pl1-Blotched*, and *Pl1-Blotched* in the presence and absence of *Spb*. I expected to see highest levels of active histone modifications in *Pl1-*

Rhoades, lowest levels in *Pl1-Blotched* and an intermediate level in *Pl1-Blotched* with *Spb*. Results of these experiments are presented in Chapter 3.

My second experimental approach was to use quantitative trait locus (QTL) mapping to determine the chromosomal regions responsible for the *Spb*-induced increase in pigment levels in *Pl1-Blotched* plants. I expected this analysis to reveal the locations of *Spb* candidate genes. Results are presented in Chapter 4.

Together, these two complementary strategies provided important insights on how *Pl1-Blotched* is regulated by *Spb*. Chapter 5 presents conclusions and considerations for future study.

CHAPTER 2. EFFECT OF *SUPPRESSOR OF PLANT BLOTCHING* (*SPB*) ON NUCLEASE SENSITIVITY OF *PL1-BLOTCHED*

Packaging of the genome into chromatin has profound effects on gene expression. The position of nucleosomes within a gene can interfere with gene expression by preventing access of transcription factors to the DNA target (Struhl, 1999). For example, nucleosomes positioned over a TATA box prevent the TATA-binding protein from targeting to the promoter of a gene (Goodrich and Tweedie, 2002). For transcription to occur, changes in chromatin structure are necessary to permit access to transcription start site.

Experimentally, chromatin organization can be monitored using small sequence-independent endonucleases, like DNase I, that can diffuse across the nuclear membrane and digest DNA in chromatin. Regions accessible to DNase I cleavage are believed to reflect a more open or relaxed packaging of the chromatin (Wurtzel et al., 1987; Bellard et al., 1989). Actively transcribed regions are more sensitive to DNase I cleavage than transcriptionally inactive chromatin. Two patterns of DNase I sensitivity are possible. First, actively transcribed genes tend to show generalized sensitivity to DNase I digestion along their entire length, relative to inactive genes (Weintraub and Groudine, 1976). Second, promoter regions of active genes, which tend to have few or no nucleosomes, can show hypersensitivity to DNase I (Wu, 1980).

Our previous studies comparing *Pl1-Rhoades* and *Pl1-Blotched* showed a differential pattern of DNase I sensitivity that was correlated with expression levels of these alleles (Hoekenga et al., 2000). The highly expressed *Pl1-Rhoades* allele was more sensitive to DNase I along the entire length of the gene than was the less expressed *Pl1-Blotched* allele. To ask whether the increase in pigmentation of *Pl1-Blotched* plants induced by the modifier *Spb* is associated with increased sensitivity to

DNase I, I performed DNase I sensitivity assays using nuclei isolated from *Pl1-Blotched* plants with and without *Spb*. As a control, assays were also performed on *Pl1-Rhoades* nuclei.

Materials and Methods

Plant Materials

Three maize lines were used in these experiments. All three stocks are homozygous dominant for all of the anthocyanin structural genes. The *Pl1-Rhoades* stock is homozygous for the fully expressed *Pl1-Rhoades* allele and for *B-I*. These plants have uniform, dark purple pigmentation in the culm, leaf sheaths and tassel. The two *Pl1-Blotched* stocks used were derived by divergent selection from a single segregating population. Both stocks are homozygous for *Pl1-Blotched* and *B-I* and have variegated pigmentation in culm, leaf sheath and tassel; however, the *Spb* stock has more pigment than non-*Spb* stock. Plants were grown in the field at the University of Missouri Genetics Farm in Columbia, MO during the summers of 2004, 2005 and 2006.

Nuclei Isolation and Nuclease Sensitivity Assays

Nuclei were isolated by published methods (Cone et al., 1993a) with slight modifications. Inner husks were harvested from ears at the time of silk emergence and used for nuclei isolation. Husks from approximately 20 ears yielded 300-360 g of tissue, which was cut crosswise into 3-cm sections and processed in batches of 50-60 g. For each batch, husks were immersed for 3 min in 50 ml of cold ether (Fisher) on ice under a ventilation hood. The ether was poured off and the husk tissue was drained and blotted on paper towels to remove excess ether. The tissue was transferred to a cold Waring blender containing 250 ml cold nuclear extraction buffer (NEB: 1 M hexylene glycol: 2 Methy-2, 4-pentanediol, Aldrich; 10 mM PIPES-KOH, pH 7.0; 10 mM $MgCl_2$; 1.8 mM β-mercaptoethanol) and blended for 3 to 5 seconds. Samples were filtered through

cheesecloth wetted with working buffer (WB: 0.5 M hexylene glycol; 10 mM PIPES-KOH, pH 7.0; 10 mM $MgCl_2$; 1.8 mM β-mercaptoethanol) into a cold 500-ml beaker containing a stir bar. The extract was held at 4˚C while the remaining aliquots were processed. All subsequent steps were performed at 4°C on ice. With stirring, 5 ml of 25% Triton X-100 were added slowly to each beaker. After 5 minutes of moderate stirring, the nuclei were filtered through Miracloth (Calbiochem) wetted with WB into 250-ml centrifuge bottles on ice. The samples were centrifuged at 2,000 rpm in a JA-10 rotor (Beckman) for 10 min and the supernatant was decanted quickly. The pellet, which contained the nuclei, was re-suspended in 10 ml of WB by swirling and gently pipetting. All six re-suspended pellets were combined into 1 bottle on ice.

Three 90% / 60% Percoll gradients were prepared: 7.5 ml of 60% Percoll (made by diluting 90% Percoll with WB) was placed in the bottom of a 50-ml polycarbonate tube on ice. Then 7.5 ml of 90% Percoll (100% Percoll, Amersham Biosciences; 0.5 M hexylene glycol; 10 mM Pipes-KOH, pH 7.0) was pipetted below the 60% Percoll taking care to not to mix the layers. Approximately 20 ml of nuclei suspension were layered over each gradient. The gradients were centrifuged in a swinging bucket rotor (JA-13.1, Beckman) at 5,000 rpm for 20 min at 4°C, and the run was terminated using low (40%) braking power to preserve the gradient integrity. To determine which band contained nuclei, the upper and lower bands were pulled separately, and aliquots were viewed under the microscope by staining nuclei with 0.1% Azure C. The nuclei usually banded at the 60%–90% interface. Collected nuclei were diluted with 2 volumes of WB in a 50 ml of polycarbonate tube and centrifuged at 2000 rpm in a JA-20 rotor (Beckman) for 10 min. The supernatant was discarded and nuclei were re-suspended in 2 ml of nuclei storage buffer (NSB: 50 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$; 2.5 mM DTT; 50% glycerol; 0.5 mM PMSF; 0.5 µg/ml antipain; 0.5 µg/ml leupeptin). Nuclei were aliquoted into microfuge tubes and stored at -80°C.

Nuclei were counted using a hemocytometer. For each nuclease sensitivity assay experiment, 10 x 10^6 nuclei were used. The nuclease sensitivity assay was adapted from published methods (Wurtzel et al., 1987; Bellard et al., 1989). Nuclease digestion buffer contained 50 mM Tris-HCl (pH 7.5), 10 mM $MnCl_2$, 50 µg/ml BSA, 340 mM sucrose and 4 mM spermidine. A series of nine 16-µl aliquots of DNase I (Gibco/BRL, Gaithersburg, MD), containing 0, 0.075, 0.15, 0.30, 0.6, 1.2, 2.4, 4.8, or 9.6 units of enzyme, were prepared with nuclease digestion buffer and stored in an ice-water bath until use. The tube with no DNase I served as a control to detect activity of endogenous nucleases. Nuclei were thawed and pelleted by centrifuging for 30 seconds at maximum speed in a bench top microfuge (Eppendorf). The pellet was re-suspended in 900 µl of nuclease digestion buffer and placed on ice for 5 min. A 100 µl aliquot of nuclei (~1 x 10^6) was added to each DNase I tube and incubated for 5 min in an ice-water bath. Naked CsCl-purified DNA was also used as a control for DNase I digestion. The amounts of DNase I used for digestion of naked DNA were 0, 0.019, 0.038 and 0.075 units. Reactions were stopped by adding two volumes of lysis buffer (350 mM NaCl; 50 mM EDTA; 1 mM Tris-HCl, pH 8.0; 7 M urea; and 2 % sarkosyl). DNA was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (100: 100: 1), precipitated with two volume of ethanol, and re-suspended in 100 µl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

DNA samples were analyzed by Southern hybridization as previously described (Cone et al., 1986). For restriction enzyme digestion, 500 ng of DNase I treated-nuclei DNA and 830 ng of DNase I treated-naked DNA were digested with *Hinc*II according to the manufacturer's instructions. Restriction digests were fractionated on 1% agarose gels and blotted to nylon membranes, which were UV-cross linked, and hybridized with probes labeled by random priming using α-^{32}P dATP. Hybridization was performed at 42°C for 24 hours and then membranes were rinsed with 2X SSC and washed with 0.1 X

SSC, 0.1% SDS at 50°C four times. The nylon membranes were placed on Fuji Phosphorimager plates (Fuji Film co. Ltd., Tokyo, Japan) and exposed for one day. Images were scanned and analyzed using ImageGauge 3.3 (Fuji Film co. Ltd., Tokyo, Japan).

To compare intensities of hybridizing bands across genotypes, band intensities were normalized to the amount of DNA loaded in each lane as follows. Ethidium bromide-stained gels were photographed using a UV-transilluminator. Images were saved in tif format. The tif files were imported into Image Gauge 3.3 and the amounts of DNA were estimated by quantifying the signal strength of each whole lane on the agarose gels. *pl1* band intensities (pixels) were normalized to the total amount of DNA by dividing the *pl1* signal by the whole-lane signal. Each normalized value was divided by the maximum normalized value to obtain a ratio for each DNase I treatment.

Probes

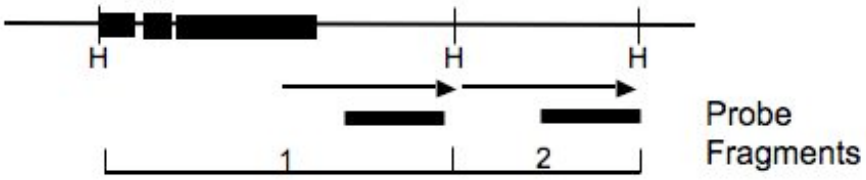
A 0.62-kb *Bgl*II-*Nsi*I fragment derived from the 3' end of the *pl1* gene was used for DNase I sensitivity assays. This *pl1*-specific probe does not cross-hybridize with the *pl1* homolog, *c1*. The probe detects two *Hin*cII fragments from *Pl1-Blotched*. Fragment 1 is 2 kb and includes most of the coding region; fragment 2 is 1.2 kb and includes a downstream repeat region (Figure 2.1A).

Results

Comparison of *Pl1-Rhoades vs. Pl1-Blotched*

Previous results from our laboratory showed that *Pl1-Rhoades* is more sensitive to DNase I digestion than is *Pl1-Blotched* (Hoekenga et al., 2000). To confirm this result and to serve as a control for current experiments, the DNase I sensitivity of *Pl1-Blotched* was compared to that of the highly expressed *Pl1-Rhoades* allele. DNase I sensitivity of DNA from the *pl1* locus in these genotypes, was monitored by digesting DNase I-treated

A



B

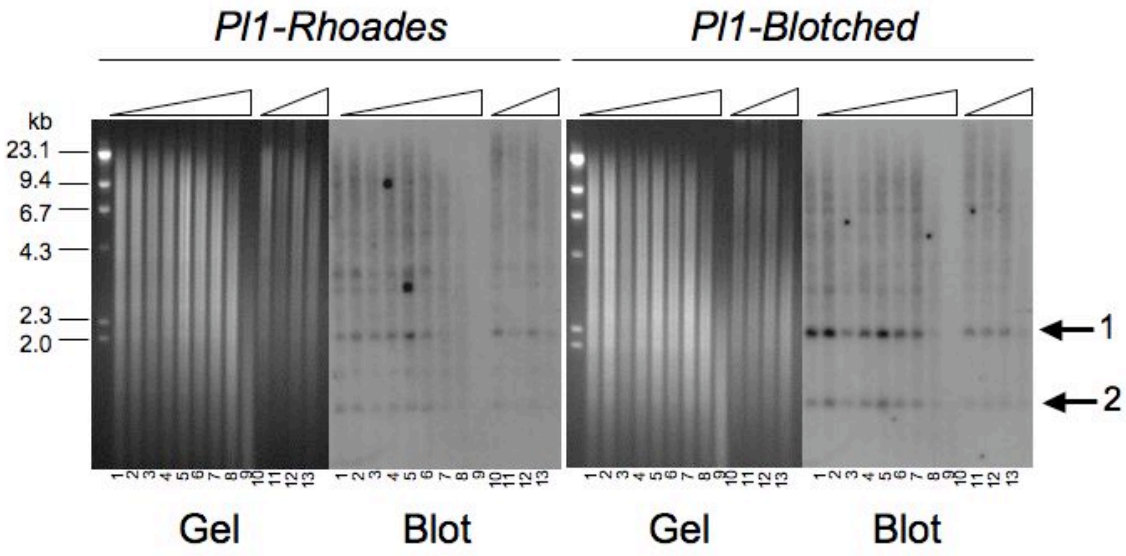


Figure 2.1. DNase I assay comparing *Pl1-Rhoades* and *Pl1-Blotched*.
A. Diagram of *pl1* gene shows positions of *Hin*cII (H) sites, probe, and fragments 1 and 2. Black boxes designate the three exons and arrows indicate tandem repeat downstream of the gene.
B. Results of DNase I sensitivity assay of husk nuclei from *Pl1-Rhoades* and *Pl1-Blotched*. Nuclei (lanes 1-9) and naked DNA (lanes 10-13) were treated with DNase I and digested with *Hin*cII. Triangles denote increasing DNase I concentrations from low on left to high on right. Digests were fractionated on agarose gels, blotted and hybridized with the probe indicated in A. For each genotype, left image shows ethidium bromide-stained agarose gel and right image shows corresponding blot. Arrows indicate positiions of fragments 1 and 2. DNaseI concentrations (units) in each lane were: 1, 0; 2, 0.075; 3, 0.15; 4, 0.3; 5, 0.6; 6, 1.2; 7, 2.4; 8, 4.8; 9, 9.6; 10, 0; 11, 0.019; 12, 0.038; 13, 0.075.

DNA with *Hin*cII and detecting the restriction fragments on Southern blots by hybridization with a probe derived from the 3' region of the gene. This probe detects two *pl1* fragments (Fig. 2.1A). The larger fragment 1 covers most of the coding region and the smaller fragment 2 covers the downstream region.

Comparison of *Pl1-Rhoades* and *Pl1-Blotched* revealed that fragment 1 is equally sensitive to DNase I digestion in both genotypes (Fig. 2.1B, 2.2). However, fragment 2 is more sensitive in *Pl1-Rhoades* than in *Pl1-Blotched* (Fig. 2.1B, 2.2); the amount of DNase I needed to digest half of the fragment 2 molecules (reduce the band intensity to 0.5) was approximately 3-fold higher in *Pl1-Blotched* than in *Pl1-Rhoades* (6 U for *Pl1-Blotched*; 2.4 U for *Pl1-Rhoades*). These results confirm our previous observations (Hoekenga et al., 2000) and once again show that *Pl1-Blotched* has more compact chromatin structure than *Pl1-Rhoades*.

Comparison of *Pl1-Blotched* with *Spb* vs. *Pl1-Blotched* without *Spb*

Because *Spb* increases the expression of *Pl1-Blotched*, I predicted that the chromatin configuration of *Pl1-Blotched* would be less condensed, and therefore more sensitive to DNase I digestion, in plants with *Spb,* compared to plants without *Spb.* Treatment of nuclei from husks of *Pl1-Blotched* plants with and without *Spb* showed that both fragments 1 and 2 were more sensitive to DNase I digestion in the presence of *Spb* (Fig. 2.3, 2.4). This difference was most obvious for fragment 2, where the amount of DNase I needed to digest half of the fragment 2 molecules differed by two- to four-fold (with *Spb*, 1.2 - 2.4 U; without *Spb*, ~ 6 U). These results indicate that the presence of *Spb* results in more relaxed chromatin packaging both upstream and downstream of *Pl1-Blotched* gene, although the effect is most pronounced in the downstream region.

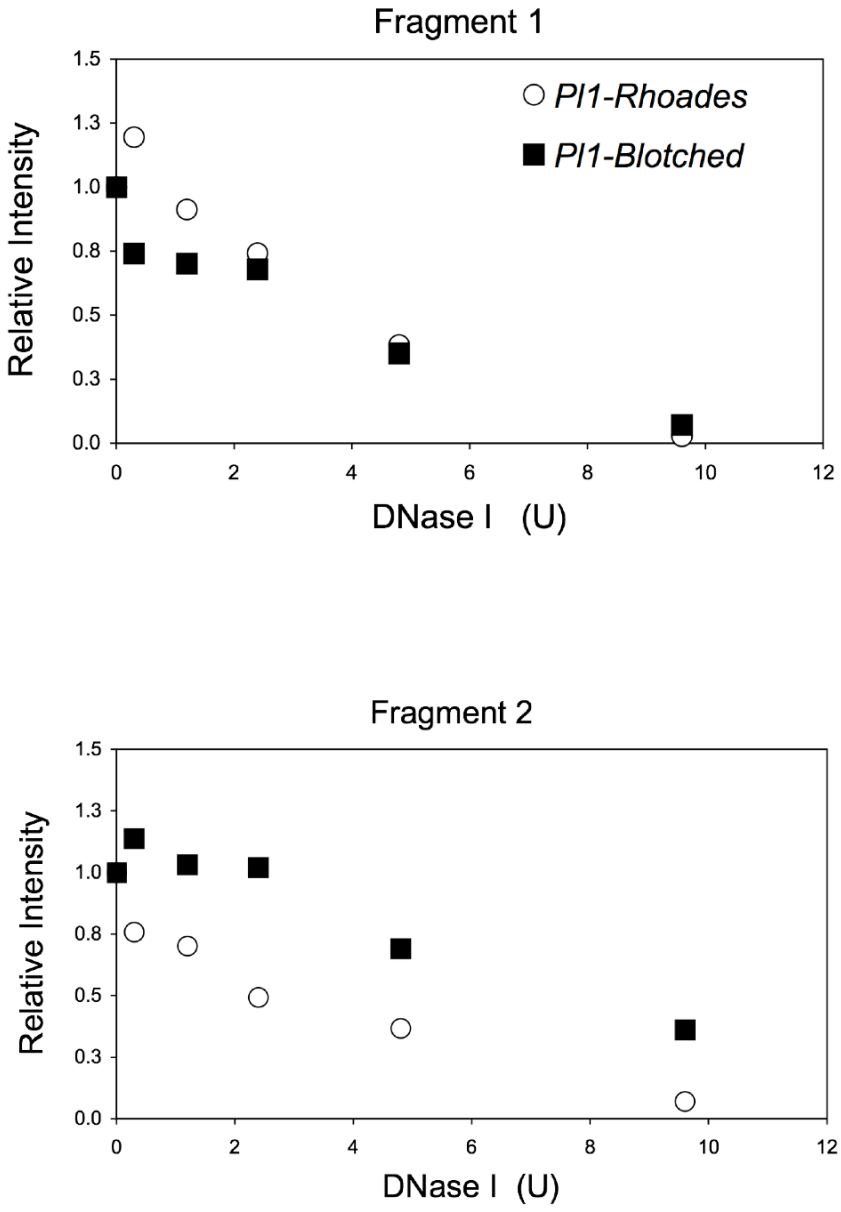


Figure 2.2. Graph of DNase I assay results, comparing *Pl1-Rhoades* and *Pl1-Blotched*. Graph shows kinetics of disappearance of fragments 1 and 2. Relative intensity of fragments 1 and 2 were calculated as described in Materials and Methods.

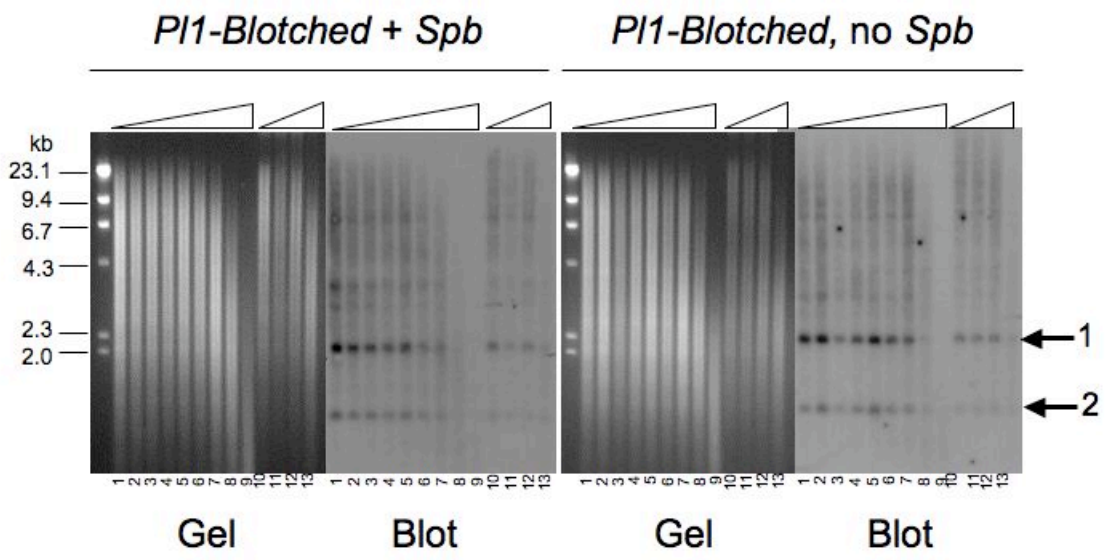


Figure 2.3. DNase I assay comparing *Pl1-Blotched* with *Spb* to *Pl1-Blotched* without *Spb.* Nuclei (lanes 1-9) and naked DNA (lanes 10-13) were treated with DNase I and digested with *Hinc*II. Triangles denote increasing DNase I concentrations from low on left to high on right. Digests were fractionated on agarose gels, blotted and hybridized with the probe indicated in Fig. 2.1 A. For each genotype, left image shows ethidium bromide-stained agarose gel and right image shows corresponding blot. Arrows indicate positiions of fragments 1 and 2. DNaseI concentrations (units) in each lane were: 1, 0; 2, 0.075; 3, 0.15; 4, 0.3; 5, 0.6; 6, 1.2; 7, 2.4; 8, 4.8; 9, 9.6; 10, 0; 11, 0.019; 12, 0.038; 13, 0.075.

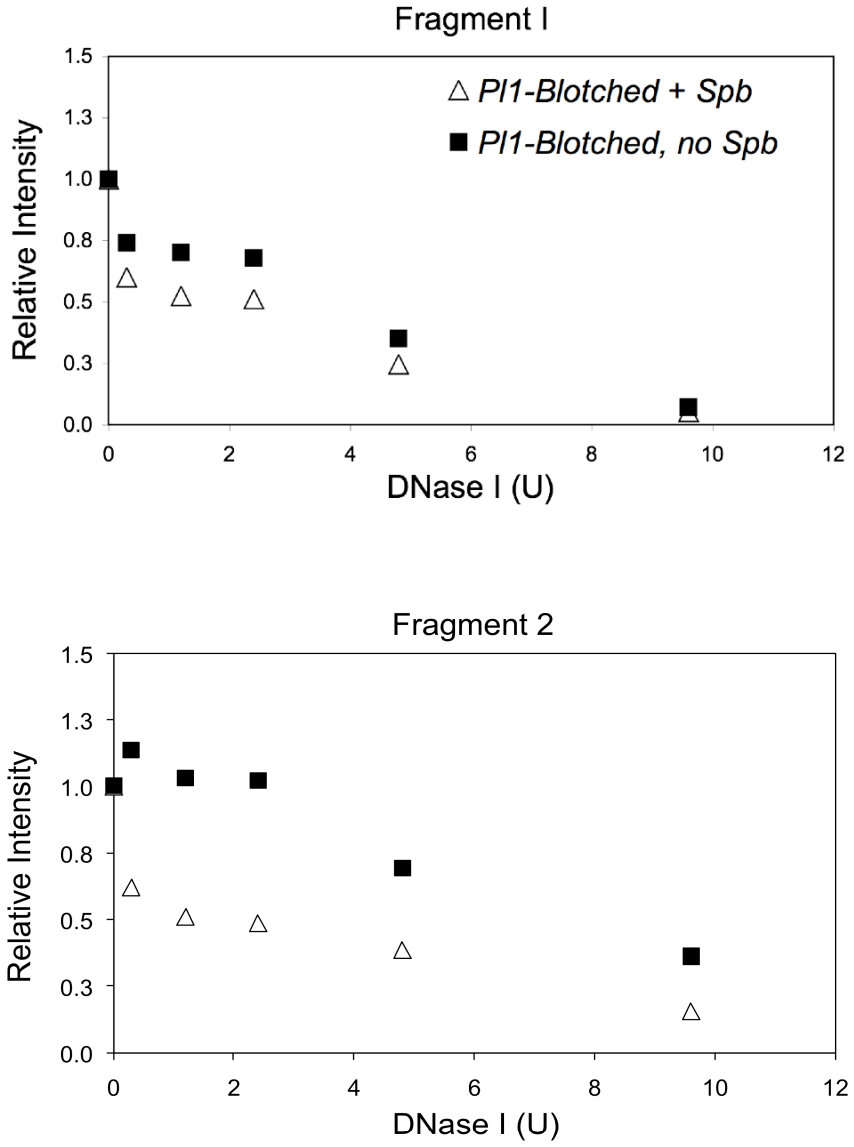


Figure 2.4. Graph of DNase I assay, comparing *Pl1-Blotched* with and without *Spb*. Graph shows kinetics of disappearances of fragments 1 and 2. Relative intensity of fragments 1 and 2 were calculated as described in Materials and Methods.

Discussion

Anthocyanin pigmentation in *Pl1-Blotched* plants with *Spb* is intermediate between the uniform, strong pigmentation of *Pl1-Rhoades* plants and the variegated pigmentation of *Pl1-Blotched* plants without *Spb*. Previous studies showed that these pigmentation differences are directly correlated with differences in *pl1* mRNA levels (Cocciolone and Cone, 1993; Hoekenga et al., 2000; Courtney, 2001), and that the differences in expression between *Pl1-Rhoades* and *Pl1-Blotched* directly reflect differences in chromatin structure (Hoekenga et al., 2000). Here, I found that the increased expression of *Pl1-Blotched* in the presence of *Spb* is due to relaxation of chromatin structure at *Pl1-Blotched*.

The chromatin structure difference in *Pl1-Blotched* induced by *Spb* was most obvious in a region downstream of the gene, which contains a 1-kb direct repeat (Fig. 2.1A). This same region also showed differential DNase I sensitivity between *Pl1-Rhoades* and *Pl1-Blotched*, with the more highly expressed *Pl1-Rhoades* allele showing higher DNase I sensitivity. Previous studies implicated this region as a site of differential methylation between *Pl1-Rhoades* and *Pl1-Blotched* (Hoekenga et al., 2000). Exactly how chromatin changes downstream of the *pl1* gene might contribute to regulating gene expression is not yet known. It is possible that the repetitive nature of this region is important. In maize, much of the genome is made up of transposable elements that are maintained in a silent state by distinct chromatin features, such as high levels of DNA methylation and histone modifications typically associated with tightly packed, transcriptionally silent chromatin. The repeat downstream of *pl1* differs from the highly repetitive fraction of the genome in that it is not repeated elsewhere and it is not heavily methylated on cytosines. Instead, methylation levels are relatively low in both the highly expressed *Pl1-Rhoades* and the less expressed *Pl1-Blotched* allele (Hoekenga et al.,

2000). Whether there are diagnostic histone modifications that distinguish the expression states of *pl1* is the subject of research presented in Chapter 3.

CHAPTER 3. HISTONE MODIFICATIONS OF *PL1-RHOADES* AND *PL1-BLOTCHED* CHROMATIN

Pl1-Rhoades, *Pl1-Blotched* with *Spb*, and *Pl1-Blotched* without *Spb* define a pigmentation hierarchy with *Pl1-Rhoades* at the top and *Pl1-Blotched* without *Spb* at the bottom. Underlying the differences in pigmentation are similarly hierarchical molecular differences in DNase I sensitivity and mRNA levels. Presumably, these relationships are determined by fundamental differences in chromatin packaging. One important determinant for chromatin packaging is histone modification. Addition of methyl or acetyl groups to histones H3 and H4 can have significant effects on gene expression, as reviewed in Chapter 1.

To ask if histone modification patterns reflect the hierarchy in pigmentation for *Pl1-Rhoades* and for *Pl1-Blotched* with and without *Spb*, I used chromatin immunoprecipitation assays (ChIP) to assess the types and abundance of histone modifications at *pl1* in the three genetic backgrounds. The ChIP assay was developed to study the association of proteins located within DNA, such as transcription factors and modified histones (Buck and Lieb, 2004). The ChIP assay combines two straightforward steps–*in vivo* formaldehyde cross-linking of intact nuclei or whole cells that freezes protein-DNA interactions, followed by immunoprecipitation of protein-DNA complexes with specific antibodies from sonicated extracts. The important feature of these experiments is the use of antibodies that recognize and bind to the protein of interest, not only in free solution, but also when assembled into chromatin.

Experiments designed to address two main questions: a) Are the differences in expression and DNase I sensitivity between *Pl1-Rhoades* and *Pl1-Blotched* correlated with differences in histone modifications? b) Is the increased expression of *Pl1-Blotched* in the presence of *Spb* mediated by changes in histone modification? If so, are the

chromatin changes induced by *Spb* similar to the modifications that distinguish *Pl1-Rhoades* from *Pl1-Blotched*?

Materials and Methods

Plant Materials

Three maize lines - one *Pl1-Rhoades* and two *Pl1-Blotched* stocks - were used in these experiments. All three stocks were homozygous dominant for *B-I* and the anthocyanin structural genes. The *Pl1-Rhoades* stock was homozygous for *Pl1-Rhoades.* One of the *Pl1-Blotched* stocks had the modifier, *Spb*, and the other did not. The non-*Spb* line was derived as described in Chapter 2. Plants were grown in the field at the University of Missouri Genetics Farm during the summer of 2006.

Nuclei Isolation and Chromatin Immunoprecipitation (ChIP) assays

Nuclei were isolated by the same protocol described in Chapter 2, with the following modifications. Nuclei to be used for ChIP assays were fixed just after isolation with formaldehyde at a final concentration of 1% for 15 min at room temperature; fixation was stopped by addition of glycine to 125 mM, followed by mixing for 5 min at room temperature. Fixed nuclei were stored at -80°C until use.

For ChIP assays, nuclei (5×10^6 nuclei per antiserum) were thawed and pelleted by spinning for 30 sec at room temperature at 14,000 rpm in a microfuge (Centrifuge 5417C, Eppendorf, Westbury, NY). Nuclei were suspended in 800 µl SDS lysis buffer (1% SDS; 10 mM EDTA; 50 mM Tris-HCl, pH 8.0; 0.5 µg/ml antipain; 0.5 µg/ml leupeptin; 1 mM PMSF). To shear the chromatin, nuclei were sonicated in 2.0 ml microtubes using a microprobe (Model W-375, Heat-Systems-Ultrasonics, Inc., 50% duty, at knob 3, pulse) seven times for 20 sec each; between bursts, tubes were held on ice for 5 min. The sonicated solution was spun at 14,000 rpm in a microfuge for 10 min at room temperature, and the supernatant was removed to a new 1.5 ml microtube. This

supernatant (crude chromatin solution) was diluted 10-fold with IP buffer (167 mM NaCl; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris-HCl, pH 8.0; 0.5 µg/ml antipain; 0.5 µg/ml leupeptin; 1 mM PMSF). Protein A agarose / salmon sperm DNA (2 ml per each ChIP experiment, Upstate Biotechnology) was equilibrated by rinsing three times with 1 volume of IP dilution buffer, and 200 µl were added to the diluted crude chromatin solution and they were incubated on a rotation wheel (Model 7637, Cole-Parmer Instrument Company, Chicago, IL) for 1 hr at 4°C. This pre-cleared chromatin solution was spun at 5,000 rpm (JA-20 rotor, Beckman) for 3 min at 4°C, and the supernatant was divided into 1-ml aliquots. To each aliquot, one antiserum was added. Two controls were used. As an input control, 0.5 ml of pre-cleared chromatin solution was saved at 4°C and then processed with the other samples from the cross-linking reversal step. A no-antibody control contained 1 ml of pre-cleared chromatin, but no antiserum. For immunoprecipitation, reactions were incubated on a rotation wheel at 4°C overnight. The next day, 60 µl of protein A agarose / salmon sperm DNA equilibrated with IP buffer were added and incubated for two hrs on the rotation wheel at 4°C. Tubes were spun for 1 min at 14,000 rpm in a microfuge and the pellets were successively washed–by suspension in 1 ml, inversion for 3 min and spinning for 1 min–once with low salt buffer (TSE-150: 150 mM NaCl; 0.2% SDS; 0.5% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.0), once with high salt buffer (TSE-500: 500 mM NaCl; 0.2% SDS; 0.5% Triton X-100; 2 mM EDTA; 20mM Tris-HCl, pH 8.0), once with LiCl wash buffer (0.25 M LiCl; 0.5% NP-40; 0.5% sodium deoxycholate; 1 mM EDTA; 10 mM Tris-HCl, pH 8.0), and twice with TE (10 mM Tris-HCl; 1 mM EDTA, pH 8.0). Then, immune complexes were eluted by suspending pellets in 500 µl fresh-prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$), vortexing briefly and incubating for 15 min at 65°C and then for 15 minutes on a rotation wheel at room temperature. Tubes were spun for 1 min at 14,000 rpm in a

microfuge and the supernatants were transferred carefully to a new tube. To reverse cross-linking, 2.5 µl of 5 M NaCl, 5 µl proteinase K (10 mg/µl) and 25 µl of 1M Tris-HCl, pH 6.5 were added to each tube (including the saved input control) and incubated at 65°C for 6 hrs. Chromatin immunoprecipitated DNA (ChIPed DNA) was extracted with one volume of phenol:chloroform:isoamyl alchohol (100:100:1) and back-extracted with 100 µl of 1M Tris-HCl, pH 6.5, pooling aqueous phases. DNA was precipitated by adding 2 µl of glycogen (15 mg/µl), 1/15 volume of 3 M sodium acetate, pH 5.5 and 1.5 volumes of 95% EtOH at 20°C overnight. Precipitated DNA was spun for 20 min at 14,000 rpm in a microfuge and the pellet was re-suspended in 50 µl of de-ionized water. DNA was stored at 4°C. Nuclei were isolated three to four times for each genotype in summer of 2006. To minimize biological variation, nuclei from multiple isolations were pooled within a genotype and aliquotted for ChIP assays.

Quantitative real-time PCR (qPCR)

ChIPed DNA was analyzed by real-time PCR (ABI 7300, Applied Biosystems, Foster City, CA; Wong and Medrano, 2005), using an absolute quantification method. Four gene regions were amplified using primers indicated in Table 3.1. Two primer sets were used for *pl1* (upstream and downstream regions); one set for *gpc4*, which served as an active-gene control; and one set for *dzs10*, which served as a silent-gene control. Amplicons produced with all four primer sets were cloned using a TA cloning kit (Invitrogen, Carlsbad, CA), plasmid DNA for each was purified (QIAprep Spin Miniprep Kit 250, QIAGEN Science, Maryland, USA); and serial dilutions of the plasmids (2.0×10^{-14} -7.8×10^{-17}g) were amplified on the same plate as ChIPed DNA and used to construct standard curves for quantification of respective targets. For real-time quantitative PCR, each reaction contains 800 nM of each forward and reverse primer, 12.5 µl of 2 x SYBR mix (Power SYBR Green PCR Master Mix (P/N: 4367659, Applied Biosystems, Foster City, CA) and 2 µl of plasmid DNA for standard curves or 2 µl of

Table 3.1. Primer sequences used for amplifying each gene after ChIP

Name	Primer sequences	Length (bp)	Amplicon (bp)	GenBank accession
Pl1-Up_f	5'-CGAAAATTTAGGCGTTTCCTGTAG-3'	24	199	L13454
Pl1-Up_r	5'-CCCTCTCTTCACCCCTTCCT-3'	20		
Pl1-Down_f	5'-GAATTTCTATTTCGTTGCGTAAAGAT-3'	26	207	
Pl1-Down_r	5'-CGGTGTGTCAACATCAATATG-3'	21		
gpc4-Up_f	5'-GCTGGTGGTGACATTTCCGCTGAT-3'	25	191	X73152
gpc4-Up_r	5'-GCCAAACCCTGCACCAACAAAATA-3'	24		
dzs10-Up_f	5'-CGAGTCATCCGGCTTATCC-3'	19	189	M23537
dzs10-Up_f	5'-CATGGCGGTGGTGTCCT-3'	17		

ChIPed DNA in total reaction volume of 25 µl. Some ChiPed DNA was diluted with de-ionized water to get proper template concentration for RT-qPCR. PCR conditions included initial incubation for 10 min at 95°C to activate polymerase, then 45 cycles of denaturation for 15 sec at 95°C, annealing for 1 min at 60°C, and data were collected at annealing step.

For each genotype, ChIP was performed two or three times with each antibody (Table 3.2). Each ChIPed DNA sample was analyzed in duplicate by real-time qPCR with four primer sets (two for *pl1*, one for *gpc4* and one for *dzs10*). PCR products were quantitated by comparison to the within-plate standard curve. The percentage of input chromatin immunoprecipitated by a specific antibody (%IP) was calculated by dividing the amount of PCR product generated from the immunoprecipitated sample by the amount of PCR product generated from the corresponding input control and then multiplying by 100.

Results

To investigate the role played by histone modification in epigenetic regulation of *Pl1-Blotched*, ChIP assays were conducted using antibodies (Table 3.3) that recognize a spectrum of modifications, some reported in other systems to be associated with active genes and others with inactive genes. Two sets of pair-wise comparisons were made. Chromatin from purple plants carrying the fully expressed *Pl1-Rhoades* allele was compared to chromatin from variegated plants carrying the poorly expressed *Pl1-Blotched* allele; and chromatin from variegated *Pl1-Blotched* plants was compared to chromatin from more pigmented *Pl1-Blotched* plants that carried the modifier *Spb*. Because few maize genes have been analyzed by ChIP, two controls were included to aid in characterization of particular histone modifications as diagnostic of active or inactive genes. As an active gene control, *gpc4* was selected; this gene, which encodes

Table 3.2. Antibodies used for ChIP assays in this study

Antibody	Experiment					
	M	**N**	**O**	**Q**	**R**	**S**
H3	√	√	√	√	√	√
H3K4me3				√	√	√
H3K4me2	√	√			√	
H3K9me2	√	√			√	
H3K9ac			√	√		√
H4-tetra-ac			√	√		√
H4K12ac				√	√	√
H3K27me2	√	√				
H4K20me2	√	√				

Table 3.3. Antisera used in this study.

Antiserum	Amount Used [a]	Company [b]	Catalog number
Anti-H3	5 µg	Abcam	ab1791
Anti-H3K4me2	5 µg	Abcam	ab7766
Anti-H3K4me3	5 µg	Abcam	ab8580
Anti-H3K9me2	5 µg	Abcam	ab1220
Anti-H3K9ac	5 µg	Abcam	ab4441
Anti-H4K12ac	5 µg	Abcam	ab1761
Anti-H4-tetra ac	20 µl (Whole serum)	Upstate	06-866
Anti-H4K20me2	5 µg	Abcam	ab14092
Anti-H3K27me2	20 µl (whole serum)	Upstate	07-421

[a] For each immunoprecipitation
[b] Abcam: Abcam Inc., Cambridge, MA; Upstate: formerly Upstate Biotechnology, now Millipore, Billerica, MA

glyceraldehyde-3-phosphate dehydrogenase, is constitutively expressed in most maize tissues (Russell and M.M., 1991; Russell and Sachs, 1992) including husks, which were used in this study (data not shown). As an inactive gene control, *dzs10* was chosen; this gene encodes one of the zein endosperm storage proteins and is expressed at high levels in endosperm (Lai and Messing, 2002), but is not expressed in husks (data not shown; Fig.3.1).

Comparison of *Pl1-Rhoades* and *Pl1-Blotched*

Methylation of histone H3K4 and H3K9

Di- and tri-methylation of H3K4 ((H3K4me2 and H3K4me3, respectively) are associated with transcriptionally active genes in many eukaryotes (Bernstein et al., 2002; Santos-Rosa, 2002; Sakamoto et al., 2004; Alvarez-Venegas and Zoya, 2005; Habu et al., 2006; Barski et al., 2007). Because *Pl1-Rhoades* is much more highly expressed than *Pl1-Blotched*, these modifications were predicted to be more abundant in *Pl1-Rhoades*. Both modifications were detected at both the upstream and downstream regions of *pl1* in *Pl1-Rhoades* and *Pl1-Blotched*. Compared to *Pl1-Blotched*, in *Pl1-Rhoades*, the H3K4me3 level was 3-fold higher in the upstream region and 1.4-fold higher in the downstream region (Figure 3.2 A and B). These H3K4me3 signals for the upstream region were directly correlated with transcriptional activities of the two alleles. By contrast, the H3K4me2 levels did not correlate well with gene expression levels; in both the upstream and downstream regions, the levels of H3K4me2 in *Pl1-Rhoades* were lower than they were in *Pl1-Blotched* (Figure 3.2 A and B).

The H3K4me3 levels of the two control genes correlated well with their gene activities. For the constitutively expressed *gpc4*, H3K4me3 levels were much higher than for the inactive *dzs10* gene in both *pl1* lines (Figure 3.2 C and D). The levels of

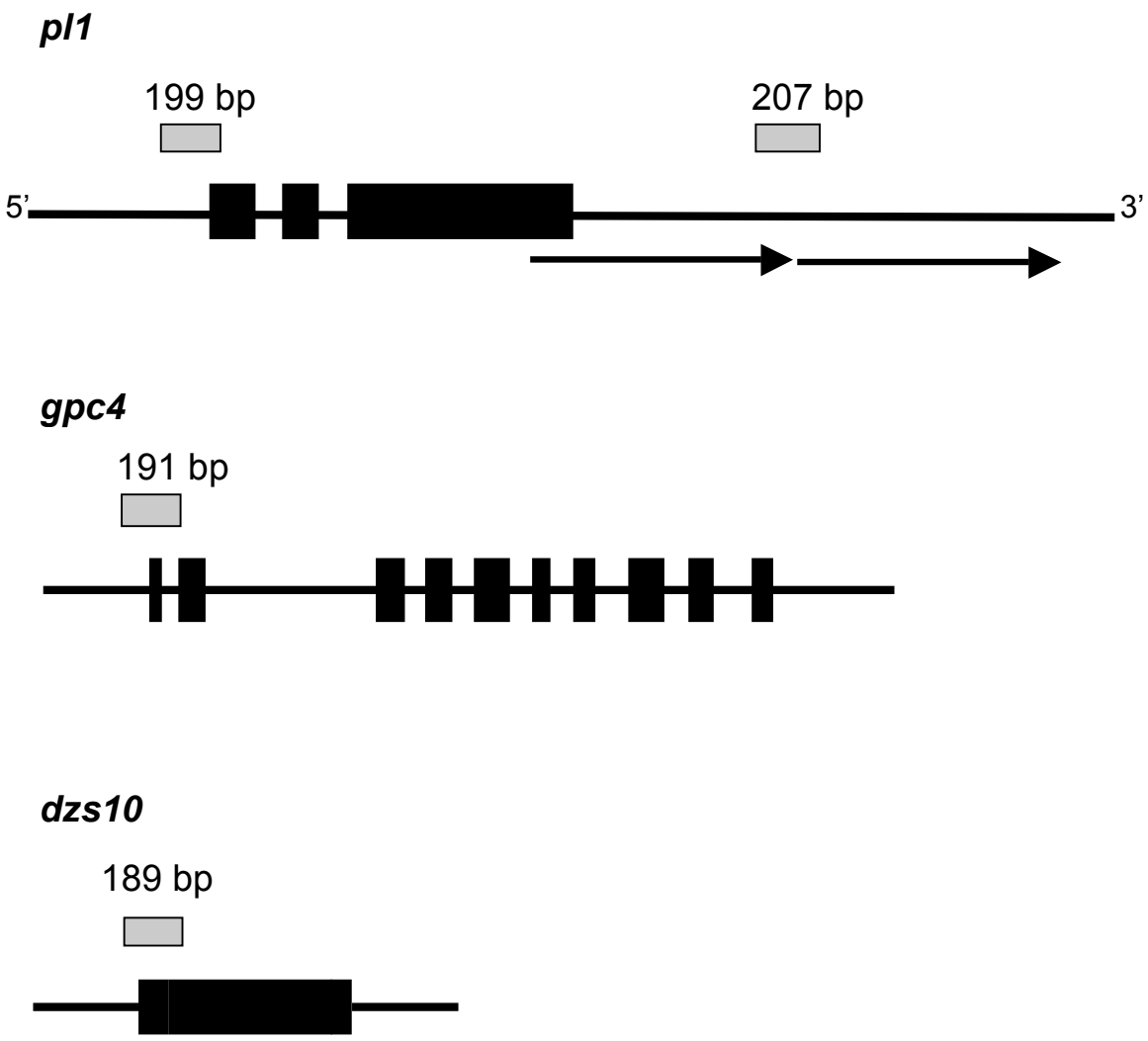


Figure 3.1. Diagrams of genes analyzed in ChIP assays. Black boxes indicate exons. Grey boxes indicate PCR-amplified regions.

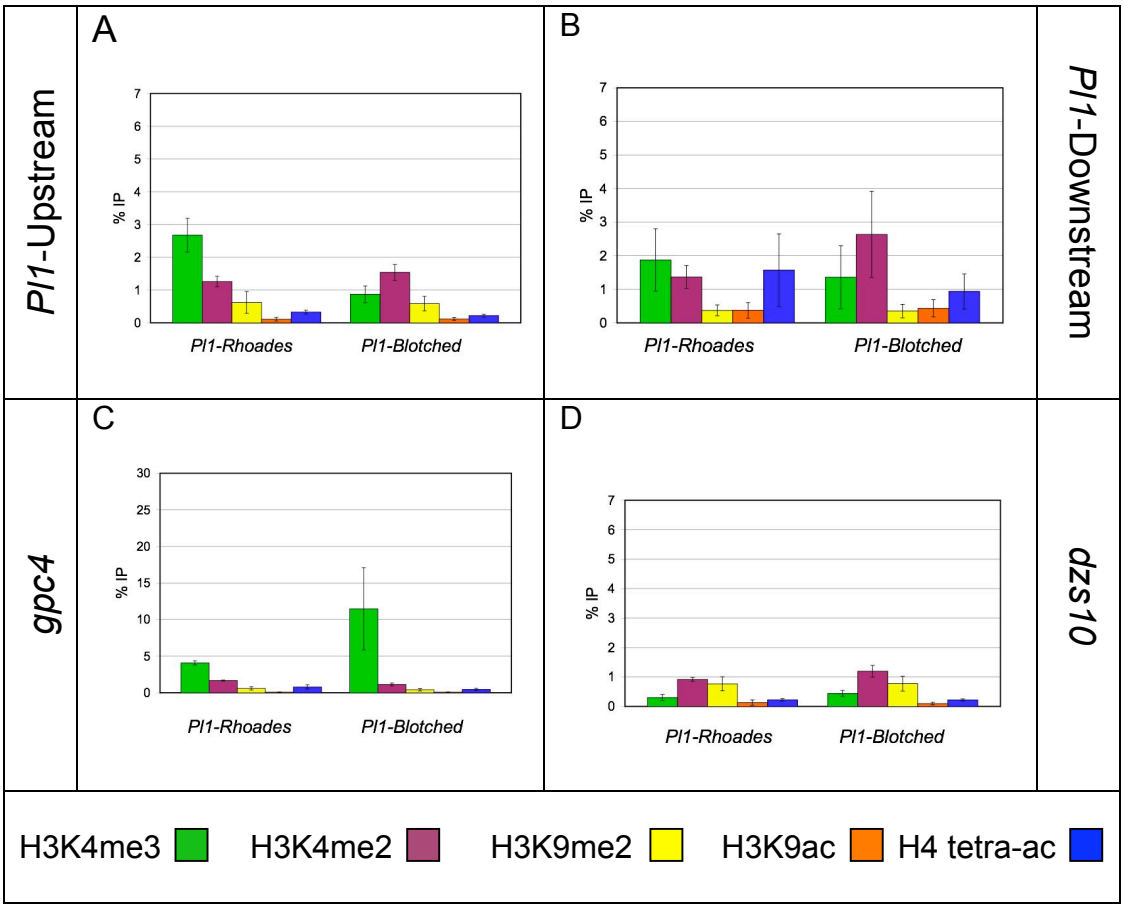


Figure 3.2. Comparison of histone modifications between *Pl1-Rhoades* and *Pl1-Blotched*. Columns depict mean % immunoprecipitation (%IP) from three experiments; error bars represent standard deviation. Note different scale for *gpc4* graph.

H3K4me2 did not differ much between *gpc4* and *dzs10*; both genes had low levels of this modification in the two *pl1* lines (Figure 3.2 C and D).

Di-methylation of histone H3 lysine 9 (H3K9me2) in many systems is considered to be a mark of inactive gene (Jenuwein and Allis, 2001; Lachner et al., 2001; Nakayama et al., 2001; Lindroth et al., 2004; Naumann et al., 2005; Ng et al., 2006). Thus, the less active genes examined–*Pl1-Blotched* and *dzs10*–were predicted to have higher levels of this modification than the active genes, *Pl1-Rhoades* and *gpc4*. However, this prediction did not hold true, as levels of this modification were low across all genes and genotypes (Figure 3.2 A, B, C and D).

Acetylation of histone H3 and histone H4

Histone acetylation, which can occur on histone H3 and histone H4, is usually associated with active gene expression (Grunstein, 1997; Johnson et al., 1998; Turner, 2000; Chua et al., 2001; Lusser et al., 2001). I examined three patterns of histone acetylation: H3K9ac, H4-tetra-ac, and H4K12ac. Because acetylation and methylation of H3K9 are mutually exclusive modifications and the levels of H3K9me2 we observed were low, one possible outcome of assaying acetylation might be that H3K9ac levels are high. However, this was not true. H3K9ac levels were low in all gene regions assayed in both genotypes (Figure 3.2). By contrast, H4-tetra-ac levels showed more variation across genes and genotypes. H4-tetra-ac levels were higher in the *pl1*-downstream regions of both *Pl1-Rhoades* and *Pl1-Blotched*, than in the respective upstream regions of each gene; and this modification was higher in *Pl1-Rhoades* than in *Pl1-Blotched* (Figure 3.2 A and B). In the control genes, H4-tetra-ac levels were low in both *gpc4* and *dzs10* in both genotypes (Figure 3.2 C and D).

Tetra-acetylation of H4 in *Arabidopsis thaliana* involves sequential acetylation of four lysines, beginning with K16, followed by K12, K8, and K5 (Earley et al., 2007). The reliable commercially available antibody specific for a single-lysine acetylation in H4

recognizes K12ac. When this antibody was used in our ChIP assays, no signals above the no-antibody control were obtained for any gene in any genotype (Table 3.5). Possible reasons for failing to detect this single modification, even though acetylation was detected with the H4-tetra-ac antibody, could be that H4 is only acetylated on K20 and/or K16 in maize or that the H4K12ac antibody does not recognize this modification in maize H4.

Methylation of Histone H3K27 or H4K20 (H3K27me2 or H4K20me2)

H3K27me and H4K20me are usually associated with inactive genes (Plath et al., 2003; Sims III et al., 2003; Ng et al., 2006). When antibodies for H3K27me2 or H4K20me2 were used for ChIP assays in this study, signals were no higher than the no-antibody controls for any gene region in any genotype (Tables 3.4 and 3.5).

Normalization to nucleosome occupany

Differences in the level of a particular histone modification, evident from direct measurements in ChIP assays, are influenced both by the absolute level of the modification and by the nucleosome occupancy on the assayed gene segment. To control for differential nucleosome occupancy, an anti-H3 antibody was used in every ChIP assay, so that histone modification-specific signals could be normalized to nucleosome occupancy by calculating the ratio of the signal obtained with each histone modification-specific antibody (Ab %IP) to the signal obtained for H3 (H3 %IP).

Nucleosome occupancy, as assessed by H3 %IP, was the same for *Pl1-Rhoades* and *Pl1-Blotched* at the upstream region, but slightly higher in *Pl1-Blotched* at the downstream region (Figure 3.3). Using nucleosome occupancy to normalize the data for histone modifications accentuated trends across the whole data set (Figure 3.4 A and B). The most notable differences in modification of the *pl1* gene were seen for two of the "active" gene modifications, H3K4me3 and H4 tetra-ac. In *Pl1-Rhoades*, the level of H3K4me3 was 7-fold higher than in *Pl1-Blotched* in the upstream region and 3-fold

Table 3.4. Summary of ChIP data for antibodies recognizing histone H3

Probe	Genotype	H3[a]		H3K4me3[b]		H3K4me2[b]		H3K9me2[b]		H3K9ac[b]		H3K27me2[c]	
		mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
Pl1-Up	*Pl1-Rhoades*	6.01	3.09	2.67	1.03	1.26	0.33	0.62	0.67	0.11	0.11	0.01	0.01
	Pl1-Blotched, no *Spb*	7.26	4.46	0.87	0.51	1.54	0.48	0.59	0.44	0.12	0.09	0.06	0.02
	Pl1-Blotched + Spb	11.68	8.13	1.38	0.43	2.16	0.35	0.56	0.44	0.37	0.47	0.06	0.06
Pl1-Down	*Pl1-Rhoades*	7.45	6.24	1.88	1.85	1.36	0.68	0.37	0.33	0.37	0.46	0.06	0.05
	Pl1-Blotched, no *Spb*	10.52	7.43	1.36	1.87	2.64	2.56	0.35	0.41	0.43	0.51	0.09	0.04
	Pl1-Blotched + Spb	26.07	13.46	2.28	2.11	4.90	2.97	1.48	1.37	0.87	0.97	0.31	0.19
gpc4	*Pl1-Rhoades*	6.26	4.09	4.07	0.57	1.66	0.12	0.58	0.42	0.08	0.06	0.00	0.00
	Pl1-Blotched, no *Spb*	4.13	1.40	11.47	11.23	1.14	0.37	0.40	0.30	0.08	0.01	0.01	0.00
	Pl1-Blotched + Spb	9.08	8.36	19.31	15.76	1.29	0.23	0.35	0.22	0.25	0.27	0.01	0.01
dzs10	*Pl1-Rhoades*	8.45	3.29	0.30	0.21	0.92	0.13	0.77	0.48	0.13	0.19	0.00	0.00
	Pl1-Blotched, no *Spb*	8.63	6.66	0.44	0.20	1.19	0.40	0.78	0.51	0.10	0.09	0.01	0.00
	Pl1-Blotched + Spb	15.36	18.85	0.47	0.20	0.99	0.30	0.49	0.36	0.20	0.30	0.02	0.01

[a] Used in six experiments
[b] Used in three experiments
[c] Used in two experiments

Table 3.5. Summary of ChIP data for antibodies recognizing histone H4

Probe	Genotype	H4-tetra-ac [a]		H4K12ac [a]		H4K20me2 [b]	
		mean	s.d.	mean	s.d.	mean	s.d.
Pl1-Up	*Pl1-Rhoades*	0.33	0.11	0.05	0.04	0.06	0.04
	Pl1-Blotched, no *Spb*	0.22	0.09	0.11	0.14	0.27	0.30
	Pl1-Blotched + Spb	0.94	0.73	0.15	0.20	0.21	0.21
Pl1-Down	*Pl1-Rhoades*	1.57	2.16	0.09	0.11	0.14	0.02
	Pl1-Blotched, no *Spb*	0.94	1.04	0.05	0.03	0.58	0.58
	Pl1-Blotched + Spb1	3.82	5.42	0.13	0.09	0.60	0.48
gpc4	*Pl1-Rhoades*	0.79	0.60	0.08	0.07	0.02	0.01
	Pl1-Blotched, no *Spb*	0.45	0.27	0.08	0.06	0.04	0.05
	Pl1-Blotched + Spb1	2.11	1.52	0.10	0.10	0.02	0.02
dzs10	*Pl1-Rhoades*	0.22	0.08	0.06	0.03	0.01	0.01
	Pl1-Blotched, no *Spb*	0.22	0.08	0.09	0.07	0.05	0.06
	Pl1-Blotched + Spb1	1.10	1.12	0.09	0.09	0.04	0.03

[a] Used in three experiments
[b] Used in two experiments

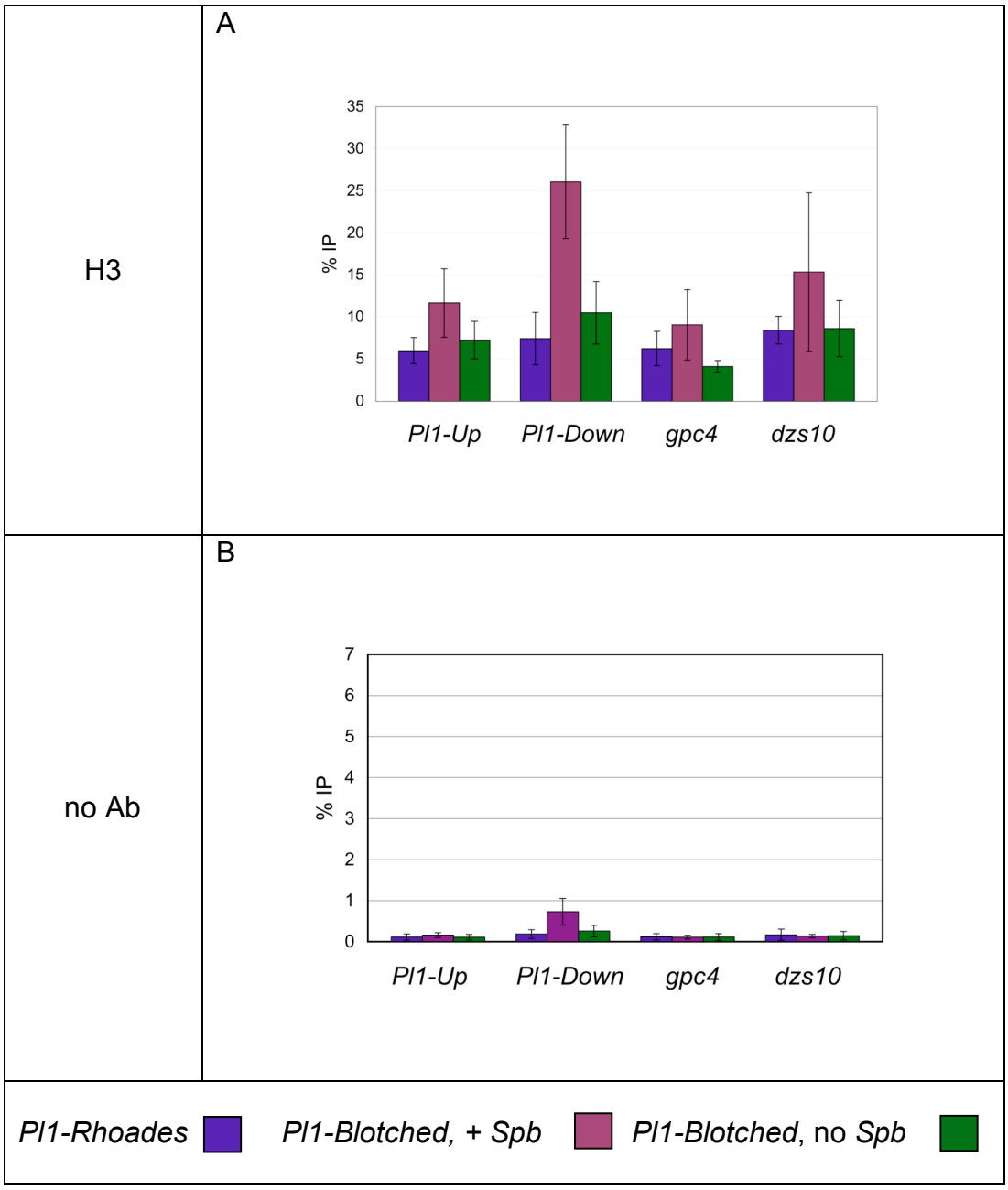


Figure 3.3. Histone H3 and no-antibody (no Ab) controls from ChIP experiments. Columns represent mean %IP from six experiments; error bars represent standard deviation.

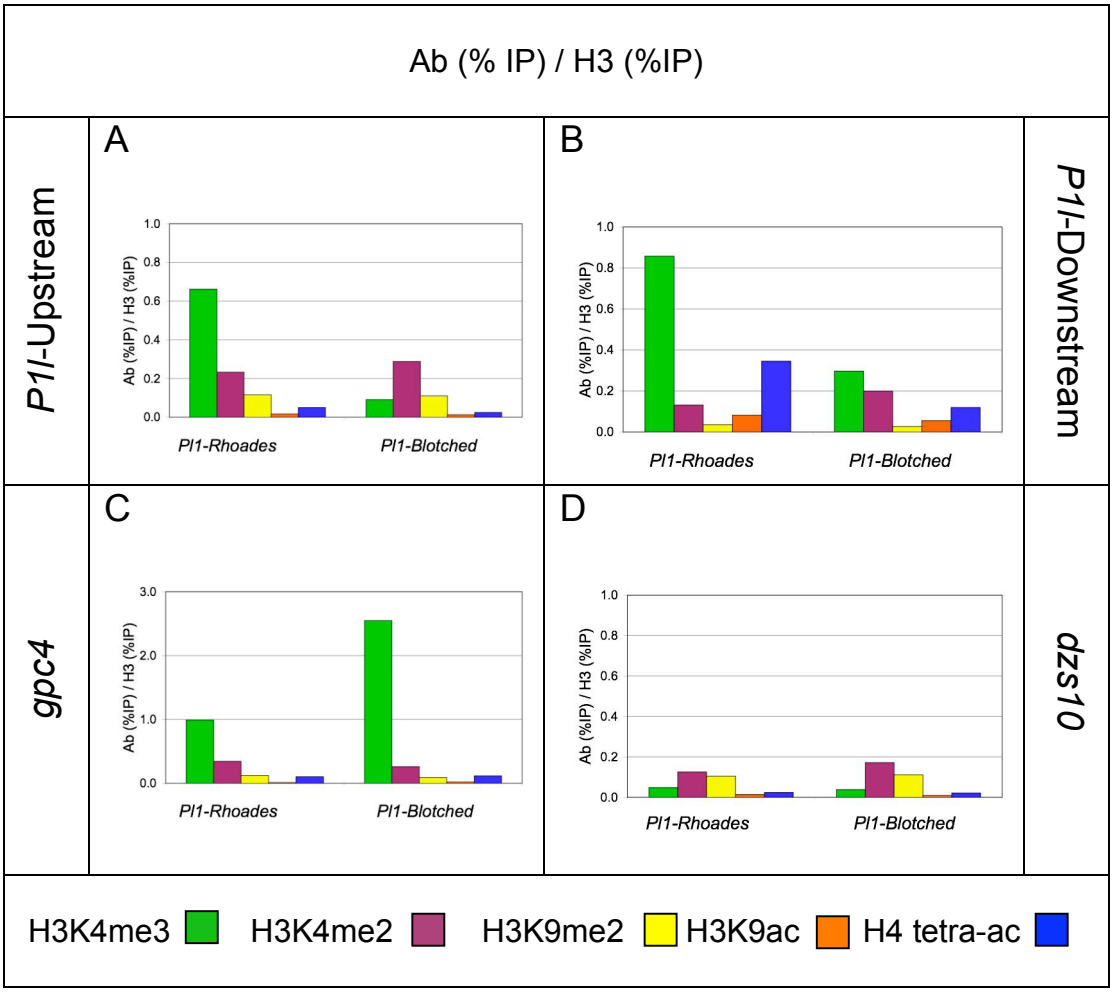


Figure 3.4. Normalized ChIP results comparing *Pl1-Rhoades* to *Pl1-Blotched*. Signal for each antibody (Ab %IP) was divided by the signal for anti-H3 antibody (H3 %IP). Columns represent ratios. Note different scale for *gpc4* graph.

higher in the downstream region of the gene. In both genotypes, H3K4me3 levels were higher downstream than upstream of the gene. The levels of H4 tetra-ac were low in the upstream region of both genotypes; however, in the downstream regions, this modification was noticeably higher in both genotypes and 2-fold higher in *Pl1-Rhoades* than in *Pl1-Blotched*. Together, these results suggest that H3K4me3 and H4 tetra-ac modifications downstream, rather than upstream, of the gene are better indicators of *pl1* gene activity.

At H3K9, di-methylation and acetylation were observed in both gene regions in both genotypes. Although these modifications were not very abundant, the relative abundance of the two showed a reciprocal relationship, as expected from the fact that these modifications are mutually exclusive. The upstream region in both *Pl1-Rhoades* and *Pl1-Blotched* had higher H3K9me2 levels and correspondingly lower H3K9ac levels, whereas the downstream region in both genotypes had lower levels of H3K9me2 and higher levels H3K9ac. The highest levels of H3K9ac were observed in the downstream region of *Pl1-Rhoades*.

For the control genes, H3K4me3 proved to be the best indicator of gene activity. This modification was high in constitutively expressed *gpc4*, but low in the inactive *dzs10* gene. In *gpc4*, the levels of H3K4me3 in *Pl1-Blotched* were 2.5-fold higher than in *Pl1-Rhoades*. This may reflect differences in expression of the *gpc* alleles carried in the two lines.

Comparison of *Pl1-Blotched* with and without *Spb*

Spb leads to increased expression of *Pl1-Blotched*. Given our observation that "active" histone modifications are enriched in the more highly expressed *Pl1-Rhoades* allele than in *Pl1-Blotched*, we predicted that the *Spb*-induced increase in *Pl1-Blotched* expression would be correlated with increased levels of "active" histone modifications. To evaluate this hypothesis, ChIP assays were performed to compare *Pl1-Blotched* with

Spb to *Pl1-Blotched* without *Spb*. In Figures 3.5 and 3.6, the data points for *Pl1-Blotched* without *Spb* are the same as those presented for *Pl1-Blotched* in the comparison to *Pl1-Rhoades* (Figures 3.2 and 3.4).

Methylation of H3K4 and H3K9

Two trends were obvious from measurements of absolute levels of H3K4 methylation. First, the levels of H3K4me3 and H3K4me2 were consistently higher downstream than upstream of *Pl1-Blotched*, in both the presence and absence of *Spb*. Second, in both gene regions, the levels of H3K4me3 and H3K4me2 were higher in *Pl1-Blotched* with *Spb* than without. The latter observation lends support to the notion that *Spb* increases the level of active gene modifications. However, this interpretation is tempered by the finding that *Spb* also led to higher levels of the canonical silent modification, H3K9me2, in the downstream region of *Pl1-Blotched*, albeit not in the upstream region. For the control genes, H3K4me3 levels were high for *gpc4* and low for *dzs10*, and neither pattern was affected by the presence of *Spb*.

Acetylation of Histone H3 and H4

In *Pl1-Blotched*, *Spb* led to higher levels of H3K9ac and H4 tetra-ac in both the upstream and downstream regions of the gene (Figure 3.5 A and B). This effect was the most dramatic for H4 tetra-ac levels, which were 4-fold higher in the presence of *Spb*. For both control genes, H3K9ac and H4 tetra-ac levels were also slightly higher in the *Spb* background.

Normalization to nucleosome occupancy

Nucleosome occupancy, assessed by immunoprecipitation with anti-H3 antibody, was two-fold higher in the downstream region of *Pl1-Blotched* when *Spb* was present (Fig. 3.3). At the other gene regions assays, a similar, but modest, trend towards higher nucleosome occupancy was seen in *Pl1-Blotched* plants with *Spb.* Normalizing histone modification levels to nucleosome occupancy showed that *Spb* had little effect on

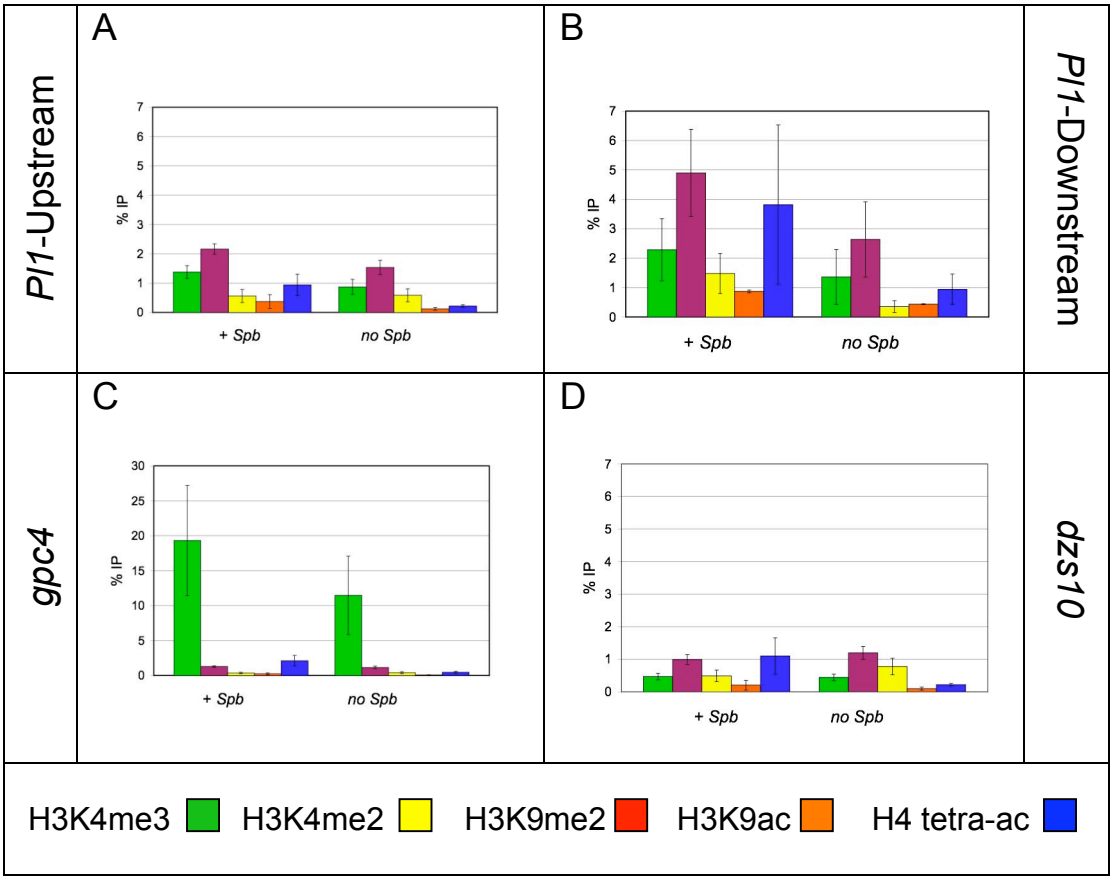


Figure 3.5. Comparison of histone modifications at *Pl1-Blotched* in lines with *Spb* (+*Spb*) and without *Spb* (no *Spb*). Columns represent mean % IP from three experiments; error bars repesent standard deviation. Note different scale for *gpc4* graph.

histone modifications in the upstream region of *Pl1-Blotched* and only modest effects on in the downstream region, evident as slight increases in H3K4me2 and H4 tetra-ac levels (Fig. 3.6 A and B). *Spb* did not alter the histone modification profiles for the two control genes (Fig. 3.6 C and D).

Discussion

Comparison of *Pl1-Rhoades vs. Pl1-Blotched*

The major differences between *Pl1-Rhoades* and *Pl1-Blotched* were higher levels of the active gene modifications, H3K4me3 and H4 tetra-ac, in *Pl1-Rhoades*. H3K4me3 was the most abundant modification in *Pl1-Rhoades* and was positively correlated with transcriptional activity. This observation corroborates studies from many other systems, which showed that H3K4me3 is associated with actively expressed genes. Unlike in animal systems, where H3K4me3 is located primarily upstream of genes (Santos-Rosa, 2002; Sakamoto et al., 2004; Barski et al., 2007), high levels of this modification were seen in both upstream and downstream regions of *Pl1-Rhoades*.

The downstream region was also the site of the second most abundant modification observed in this study, H4 tetra-ac. The level of this modification was directly correlated with gene activity, as *Pl1-Rhoades* had much higher levels than *Pl1-Blotched*. Perhaps the localization of these abundant active gene modifications in the downstream of the gene reflects a pattern common in plants, as in *Arabidopsis*, H3K4me3 is also found at upstream and downstream regions (Alvarez-Venegas and Zoya, 2005).

Comparison of *Pl1-Blotched* with and without *Spb*

At the outset of this study, I predicted that the levels of expression of *Pl1-Blotched* with *Spb*, which is intermediate between that of *Pl1-Rhoades* and *Pl1-Blotched*

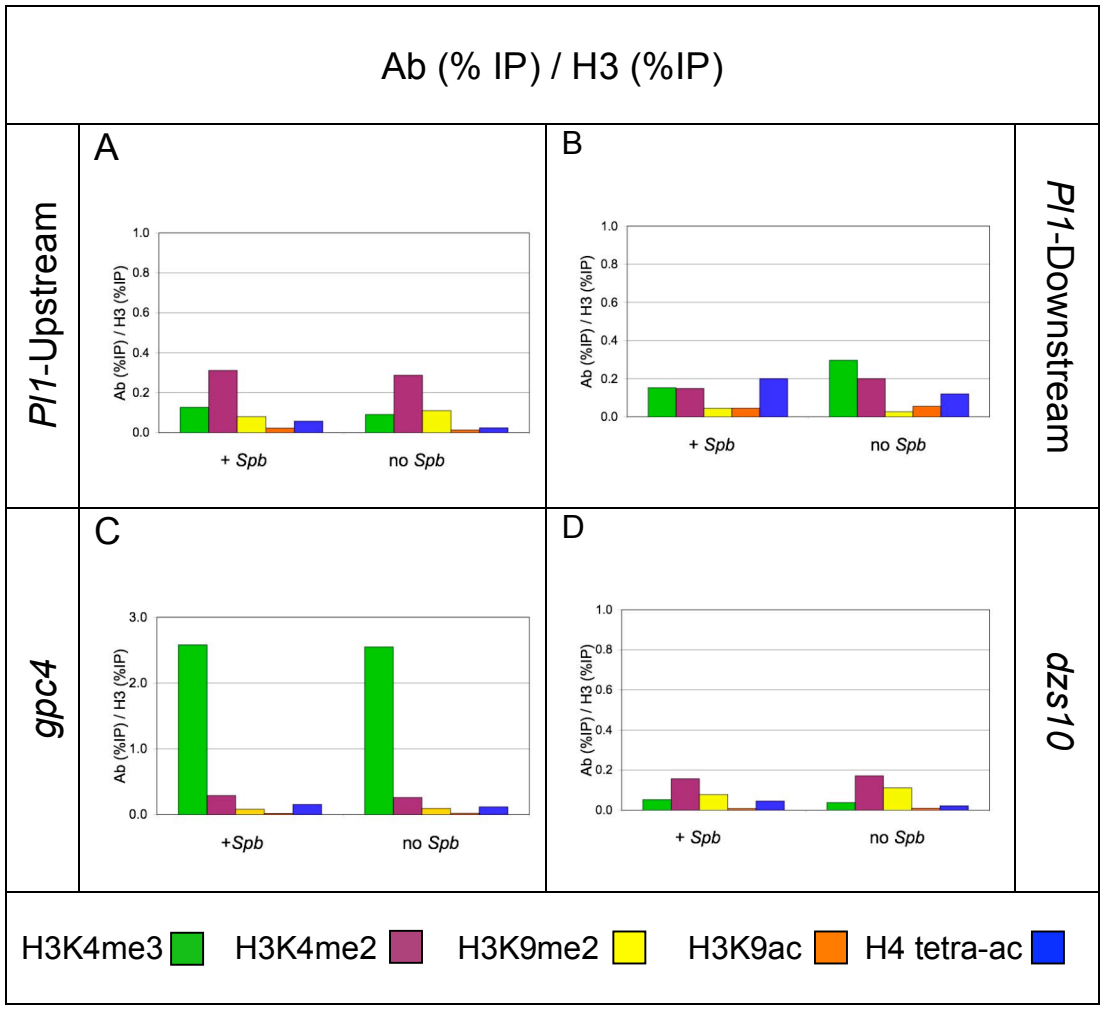


Figure 3.6. Normalized ChIP results comparing *Pl1*-Blotched with *Spb* (+ *Spb*) to *Pl1-Blotched* without *Spb* (no *Spb*). Signal for each antibody (Ab %IP) was divided by the signal for anti-H3 antibody (H3 %IP). Columns represent ratios. Note different scale for *gpc4* graph.

without *Spb*, would be reflected by intermediate levels of modifications typically associated with active genes, e.g., for H3K4me3 and for H4 tetra-ac. However, this prediction was not upheld. Higher levels of H4 tetra-ac were observed in *Pl1-Blotched* with *Spb* than without *Spb*, but the levels of H3K4me3 did not differ at all between these lines. Thus, the major histone modification difference that distinguishes *Pl1-Rhoades* from *Pl1-Blotched* -H3K4me3 level- is not what distinguishes *Pl1-Blotched* with *Spb* from *Pl1-Blotched* without *Spb* .

Instead, the main difference detected between *Pl1-Blotched* with and without *Spb* was with the anti-H3 antibody used as a nucleosome occupancy control. The presence of *Spb* led to a modest increase in H3 signals upstream of the *Pl1-Blotched* gene and 2- to 3-fold higher H3 signals in the downstream region. This suggests that *Spb* regulates nucleosome occupancy.

In other eukaryotes, changes in nucleosome occupancy and nucleosome positioning have been implicated in the regulation of gene expression (Yuan et al., 2005; Hogan et al., 2006; Lin et al., 2007) and the inherently dynamic nature of the nucleosome-DNA interaction permits flexibility in nucleosome positioning to allow modulation by other factors (Mellor, 2006; Rando and Ahmad, 2007). Usually, promoter regions are nucleosome-free, with ~200 bp upstream region of the transcription start site flanked on both sides by positioned nucleosomes (Yuan et al., 2005; Mito et al., 2007). This nucleosome positioning is modulated by protein complexes with ATP-dependent chromatin remodeling activity, In yeast (*Saccharomyces cerevisiae*), the chromatin remodeling protein, Isw2, functions to reposition nucleosomes at the interface between genic and intergenic sequences at both the 5’ and 3’ ends of genes (Whitehouse et al., 2007). Near the transcription start site, this Isw2-directed shift in nucleosome position enforces directionality on transcription, thereby preventing transcription initiation from cryptic sites. The function of Isw2 at the 3' end of genes is unknown (Arndt, 2007).

A link between chromatin remodeling and histone H4 acetylation has been found for two families of chromatin remodeling proteins. In yeast, *Drosophila* and *Xenopus laevis*, chromatin remodeling by ISWI chromatin remodeling factors requires H4 acetylation, but not H3 acetylation (Clapier et al., 2001; Hamiche et al., 2001; Kang et al., 2002; Schwanbeck et al., 2004; Durant and Pugh, 2007). Also in yeast, NuA4 acetylates histone H4, which is bound by the bromodomain proein, bdf1, which in turn recruits the general transcription factor, TFIID, and chromatin remodeling complex (SWR-C; Durant and Pugh, 2007).

In our system, the *Spb*-induced change in nucleosome occupancy of *Pl1-Blotched* is associated with differences in H4 tetra-ac. This parallel to chromosome remodeling activities in other systems makes it tempting to speculate that *Spb* plays a role in chromatin remodeling. If this is true, then the genes associated with the *Spb* activity might encode maize chromatin remodeling factors, histone acetyltransferases and bromodomain proteins. The studies presented in Chapter 4 address this possibility.

Similarities in histone modifications across all genes

Several of the histone modifications we examined were similar across all genes. H3K4me2 was detected at both upstream and downstream regions in *Pl1-Rhoades* and in *Pl1-Blotched* with and without *Spb*, but this modification was not positively correlated with transcriptional activity. In the less expressed *Pl1-Blotched*, the levels of H3K4me2 were higher than in the fully expressed *Pl1-Rhoades*. This may reflect the fact that H3K4me3 and H3K4me2 occur at the same site, such that if levels of H3K4me3 are high, then H3K4me2 are likely to be lower. The ratio of H3K4me3 to H3K4me2 then might be used as an activity index. To see if this is true for *pl1*, I calculated the H3K4m3 to H3K4m2 ratio at the upstream and downstream gene regions for *Pl1-Rhoades* and for *Pl1-Blotched* with and without *Spb*. As shown in Table 3.6, the ratio for the highly

Table 3.6. H3K4me3 to H3K4me2 ratios

Gene region	***Pl1-Rhoades***	***Pl1-Blotched***	***Pl1-Blotched* with*Spb***
Pl1-Upsteam	2.123137	0.565161	0.63625
Pl1-Downstream	1.373715	0.516039	0.466293
dzs10-Upstream	0.32816	0.368679	0.472931

expressed *Pl1-Rhoades* allele was 3- to 4-fold higher than for *Pl1-Blotched*. The *Pl1-Blotched* ratios were low and similar to that for the inactive *dzs10*.

In *Arabidopsis*, H3K4m2 at the *SUPERMAN* gene was present in genic regions no matter whether the gene was in an active or inactive state; in contrast, the modification was not present in intergenic regions (Alvarez-Venegas and Zoya, 2005). Based on these observations H3K4me2 was suggested as a general mark that distinguishes genes from non- transcribed sequence in *Arabidopsis*. In our results, H3K4me2 was detected at all genes examined, even the ones with lowest activity. Thus, like in *Arabidopsis*, H3K4me2 may be a general mark for genic regions in maize. However, before this conclusion can be made, H3K4me2 on more regions, both genic and intergenic regions need to be investigated.

The canonical silent gene mark, H3K9me2, was detected in all genes examined, regardless of whether the genes were active or not. Although the H3K9me2 has been associated with gene silencing and heterochromatin formation in yeast and *Drosophila* (Bannister et al., 2001; Jenuwein and Allis, 2001; Lachner et al., 2001; Nakayama et al., 2001; Noma et al., 2001; Zhang and Reinberg, 2001; Jacobs and Khorasanizadeh, 2002), in my study H3K9me2 was present in euchromatin and my results are consistent with the results of the cytological immunostaining in maize, in which H3K9me2 was found in euchromatic chromosome arms, not in heterochromatin associated with knobs and pericentromeres (Shi and Dawe, 2006).

My results also confirm other aspects of the immunostaining of maize chromosomes (Shi and Dawe, 2006). I did not detect H4K20 methylation in any of the genes examined here, confirming the previous conclusion that this modification is not present in maize. Also, I failed to detect H3K27 methylation at any of the genes I analyzed; this fits with the previous data showing that this canonically silent modification is only localized in a few discrete regions of the maize genome.

Summary

In conclusion, I have shown that chromatin-level regulation distinguishes expression states of *pl1*. Changes in the level of active chromatin modifications are the main mechanism distinguishing *Pl1-Rhoades* from *Pl1-Blotched*. By contrast, the chromatin changes in *Pl1-Blotched* induced by *Spb* are alterations in nucleosome occupancy and increased histone H4 acetylation. These latter features suggest a role for *Spb* in chromatin remodeling.

CHAPTER 4. QUANTITATIVE TRAIT LOCUS (QTL) MAPPING OF *SPB*

Results of molecular studies aimed at understanding the effect of *Spb* on *Pl1-Blotched* support the idea that *Spb* regulates the chromatin organization of *Pl1-Blotched* (Chapter 2 and Chapter 3). Understanding exactly how this regulation occurs will require cloning *Spb*. Map-based cloning has been used recently to isolate several maize genes (Bortiri et al., 2006) and a similar strategy should prove useful for cloning *Spb*.

Previous mapping of *Spb* showed that the increased pigmentation does not segregate as a single dominant locus in an F_2 derived from crossing *Pl1-Blotched* with *Spb* by a less pigmented line (Courtney, 2001). Instead, the phenotype segregates as a quantitative trait, which shows continuous variation in phenotypic expression resulting from the collective effects of multiple genes, modified environmental effects, and interactions of genotype and environment (Edwards et al., 1987). The development of polymorphic genetic markers that span the whole genome has made it possible for quantitative and molecular geneticists to investigate QTL accurately. In these studies, QTL is identified as significant statistical associations between genotypic values and phenotypic variability among the segregating progeny (Beavis, 1998). Such QTL analysis can identify genes that regulate or control variation in phenotypic expression (McMullen, 2003).

To identify what gene or genes are responsible for the *Spb* influence on *Pl1-Blotched*, QTL mapping was used to identify genomic regions contributing to the high-pigmentation phenotype associated with *Spb*. Chromatin genes in these regions can be further studied as potential *Spb* candidates.

Materials and Methods

Plant materials

To generate an F_2 population for QTL mapping of *Spb*, highly pigmented *Pl1-Blotched* with *Spb* plants were crossed by plants of a less pigmented stock generated in the Cone laboratory (designated *Y1 Pl1-Blotched Su2* and referred to here as the non-*Spb* stock). Both lines are homozygous recessive for *c1,* homozygous for *B-I*, and homozygous dominant for the anthocyanin structural genes. F_1 plants were self-pollinated to produce F_2 ears. F_2 individuals were grown in the field at the University of Missouri Genetics Farm in Columbia, MO in the summer of 2003, and self-pollinated to produce $F_{2:3}$ ears.

Phenotype measurements

Anthocyanins were measured from leaf sheaths from 189 F_2 plants grown in 2003. Approximately two weeks after silk emergence, the leaf sheath attached to the second node below the first ear node was removed from each plant by cutting at the point of attachment to the node. Leaf sheaths were labeled and stored in plastic bags at -20˚C. From each sheath, a 1- x 2-cm, rectangle, which spanned the midline at the base of the sheath, was cut and placed in 2 ml of 1% HCl in 95% ethanol in a 15-ml capped tube at room temperature for 16 hrs in the dark. The absorbance of the resulting extracts was measured at 530 nm using a spectrophotometer (LKB Ultrospec II 4050, Ontario, Canada).

DNA isolation and genotype determination

For DNA isolation, leaf tissue was collected from 20 $F_{2:3}$ seedlings at the V3-V4 stage and bulked to reconstitute the F_2 genotypes. Tissue was frozen and DNA was prepared by a rapid isolation method (Cocciolone and Cone, 1993). For genotype

determination, a combination of restriction fragment length polymorphism (RFLP) (Lander and David, 1989) and simple sequence repeat (SSR) markers was used. Because we believe that *Spb* is a chromatin-level modifier, I chose RFLP markers derived from previously mapped chromatin genes (http://chromdb.org and http://maizegdb.org). SSR markers were derived from the publicly available collection (http://maizegdb.org). Parental and F_1 DNAs were used to screen for RFLP and SSR polymorphisms. In all, around 50 RFLP and over 1,000 SSR were screened. Genotyping with 20 RFLP markers was performed by digesting the F_2 DNA with one of six restriction enzymes, fractionating digests on 1% agarose gels, transferring DNA to nylon membranes, and hybridizing with radioactively labeled probes as described in Chapter 2. Genotyping with 93 SSR markers was performed using the following PCR conditions; 10 cycles, beginning with 95°C for 1 min, 65°C for 1 min, 72°C for 1.5 min with a 1°C decrement per cycle; and then 34 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min. Genotypes were scored manually (A, homozygous for allele derived from *Spb* parent; B, homozygous for allele from non-*Spb* parent; H, heterozygous) and independently verified.

Data analysis

MAPMAKER/EXP verson 3.0b was used to construct linkage maps using genotype data from 232 plants (Lander et al., 1987). QTL Cartographer version 1.16c (Basten et al., 1999) was used for QTL analysis. Experiment-wise significance thresholds at $p = 0.05$ were determined by analysis of 1,000 permutations of the data (Churchill and Doerge 1994). The statistical program EPISTACY was used to test for the presence of epistatic interactions between pairs of markers (Holland, 1998). Two methods were used for building multi-locus models (MLM). In the first method, stepwise regression at $p < 0.05$ by SAS PROC REG was used to build MLM, incorporating the nearest markers to single-effect QTL, interactions, and markers involved in the

interactions (SAS Institute, Inc., Carey, NC, 1998). Markers were added to the model in order of increasing *p*-value (forward regression in) and were removed if their significance while in the model exceeded 0.05 (backward regression out). The second method used to build a MLM was the SAS GLMSELECT experimental procedure (SAS v. 9.1, Cary, NC). The GLMSELECT procedure performs effect selection in the framework of general linear models, and compares most closely to REG and GLM. The genetic terms entered into GLMSELECT included the SSR marker most closely associated with each single-effect QTL (i.e., those identified by QTL Cartographer with a LOD score of 3.5 or greater) and the two-locus interactions (identified by EPISTACY). Stepwise selection was performed based on the adjusted R-square statistic, with a significance level of $p = 0.05$ for entry into and for retention in the model. Genotype class means were obtained through PROC MEANS using the CLASSES statement of SAS.

Locating chromatin candidate genes in QTL regions

To determine what chromatin genes are located in the QTL regions, chromatin gene transcript sequences were retrieved from ChromDB.org and compared by BLASTN to BAC sequence data from Maizesequence.org. Then the BAC sequences were matched to BAC contig and contigs were matched to chromosomal positions as recorded at Maizesequence.org. Date of the comparison was 2/12/08. The following filters were applied to the data: BAC sequences with bit scores < 199 were excluded. BAC contigs corresponding to the QTL regions included were: 2.05 contigs 82-91 (between *umc2007* and *bnlg1036*); 5.03 contigs 236-242 (between *bnlg2323* and *umc1155*); 6.05 contigs 262-285 (between *umc2311* and *umc1020*); 7.02 contigs 296-298 (between *mmc0171* and *umc1978*). Finally, redundant query ID for each QTL region was removed, retaining the match with the highest bit score. The resulting table included 126 genes (Appendix 1).

Results

Phenotypic analysis

An F_2 mapping population was generated from crossing two *Pl1-Blotched* lines, one with *Spb* and one without this modifier. The population was planted and phenotypically scored in the summer field season in 2003. Pigment levels of 189 F_2 plants exhibited a non-normal continuous distribution with evidence of transgressive segregation (Fig. 4.1). Approximately 20% of the plants (37/189) had anthocyanin levels lower than the non-*Spb* parent (mean 4.4) and about 13% of the plants (25/189) had anthocyanin levels higher than the *Spb* parent (mean 34.5).

Linkage maps

A linkage map was generated for the F_2 population. Genotypes of 232 F_2 individuals were determined for a total of 113 RFLP and SSR markers. Segregation distortion was observed for 15 of the 113 markers, but in no case was distortion seen for adjacent markers. The resulting map had a total genetic distance of 1298 cM with an average interval length 12 cM. The largest non-polymorphic region was on chromosome 6 and included the location of *pl1*. This lack of polymorphism probably reflects the history of the two mapping parents, which were both selected to contain *Pl1-Blotched* (likely the same allele); thus, in generating stocks homozygous for *Pl1-Blotched*, adjacent chromosomal regions were also "fixed". Otherwise, marker order and approximate distances were consistent with the IBM2 map at MaizeGDB (http://maizegdb.org)

QTL detection

Four major effect QTL were detected with LOD values above the arbitrary threshold of 3.5 used by QTL Cartographer (Table 4.1; Fig. 4.2). LOD is likelihood ratio

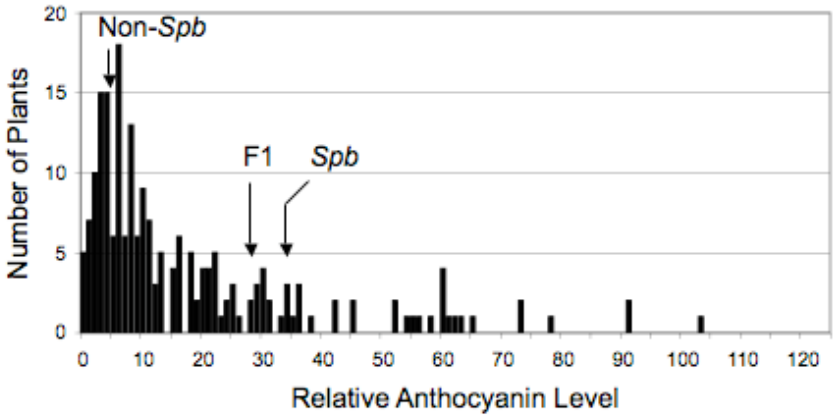


Figure 4.1. Phenotypic distribution in the F2 population. Anthocyanin levels for the parents and the F1 are indicated with arrows.

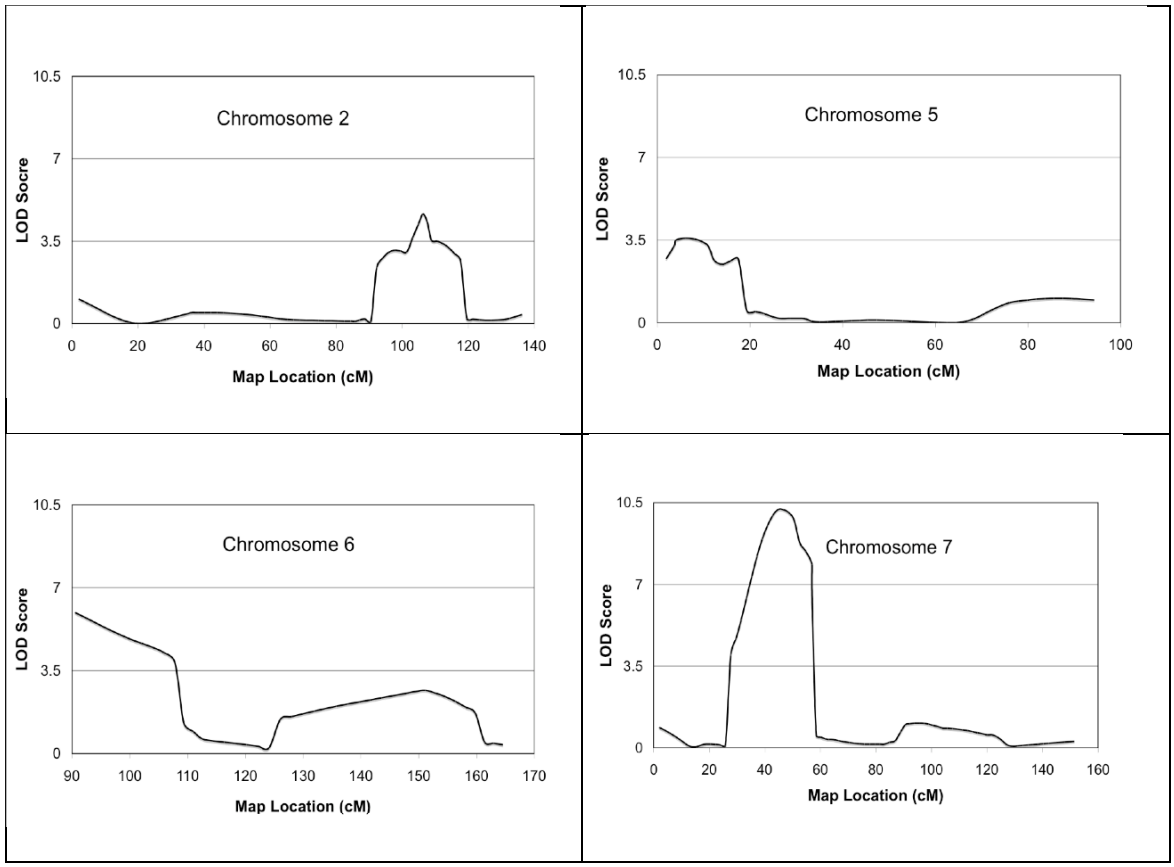


Figure 4.2. LOD scores of QTL. Distances are measured from the terminal marker on the short arm of each chromosome. LOD score was calculated by dividing the likelihood ratio by 4.605. LOD of 3.5 was used as the arbitrary significance threshold.

Table 4.1. QTL determined by QTL Cartographer

Bin[a]	Nearest Marker	Position (cM)	LOD	Genetic Effect[b]		Partial R^2	Donor parent
				A	D		
2.05	*umc0401*	104.84	4.7	7.4537	-4.262	0.0813	*Spb*
5.03	*umc1274*	5.08	3.6	-5.8106	3.7859	0.0603	Non-*Spb*
6.05	*umc1352*	0.01	6.0	9.1713	1.5969	0.1045	*Spb*
7.02	*umc1016*	43.77	10.2	-11.4413	-7.6524	0.2267	Non-*Spb*

[a] Chromsome.bin number
[b] A, additive; D, dominance

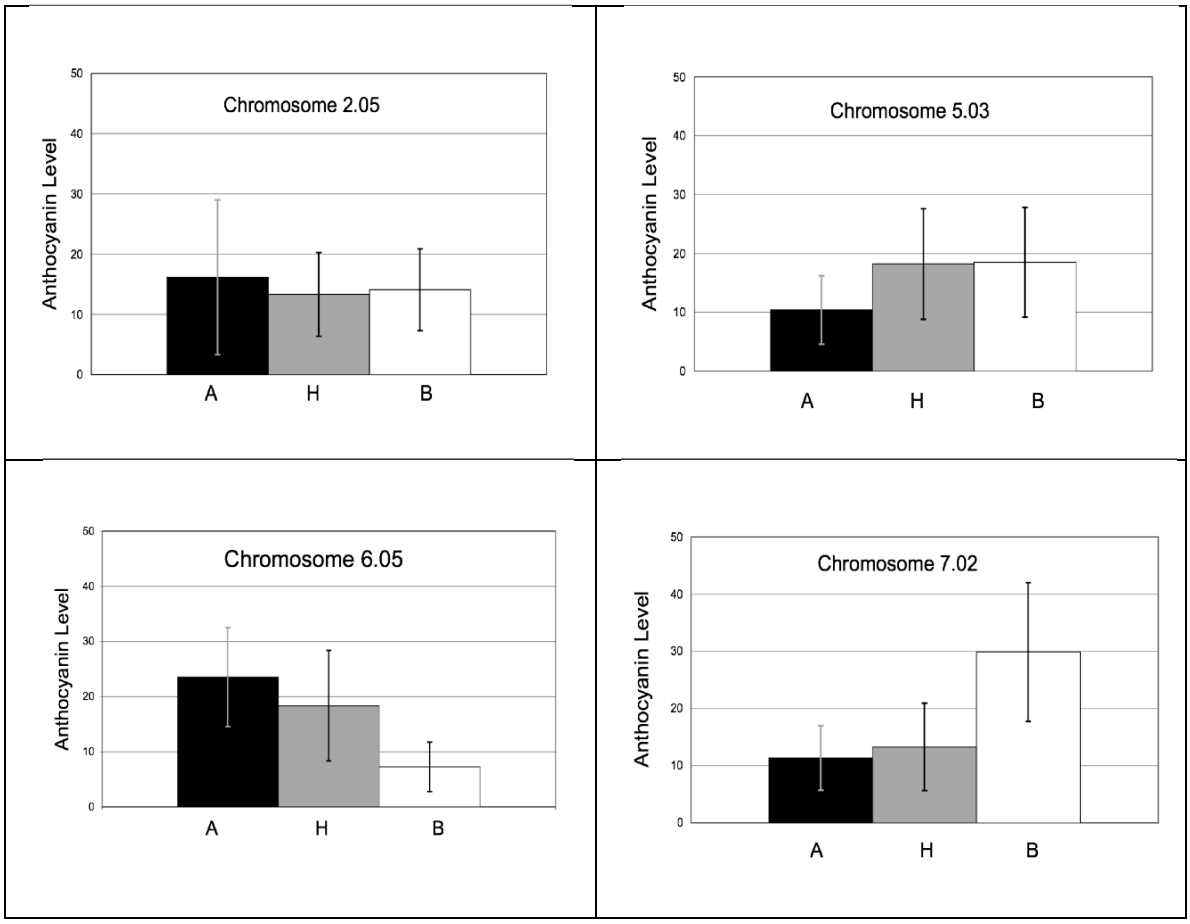


Figure 4.3. Mean anthocyanin phenotypes for genotype classes for the four QTL. Genotype designations: A, homozygous for alleles from *Spb* parent; H, heterozygous; B, homozygous for alleles from the non-*Spb* parent.

(LR)/4.6032. The QTL with the largest effect was located near marker *umc1016* on chromosome 7 in bin 2, e.g., 7.02 (maize chromosomes are divided into ~20 cM segments called bins, which are numbered from the tip of the short arm). This QTL explained approximately 23% of the phenotypic variation. Permutation analysis established an experiment-wise threshold LOD value of 7.8 at significance level p=0.05; thus this QTL was the only one supported by permutation analysis. Plants homozygous for the *umc1016* allele from the non-*Spb* parent had pigment levels approximately three-fold higher than heterozygotes or plants homozygous for the allele from the *Spb* parent (Fig. 4.3D). Thus, this QTL acts as a recessive enhancer of pigmentation. The location of this QTL is close to that of a previously described anthocyanin gene, *intensifier1* (*in1*), which acts in a recessive manner to intensify pigmentation.

The QTL with the second largest effect was on chromosome 6.05 near marker *umc1352* (Table 4.1). This QTL explained approximately 10% of the phenotypic variation. The *umc1352* allele contributing to increased pigmentation is from the *Spb* parent and appears to act in an additive fashion. The two remaining QTL explained approximately equal amounts of the phenotypic variation, e.g., 8% for the QTL on 2.05 near *umc0401* and 6% for the QTL on 5.03 near *umc1274*. The 5.03 QTL has a dominant effect and increases pigmentation by nearly two-fold when homozygous or heterozygous (Fig. 4.3B). This QTL region also includes a known anthocyanin gene, *anthocyaninless2* (*a2*), which is one of the structural genes in the biosynthetic pathway.

Twenty-six epistatic interactions were found (Table 4.2). These involved a total of 27 loci, including two of the single-effect QTL. Two multi-locus models (MLM) were made. The first, which was built by step-wise regression (Table 4.3), incorporated all four QTL, one interaction between the 2.05 and 7.02 QTL, one interaction between non-QTL loci, and the two interacting loci; this model explained 32% of the phenotypic variation. The second MLM, built using GLM SELECT (Table 4.4), incorporated all four

Table 4.2. Two-way epistatic interactions as determined by Epistacy

Locus1	Bin	Locus2	Bin	partial R^2
bnlg1866	1.03	*hon105*	1.07	0.10415
bnlg1866	1.03	*ph021*	4.03	0.11194
chr106	1.10	*umc1080*	2.06	0.09393
hxa102	1.10	*bnlg131*	1.11	0.09963
hxa102	1.10	*umc2007*	2.04	0.10495
hxa102	1.10	*mmc0401*	2.05	0.11363
hxa102	1.10	*umc1108*	2.06	0.10428
hxa102	1.10	*bnlg1036*	2.06	0.09789
mmc0401	2.05	*bnlg2132*	5.02	0.12181
mmc0401	2.05	*mmc0171*	7.00	0.09262
mmc0401	2.05	*umc1545*	7.00	0.09953
mmc0401	2.05	*umc1016*	7.02	0.10563
mmc0411	7.02	*umc1001*	7.03	0.15429
umc1015	7.03	*chr111*	7.00	0.10978
umc1015	7.03	*umc1001*	7.03	0.15521
umc1080	2.06	*bnlg1318*	4.01	0.09552
umc1080	2.06	*umc2281*	4.03	0.10242
umc1166	1.02	*hac101*	3.04	0.10244
umc1166	1.02	*umc1449*	3.04	0.09785
umc1431	1.09	*umc2007*	2.04	0.09962
umc1431	1.09	*mmc0401*	2.05	0.09867
umc1545	7.00	*bnlg2132*	5.02	0.08731
umc1976	1.02	*umc1967*	9.01	0.11505
umc1983	7.02	*umc1001*	7.03	0.16335
umc2007	2.04	*umc2281*	4.03	0.10962
umc2007	2.04	*umc1545*	7.00	0.10242

[a] Chromosome.bin number

Table 4.3. MLM determined by step-wise regression

Bin	Marker	df	Parameter	Std. Error	t-value	P-value
6.05	umc1352	1	-8.55986	2.11251	-4.05	<.0001
7.02	umc1016	1	12.35803	3.31289	3.73	0.0003
2.05 x 7.02	mmc0401_umc1016	1	-3.85642	2.66680	-1.45	0.1505
5.03	umc1274	1	5.21835	1.73923	3.00	0.0032
1.10 x 2.06	hxa102_umc1108	1	-1.13197	2.80919	-0.40	0.6876
2.05	mmc0401	1	-1.92210	3.43240	-0.56	0.5764
1.1	hxa102	1	-3.11177	3.58152	-0.87	0.3865
2.06	umc1108	1	-0.14334	3.41214	-0.04	0.9666
Total R^2	**0.3242**					

Table 4.4. MLM determined by GLM SELECT

Bin	Marker
7.02	umc1016
2.05	mmc0401
6.05	umc1352
5.03	umc1274
1.10 x 2.06	hxa102*umc1108
7.03 x 7.03	umc1015*umc1001
2.06 x 4.03	umc1080*umc2281
1.03 x 4.03	bnlg1866*ph021
2.05 x 7.02	mmc0401*umc1016
2.05 x 7.00	mmc0401*umc1545
2.05 x 7.00	mmc0401*mmc0171
Total R^2	**0.3499**

QTL and a total of seven interactions. The two interactions identified in the first MLM were included in this model, as well as two more interactions involving the 2.05 QTL and three interactions between non-QTL loci. This model explained 35% of the phenotypic variation.

Discussion

I set out to map the location of gene(s) responsible for *Spb*-induced epigenetic alterations in *Pl1-Blotched*. The results indicate that the effect of *Spb* involves multiple genes and multiple gene interactions. This makes *Spb* different from previously characterized modifiers of epigenetic phenomina in maize, such as *mediator of paramutation1* (*mop1*, Dorweiler et al., 2000; Alleman et al., 2006), *required to maintain repression (rmr*) *1, 2 and 6*, (Hollick and Chandler, 2001; Hollick et al., 2005; Hale et al., 2007) which are single genes.

Two of the QTL regions contain anthocyanin genes. The chromosome 7.02 QTL contains the *in1* gene. *In1* codes for b-HLH protein related to the anthocyanin regulatory protein R1/B1 (Burr et al., 1996). The IN1 protein is thought to function as a repressor, as recessive mutants lead to higher than normal levels of pigmentation (www.maizegdb.org). In my F_2 population, the allele for the 7.02 QTL that contributes to higher pigment levels is from the non-*Spb* parent, raising the possibility that this line is homozygous recessive for *in1*. Supporting this idea is the observation that the genetic behavior of this QTL is like that of *in1*, e.g., when the allele from the non-*Spb* parent is homozygous, pigmentation is higher than in the heterozygote or the other homozygote (Fig. 4.3D).

The 5.03 QTL region contains *a2*, a structural gene, which encodes one of the anthocyanin biosynthetic enzymes. The allele contributing to higher pigment for this QTL is also from the non-*Spb* parent. Both mapping parents have functional *a2* alleles,

because both produce anthocyanin pigments. Conceivably, the allele from the non-*Spb* parent produces a more active or more stable enzyme, which can lead to more efficient catalysis and thus more pigment.

Our previous studies established that *Spb* leads to four types of epigenetic change at *Pl1-Blotched*. The first is a modest change in DNA methylation (Courtney, 2001); the second effect is relaxation of chromatin structure (Chapter 2); the third is altered nucleosome occupancy or positioning; and the fourth is an increase in histone H4 acetylation. Together, these observations suggest that the best chromatin gene candidates are DNA methyltransferases, histone acetyltransferases, histone deacetylases, chromatin remodeling proteins, and proteins involved in nucleosome sliding or replacement. Chromatin genes located in the four QTL regions were identified by BLASTN comparison of chromatin-gene transcript sequences (chromdb.org) to the DNA sequences of maize BAC clones sequenced by the Maize Genome Sequencing Consortium (maizesequence.org) and then by matching identified clones to their BAC contigs and mapped chromosomal position. A list of the 126 chromatin genes identified in BLASTN searches with bit score over 200 are listed in Appendix 1.

A subset of those genes (32) fall into the categories considered to be the best chromatin gene candidates for *Spb* (Table 4.5). Of special interest are the genes that code for proteins in the same classes as the yeast chromatin remodeling complexes that mediate histone H4 acetylation-dependent chromatin remodeling (Kang et al., 2002; Durant and Pugh, 2007). The maize genes and their proposed functions are: *nfe*, ATP-dependent nucleosome sliding; *arp, che,* and *chr*, ATP-dependent chromosome remodeling proteins, including those related to SNF2; *hac* and *hag*, histone acetyltransferases; *brd* and *gte*; proteins with bromodomains capable of binding acetylated lysines and interacting with chromatin remodeling proteins. These genes are prime candidates for future analyses.

Table 4.5. Selected chromatin gene candidates in QTL region

Gene	Location[a]	Protein description[b]
arp105	2	Nuclear actin-related protein; component of chromatin remodeling complex
arp108	5	
brd104	7	Single bromodomain-containing protein; similar to human BRD7; bromodomains can interact specifically with acetylated lysine
che101	2, 6	Chromatin remodeling, subunit E
chr130	2	Chromatin remodeling complex subunit R; SNF2 super family
chr139	2	
chr150	2, 5, 6	
chr151	7	
chr153	2, 5, 6, 7	
chr160	6	
chr163	6	
chr164	6	
dmt101	7	DNA methyltransferase; Class I, related to mammalian DNMT1
dmt103		DNA methyltransferase; Class III, with rearranged catalytic domains, related to mammalian DNMT3
dmt107	2	DNA methyltransferase; Duplicate of dmt103
gta101	6	Global transcription factor; Transcription elongation-nucleosome displacement proteins; Spt5 homolog
gta106	6	
gta107	6	
gte102	5	Global transcription factor group E (Bdf1, BRD4 and BRD2 (RING3) homologs)
gte109	2	
gte110	6	
hac113	2,5,6	Histone acetyltransferase; related to CREB-binding protein; co-activator
hac114	6	
hag102	7	Histone acetyltransferases (GNAT superfamily)
hda101	5	Histone deacetylase; Class I; Rpd3/HDA1 superfamily;
hda110	7	Histone deacetylase; Class I; Rpd3/HDA1 superfamily;
hda116	5	Histone deacetylase; Class I; Rpd3/HDA1 superfamily;
hda119	5	Histone deacetylase; Class I; Rpd3/HDA1 superfamily;
hdt103	6	Histone deacetylases; plant-specific HD2 family
nfe101	5	Nucleosome positioning factor; ATP-dependent chromatin assembly factor involved in nucleosome sliding
nfe102	5	
tafv101	2	TATA binding protein associated factor 5 protein

[a] Chromosomal location; multiple entries reflect uncertainty
[b] Descriptions from ChromDB.org

CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS

This study was carried out to characterize the epigenetic mechanisms that control expression of the maize *purple plant1* (*pl1*) gene at the chromatin level. Using DNase I sensitivity assays and ChIP assays, I made two pair-wise comparisons: *Pl1-Rhoades* to *Pl1-Blotched*, and *Pl1-Blotched* in the presence and absence of *Spb*.

Pl1-Rhoades and *Pl1-Blotched* have very similar gene sequences, but different levels of expression. Previous studies showed that differential expression is correlated with altered patterns of DNA methylation and nuclease sensitivity. My results showing that *Pl1-Rhoades* has higher levels of histone modifications that are usually associated with active genes confirm that the expression differences are due to distinct epigenetic features.

Interestingly, these epigenetic features occur both upstream and downstream of the *pl1* gene. For example, DNA methylation is altered at the *Msp*I/*Hpa*II site in the downstream region, and nuclease sensitivity is higher at this region in *Pl1-Rhoades* compared to *Pl1-Blotched* (Hoekenga et al., 2000; Chapter 2). Also, I found that the higher levels of active histone modifications–H3K4me3 and H4 tetra-ac–in *Pl1-Rhoades* were evident in upstream and downstream regions (Chapter 3). In fact, all of the epigenetic differences between *Pl1-Rhoades* and *Pl1-Blotched* are more obvious at the downstream region of *pl1* gene, indicating that this region of the *pl1* gene is important for regulation of gene expression.

The same trend is evident from analysis of the effect of *Spb* on *Pl1-Blotched*. In the presence of *Spb*, *Pl1-Blotched* has an altered DNA methylation pattern both upstream and downstream of the gene (Courtney, 2001). DNase I sensitivity assays revealed higher nuclease sensitivity in the presence of *Spb*, especially in the downstream region (Chapter 2). The results of the ChIP assays showed that *Spb* has

two effects on *Pl1-Blotched*: In the presence of *Spb*, H4 tetra-ac is more enriched in the downstream region of *Pl1-Blotched* than when *Spb* is absent (Chapter 3). Also, the presence of *Spb* leads to a detectable increase in nucleosome occupancy downstream of *Pl1-Blotched*, which probably reflects a shift in the position of nucleosomes in this region, and perhaps across the whole gene. Together, these results suggest that *Spb* increases *Pl1-Blotched* expression by chromatin remodeling, leading to a more relaxed structure that is more accessible to the transcriptional machinery.

How chromatin changes in the downstream region of the *pl1* gene contribute to regulating gene expression is not yet known. This region is unusual because it contains a 1-kb direct repeat (Fig. 3.1). In plant systems, repeat sequences are targets for DNA methylation (Melquist and Bender, 2003) and / or gene silencing mediated by the small RNA (sRNA) pathway (Matzke and Birchler, 2005). In *Arabidopsis*, a recent study of *FLOWERING LOCUS* (*FLC*) gene showed that sRNA-mediated chromatin silencing was directed to the 3' region of the gene (Swiezewski et al., 2007). To ask if sRNAs are associated with the repeats in the *pl1* gene, I used the *Pl1-Rhoades* gene sequence to query a maize sRNA database (http://csrdb.ucdavis.edu/smrnas/). That search detected sRNAs with sequence identity to three regions known to be repeated in *Pl1-Rhoades*. These include two transposable elements in the upstream region: *doppia* [-80 to – 430 bp upstream from the transcription start site (TSS; Hoekenga et al., 2000)], and *ins1* [-500 bp upstream from the TSS (Hoekenga et al., 2000)]. These elements belong to the inverted-repeat class of DNA transposons, and the sRNAs detected for these elements are homologous to the inverted repeats. The other repeated region of *Pl1-Rhoades* identified as having corresponding sRNAs was the tandem repeat in the downstream part of the gene. It is tantalizing to speculate that some or all of these sRNA classes play a role in regulating *pl1* expression. However, the *pl1* allele in the line from which the sRNA database was constructed is unrelated to the *pl1* alleles

I analyzed. The databases were constructed by analyzing sRNAs from the inbred line B73, which contains a sun-red *pl1* allele (Cone et al., 1993b). This allele has the upstream *ins1* and downstream tandem repeat like *Pl1-Rhoades* (and *Pl1-Blotched*); however, it lacks the *doppia* element. Thus, the *doppia*-related sRNAs detected in our search cannot be derived from this *pl1* allele. Both *doppia* and *ins1* elements are found in other locations in the maize genome; therefore, it is not clear whether the *ins1*-related sRNAs I detected are from *pl1*.

By contrast, the tandem repeat downstream of *pl1* is not repeated elsewhere in the genome. Detection of sRNAs related to this repeat prompts the hypothesis that this downstream repeat is involved in sRNA-mediated gene silencing. If this idea is true, I predict that abundance of sRNAs homologous to the repeat should be inversely correlated to expression levels of *pl1*. *Pl1-Rhoades* should have lowest abundance, *Pl1-Blotched* should have the highest levels of these sRNAs, and *Pl1-Blotched* with *Spb* should have intermediate levels. This idea could be tested directly measuring sRNA abundance by RNA blot analysis or by RNA profiling of the three lines. A genetic test would be to introduce *Pl1-Blotched* into lines that carry mutations in components of the small RNA pathway, e.g., *mediator of paramutation1* (*mop1*; Dorweiler et al., 2000; Alleman et al., 2006) or *required to maintain repression1, 2 or 6* (*rmr1, rmr2, rmr6*; Hollick and Chandler, 2001; Hollick et al., 2005; Hale et al., 2007). The prediction is that these mutants, which interfere with various steps in sRNA production, should lead to increased expression, and thereby increased pigmentation, of *Pl1-Blotched*.

The *doppia* element upstream of *Pl1-Rhoades* and *Pl1-Blotched* is the site of altered DNA methylation between *Pl1-Rhoades* and *Pl1-Blotched*, and between *Pl1-Blotched* with *Spb* and without *Spb* (Hoekenga et al., 2000; Courtney, 2001). Because *doppia* is repeated in the genome, probing for sRNAs from this element will not be feasible, but it should be possible to investigate what kind of histone modifications are

associated with this *doppia*. ChIP assays can be performed and immunoprecipitated DNA analyzed with primers that flank the *doppia* element. Anchoring the downstream primer in *pl1*-specific sequences would allow analysis of this single element. Such a study could provide insights into how epigenetic features of this element, which may form part of the promoter for *Pl1-Rhoades* and *Pl1-Blotched*, are correlated with gene expression.

One additional region of *pl1* needs to be examined by ChIP assays, e.g., the coding region. In my study, I did not initially analyze the coding region of *pl1*, because of its high sequence similarity to the duplicate gene, *c1* (Cone et al., 1993a). However, it should be possible to design *pl1*-specific primers in the coding region by taking advantage of sequence polymorphisms in the introns. Because transcribed coding regions have different types of histone modifications than flanking regions (Li et al., 2007), analysis of ChIP assays with primers specific for the *pl1* coding region will furnish a more complete picture of the epigenetic features associated with *pl1* gene regulation.

In this study, I hypothesized that *Spb* is an epigenetic modifier that increases *Pl1-Blotched* expression by changes in the chromatin of the gene. The results of ChIP assays, which showed higher level of H4 acetylation and altered nucleosome positioning in the *Pl1-Blotched* in the presence of *Spb*, support this hypothesis. Moreover, my data suggest that *Spb* is involved in chromatin remodeling.

In many eukayotes, chromatin remodeling involves interaction of multiple proteins (Kang et al., 2002; Durant and Pugh, 2007; Suganuma et al., 2008). The likely involvement of *Spb* in chromatin remodeling, together with the QTL mapping results indicating that the *Spb* effect on pigmentation of *Pl1-Blotched* plants is due to interactions among multiple loci, suggest that *Spb* may encode multiple proteins that act together to alter chromatin organization. Consistent with this idea is the fact that the QTL regions identified in my analyses contain chromatin genes with functions in

epigenetic processes likely to distinguish *Pl1-Blotched* with *Spb* from *Pl1-Blotched* without *Spb*. The *caveat* is that the maize genome contains over 350 chromatin genes (ChromDB.org); thus, virtually any QTL interval, whether linked to the *Spb* effect or not, will contain chromatin genes. Therefore, future analyses need to focus on fine structural mapping to narrow the field of likely chromatin gene candidates. Then, the best candidate chromatin genes can be evaluated by molecular and biochemical analyses to determine their functions in regulating *pl1* expression.

Two anthocyanin genes underlying QTL, *a2* on chromosome 5 and *in1* on chromosome 7, deserve more study. To ask if an *a2* polymorphism influences the pigmentation of *Pl1-Blotched,* the *a2* gene is being sequenced from both mapping parents. Polymorphisms can be developed into markers, which can aid in the fine structural mapping. Alternately, enzyme activities for the functional *a2* alleles can be assayed in the two parents to determine whether one of the alleles produces a more active enzyme (Nakajima et al., 2001). For *in1*, an SSR polymorphism was detected in the QTL mapping. Whether this polymorphism is associated with any functional difference in the parents and F_2 is not clear. The non-*Spb* parent was the source of the chromosome 7 effect contributing to high pigmentation; however, this parent was not known to possess the *in1* allele. To test for *in1* genetically, both mapping parents are being crossed to *in1* testers.

The QTL mapping also implicated an interval containing *pl1* as an important determinant of pigmentation differences in the F_2 population. At first glance, this observation is puzzling, because both parents of the mapping population contain the same *pl1* allele, *e.g., Pl1-Blotched*. However, it is possible that *Pl1-Blotched* plants with *Spb* have a distinct pattern of *pl1* epigenetic modification that correlates with expression differences. This leads to the question, does this QTL represent an epigenetic polymorphism at *Pl1-Blotched*? To investigate whether the QTL effect on chromosome

6 is due to an epigenetic determinant associated with the *pl1* gene itself, DNA methylation levels at various sites will be assessed in the parents. If differences are found, then methylation levels can be measured in the F_2 individuals. These could be scored as a quantitative trait and analyzed by QTL Cartogropher. An additional approach to understanding how polymorphisms at *pl1* might contribute to the QTL mapping analysis would be to treat expression levels at *pl1* as an expression QTL (eQTL; West et al., 2007). Because anthocyanins are the final product of a biosynthetic pathway that includes the regulators *pl1* and *b1*, as well as seven structural genes, one way to identify the contribution of *pl1* to the phenotype would be to measure *pl1* mRNA levels in the F_2 by real-time PCR and use these measurements as a trait in QTL mapping. If the QTL identified in these alternate approaches are identical to the QTL identified using anthocyanin levels as the trait, then one could conclude that the QTL reflects a direct relationship between *Pl1-Blotched* expression and pigment. If the QTL identified using epigenetic or expression polymorphisms of *pl1* as the trait are a subset of the originally identified QTL, then one could conclude that the additional original QTL might be involved in steps that are not directly related to *Pl1-Blotched* expression.

In maize several epigenetic phenomena, including the kind of phenotypic variegation I studied, are well known (Das and Messing, 1994; Hollick et al., 1997; Chandler et al., 2000; Dorweiler et al., 2000; Hollick et al., 2000; Alleman et al., 2006; Gehring et al., 2006; Hale et al., 2007). However, the underlying molecular mechanisms have been understudied. My research provides important insights into the chromatin-level control of a maize regulatory gene and opens new avenues to study the relationship between *pl1* gene expression and sRNA pathway. Also, my research has provided a launching point for future studies aimed at evaluating chromatin gene candidates to discover the identity of the epigenetic modifier, *Spb*.

Appendix 1: Chromatin genes with locations in the four QTL chromosomal regions

Query id	Subject id	% identity	alignment length	mismatch	gap openings	e-value	bit score	clone name	chr	contig
ago106	AC209716.1	93	516	34	1	0	737	c0222G10	2	ctg91
ago106	AC207639.1	92	519	36	3	0	666	c0214K07	5	ctg240
ago106	AC194928.2	92	519	32	2	0	714	b0325K21	6	ctg276
ago106	AC196184.3	94	196	9	2	6.00E-74	285	c0145K10	7	ctg296
ago113	AC204289.2	100	553	0	0	0	1096	c0424A18	6	ctg267
arid101	AC203304.2	100	465	1	0	0	914	c0059D20	6	ctg274
arid102	AC203304.2	88	564	61	2	2.00E-157	561	c0059D20	6	ctg274
arid104	AC203920.2	92	712	59	0	0	944	c0054O18	2	ctg89
arid104	AC211187.1	93	232	16	1	1.00E-83	317	c0329I07	7	ctg296
arp105	AC197017.3	100	1196	0	0	0	2288	c0463C04	2	ctg84
arp108	AC205703.1	100	255	0	0	2.00E-140	505	c0452N17	5	ctg242
arp108	AC211466.1	94	228	6	1	2.00E-94	353	c0149G11	6	ctg263
brd104	AC193503.3	94	132	2	1	2.00E-48	200	c0023H19	7	ctg296
che101	AC196468.3	93	261	17	2	5.00E-94	351	b0381D11	2	ctg84
che101	AC209357.1	95	236	10	1	1.00E-100	373	c0109D04	6	ctg271
chr130	AC210204.1	97	205	6	1	1.00E-91	343	c0154I18	2	ctg91
chr139	AC204585.2	97	331	10	0	1.00E-161	577	c0319H14	2	ctg89
chr150	AC191353.2	99	165	1	0	2.00E-84	319	c0160I01	2	ctg89
chr150	AC202886.2	100	102	0	0	4.00E-49	202	c0484H21	5	ctg242
chr150	AC204418.2	100	456	0	0	0	904	c0255H16	6	ctg281
chr150	AC206841.1	90	240	21	1	9.00E-75	287	c0486P12	7	ctg296
chr151	AC211187.1	90	213	19	1	2.00E-63	250	c0329I07	7	ctg296
chr153	AC177872.1	92	475	39	0	2.00E-178	632	b0578C09	2	ctg90
chr153	AC206982.1	92	475	34	2	3.00E-177	628	c0166B11	5	ctg240
chr153	AC187242.3	91	477	41	0	6.00E-175	620	c0524G10	6	ctg269
chr153	AC205131.2	92	182	11	1	2.00E-60	240	c0265H18	7	ctg296

Query id	Subject id	% identity	alignment length	mismatch	gap openings	e-value	bit score	clone name	chr	contig
chr160	AC187073.4	96	148	6	0	5.00E-62	246	c0040M15	6	ctg275
chr163	AC194350.2	100	798	0	0	0	1534	b0464O19	6	ctg281
chr164	AC199493.2	87	230	30	0	1.00E-53	218	c0222J23	2	ctg91
chr164	AC200856.3	94	233	11	1	2.00E-92	347	b0581M23	6	ctg282
cpc101	AC200139.3	98	210	5	0	1.00E-101	377	c0064A15	6	ctg281
dmt101	AC211651.1	100	1482	3	1	0	2851	b0511C20	7	ctg296
dmt103	AC207355.1	91	205	17	1	4.00E-65	256	c0123M15	2	ctg87
dmt107	AC207355.1	95	258	11	1	1.00E-98	367	c0123M15	2	ctg87
dng101	AC196400.3	90	1451	132	3	0	1729	b0608F20	5	ctg237
dng102	AC209637.1	91	3942	340	5	0	4938	c0342D14	2	ctg82
dng102	AC196400.3	100	4207	0	0	0	8292	b0608F20	5	ctg237
dng102	AC187059.2	91	3939	344	9	0	4801	b0468L16	6	ctg269
drb104	AC207533.1	100	588	0	0	0	1166	b0205P03	2	ctg91
gta101	AC205026.2	99	369	3	1	0	692	c0295N20	6	ctg280
gta106	AC205026.2	90	327	25	2	8.00E-104	383	c0295N20	6	ctg280
gta107	AC205026.2	94	356	21	0	2.00E-150	539	c0295N20	6	ctg280
gte102	AC184782.3	100	1280	0	0	0	2537	c0206K03	5	ctg242
gte109	AC209878.1	100	820	0	0	0	1626	c0321H02	2	ctg89
gte110	AC209702.1	100	530	0	0	0	961	c0153F14	6	ctg269
hac113	AC206552.1	96	651	25	0	0	1092	c0092J01	2	ctg89
hac113	AC214350.1	97	654	21	0	0	1130	c0448A09	5	ctg238
hac113	AC212915.1	98	655	13	1	0	1179	c0070E19	6	ctg269
hac114	AC205344.2	97	414	14	0	0	710	c0064D08	6	ctg285
hag102	AC206248.1	96	788	27	1	0	1332	c0342B08	7	ctg296
hag102	AC206248.1	99	160	1	0	2.00E-81	309	c0342B08	7	ctg296
hda101	AC204652.2	95	552	29	0	0	864	c0112O02	5	ctg236
hda110	AC188989.3	100	682	0	2	0	1279	b0631G16	7	ctg296

Query id	Subject id	% identity	alignment length	mismatch	gap openings	e-value	bit score	clone name	chr	contig
hda116	AC204652.2	85	518	75	2	9.00E-109	400	c0112O02	5	ctg236
hda119	AC204652.2	100	509	0	1	0	993	c0112O02	5	ctg236
hdma101	AC207460.2	85	406	51	5	9.00E-76	291	b0599N20	2	ctg86
hdma101	AC187409.4	84	307	48	0	1.00E-56	228	c0008H09	6	ctg270
hdt103	AC204527.2	93	225	7	1	1.00E-88	333	c0541P18	6	ctg264
hen101	AC209680.1	97	158	2	1	3.00E-69	270	c0175O07	6	ctg283
hen101	AC188989.3	100	661	0	0	0	1310	b0631G16	7	ctg296
hira101	AC209716.1	96	609	15	1	0	1025	c0222G10	2	ctg91
hira101	AC195813.3	96	567	14	1	0	967	b0638L02	5	ctg238
hira101	AC186899.4	96	601	16	1	0	1001	c0042J07	6	ctg279
hira101	AC200329.2	95	502	14	1	0	821	c0010P21	7	ctg296
hmga104	AC190807.2	98	162	4	0	2.00E-75	289	b0502C16	2	ctg91
hmga104	AC190526.3	98	156	3	0	3.00E-74	285	c0147L04	5	ctg236
hmga104	AC204760.1	97	156	4	0	6.00E-72	278	c0460A24	6	ctg281
hmga104	AC207070.1	97	156	5	0	2.00E-69	270	c0507D22	7	ctg296
hmta101	AC208028.1	99	559	7	0	0	1053	c0032K02	6	ctg285
hta102	AC211187.1	89	460	42	2	9.00E-141	505	c0329I07	7	ctg296
hta103	AC211187.1	100	733	0	0	0	1453	c0329I07	7	ctg296
hta104	AC211187.1	88	398	48	0	1.00E-111	408	c0329I07	7	ctg296
hta105	AC211187.1	89	464	41	2	1.00E-145	521	c0329I07	7	ctg296
hta108	AC211187.1	90	462	35	2	1.00E-158	565	c0329I07	7	ctg296
hta110	AC211187.1	85	313	47	0	4.00E-63	248	c0329I07	7	ctg296
hta116	AC211187.1	91	399	37	0	2.00E-138	498	c0329I07	7	ctg296
htb105	AC197498.3	95	142	6	1	3.00E-54	218	c0120I15	5	ctg238
htr113	AC210022.1	100	449	1	0	0	882	c0506A12	5	ctg240
hupa101	AC209952.1	89	200	13	2	2.00E-52	214	c0415E22	5	ctg236
hupa101	AC206319.1	95	195	9	0	6.00E-83	315	b0152M19	6	ctg271

Query id	Subject id	% identity	alignment length	mismatch	gap openings	e-value	bit score	clone name	chr	contig
hupb101	AC206279.1	100	486	0	0	0	922	b0185M10	6	ctg269
hupb102	AC188614.3	96	129	5	0	2.00E-53	216	c0009J11	7	ctg296
jmj104	AC206185.1	88	416	52	0	6.00E-112	412	c0264H14	6	ctg281
jmj109	AC191119.2	97	541	12	1	0	955	c0112O22	2	ctg86
mbd101	AC195854.1	90	430	44	0	5.00E-140	504	c0257N06	6	ctg285
mbd114	AC190518.1	97	164	5	0	2.00E-74	285	c0184M16	2	ctg86
mbd114	AC207739.1	93	177	11	1	3.00E-63	248	c0029A19	5	ctg237
mbd114	AC210091.1	96	133	4	1	1.00E-53	216	b0471K09	6	ctg264
mbd114	AC208985.1	98	177	3	1	3.00E-82	311	b0269L06	7	ctg296
mbd116	AC205912.1	96	182	8	0	2.00E-77	297	c0112J21	7	ctg298
mbd120	AC195854.1	100	506	0	0	0	1003	c0257N06	6	ctg285
nfa101	AC213875.1	94	187	11	0	1.00E-73	283	b0156B22	6	ctg269
nfa103	AC212215.1	91	208	17	2	2.00E-62	246	c0326L22	5	ctg240
nfa104	AC212215.1	100	232	0	0	6.00E-127	460	c0326L22	5	ctg240
nfa105	AC213875.1	100	374	0	0	0	741	b0156B22	6	ctg269
nfe101	AC185432.3	94	421	27	0	7.00E-175	620	c0473M16	5	ctg240
nfe102	AC185432.3	100	421	0	0	0	835	c0473M16	5	ctg240
nfe102	AC187238.4	88	196	23	0	2.00E-50	206	c0527N10	6	ctg262
pafb101	AC209862.1	93	176	12	1	8.00E-60	238	c0027D03	7	ctg296
pafc101	AC177834.4	84	482	78	0	4.00E-89	337	c0043M11	2	ctg91
pafc101	AC210196.1	100	103	0	0	4.00E-49	204	c0498C10	5	ctg238
pafc101	AC210424.1	99	3833	53	0	0	7178	c0537C23	6	ctg279
pafc101	AC205131.2	81	524	101	0	3.00E-59	238	c0265H18	7	ctg296
pge102	AC204585.2	93	202	8	2	4.00E-71	276	c0319H14	2	ctg89
pge102	AC212823.1	91	203	12	2	3.00E-62	246	c0051P17	5	ctg238
pge102	AC187080.3	92	193	9	1	4.00E-68	266	c0195F06	6	ctg271
pge102	AC212126.1	90	186	12	1	8.00E-57	228	c0477A09	7	ctg296

Query id	Subject id	% identity	alignment length	mismatch	gap openings	e-value	bit score	clone name	chr	contig
rdr103	AC200616.2	100	240	1	0	3.00E-129	468	c0056E17	2	ctg86
sdg101	AC195845.3	100	991	0	1	0	1949	b0422L22	7	ctg298
sdg102	AC206186.1	97	439	9	2	0	678	c0260M04	6	ctg271
sdg110	AC207739.1	94	492	29	2	0	714	c0029A19	5	ctg237
sdg124	AC196177.3	100	708	0	0	0	1403	c0230A10	6	ctg270
sdg136	AC203818.2	92	2178	160	6	0	2888	c0028M17	2	ctg91
sdg136	AC193941.3	100	2164	0	1	0	4274	b0246B17	6	ctg283
sdg137	AC203818.2	100	1668	0	0	0	3253	c0028M17	2	ctg91
sdg137	AC193941.3	91	1468	124	2	0	1853	b0246B17	6	ctg283
sdg138	AC208671.1	87	294	36	1	2.00E-72	281	c0476M06	6	ctg276
sdg141	AC206186.1	96	248	11	0	2.00E-110	404	c0260M04	6	ctg271
sdg145	AC202147.2	100	828	0	0	0	1641	b0340E13	5	ctg238
sdg146	AC213828.1	98	355	7	0	0	648	c0193N19	2	ctg86
sdg146	AC197830.3	97	353	12	0	5.00E-170	605	b0533J12	5	ctg242
sdg146	AC194867.2	96	479	20	1	0	775	c0271D07	6	ctg269
sdg146	AC187476.2	95	356	17	0	6.00E-160	571	b0276M06	7	ctg298
sgs101	AC200561.3	100	571	0	0	0	1132	c0036M13	6	ctg264
swdc101	AC195231.1	98	661	10	1	0	1215	c0230M16	6	ctg269
tafv101	AC209207.1	93	155	11	0	2.00E-54	220	c0135O02	2	ctg89

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VITA

I was born in South Korea and was educated there. I moved to the US to pursue a Ph.D. Looking back on my life, I realized that I have been fortunate in meeting many excellent scientists. My interest in science was recognized by a science teacher in my junior high school period. During that time she assigned me to help other classmates learn science. I majored in biology at the Ewha Womans University in Seoul Korea. I realized that I had a passion about doing research while the graduate school in the Yonsei University (Seoul, Korea). In addition, I enjoyed teaching science due to interactions with students in the class. I was pleased when students showed an interest in science and became good scientists. After four years of teaching, I came to the United States. At the University of Missouri (Columbia, MO), I met Karen Cone who is an excellent mentor. I learned so much from her in many aspects: how to write about and explain my research, how to cooperate with other scientists, and what to do in order to be a good adviser and so on. In the future, I hope that I have an opportunity to influence other young scientists as they pursue their studies.