THE EFFECTS OF HISTONE ACETYLATION ON THE MAIZE ALLELE *PL1-BLOTCHED*

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

THE EFFECTS OF HISTONE ACETYLATION

ON THE MAIZE ALLELE *PL1-BLOTCHED*

presented by Paul Ladipo,

a candidate for the degree of Master of Arts,

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

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THE EFFECTS OF HISTONE ACETYLATION ON THE MAIZE ALLELE *PL1-BLOTCHED*

ABSTRACT

Covalent modifications of DNA and nucleosomal histone proteins associated with eukaryotic chromatin have the potential to alter expression of a gene without change in its DNA sequence. One gene regulated through this so-called epigenetic process is the *Pl1-Blotched* gene of maize. *Pl1-Blotched* is a allele of the *purple1 (pl1)* gene, which encodes a transcription factor that activates synthesis of purple anthocyanin pigments. *Pl1-Blotched* is unusual in that it leads to variegated, rather than uniform, pigmentation. At the molecular level, this phenotype is associated with low expression of *pl1* mRNA, a novel pattern of DNA methylation and condensed chromatin structure. To ask if acetylation of nucleosomal histone proteins might be involved in regulating the chromatin organization of *Pl1-Blotched*, this allele was crossed into 40 lines containing transgenes that target reduction of various histone acetyltransferase and histone deacetylase genes. Some of the lines led to altered *Pl1-Blotched* pigmentation. Detailed analysis of molecular changes underlying the altered pigmentation resulted in models that implicate both histone acetyltransferase and histone deacetylase genes in controlling expression of *Pl1-Blotched*. These findings lay the foundation for future studies aimed at further understanding the interplay between histone modification and regulation of gene expression.

Chapter 1

INTRODUCTION

Epigenetics

The term epigenetics is used to describe a pattern of gene expression that is caused by mechanisms other than differences in DNA sequence (Henikoff and Matzke, 1997). Almost all eukaryotes, including yeast, fruit flies, humans, and plants use some form of epigenetic regulation of gene expression. Eukaryotes utilize this pattern of expression through changes in chromatin packing. Open, loosely packed chromatin is accessible and actively transcribed, whereas closed, tightly packed chromatin is less accessible and associated with gene silencing. Examples of this type of gene expression include paramutation in maize and X-inactivation in mammals.

In paramutation, gene silencing is induced by interactions between related genes (Hollick et al., 1997; Chandler and Stam, 2004). The maize *booster (b1)* gene provides an excellent example. An allele of *B*, *B-I*, is known to paramutate to a less expressed state called *B′*, but only if paired with another paramutagenic *B′* allele. As a result, individuals expressing these alleles have significantly reduced levels of expression. The *B′* state is mitotically and meiotically stable. It never reverts to the fully expressed *B-I* state. Genetic and molecular studies have shown that *B′* and *B-I* differ in their chromatin organization, but not in DNA sequence (Stam et al., 2002).

X-inactivation in mammals is when one of the two X chromosomes in a developing female embryo becomes inactivated. Because females have two copies of the X chromosome and males only have one, there is initially an imbalance in the copy number of genes on the sex chromosomes. When one of the X-chromosomes becomes

transcriptionally silent, dosage compensation occurs, ensuring that this initial difference in copy number does not impede normal embryonic development (Henikoff and Matzke, 1997).

Many studies have demonstrated that epigenetic gene silencing is accompanied by changes in chromatin structure. The basic unit of chromatin is the nucleosome, which consists of approximately 145 base pairs of DNA wrapped around a histone octamer. This octamer is made up of two subunits each of histones H2A, H2B, H3, and H4 (Olins and Olins, 1974). Both the DNA and histone components of chromatin can be modified to produce changes in chromatin structure, as detailed below. The pattern of histone modification is thought to constitute a complex histone code capable of regulating transcriptional activity of a gene (Jenuwein and Allis, 2001).

DNA methylation

Eukaryotes make use of cytosine methylation as a major component of epigenetic regulation. In plants, DNA methyltransferases add methyl groups to cytosines in CG, CNG, or CGG contexts (Bartee, 2001; Finnegan and Dennis, 1993; Cao et al., 2000; Lindroth et al., 2001). Heavily methylated DNA serves as a binding site for methylcytosine binding proteins. These proteins associate with and recruit histone deacetylases, which serve to condense chromatin and inactivate DNA (Jones et al., 1998; Nan et al., 1998).

Histone methylation

Methylation of lysine residues in the amino terminal histone tails by histone methyl transferases can be used to activate or repress transcription (Jenuwein and Allis, 2001). Trimethylation on lysine 4 of histone H3 (H3K4me3), for example, is associated

with transcriptionally active genes. By contrast, H3K9me2 is a hallmark of transcriptionally silent genes, especially those in heterochromatin. This contrast in expression is thought to be is due to the fact that histone modifications serve as binding sites for chromatin remodeling proteins. Several recent reports established that the PHD protein domains in a number of chromatin modifiers recognize and bind to H3K4me3 (Li et al., 2006; Peña et al., 2006; Shi et al., 2006; Wysocka et al., 2006).

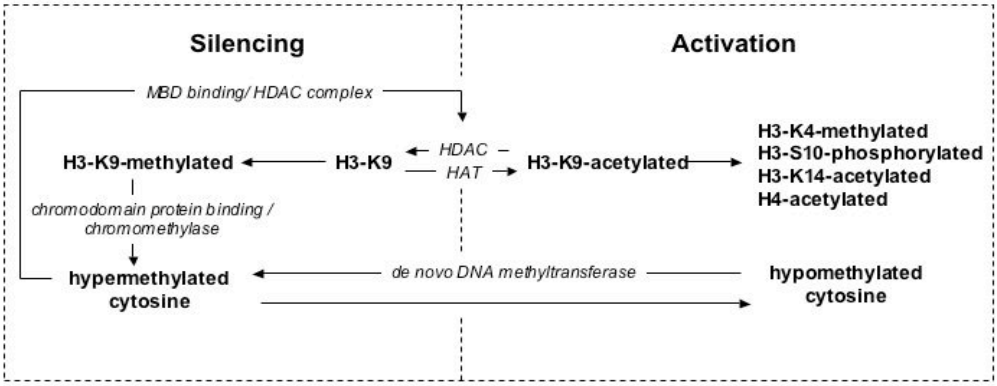
Histone acetylation/deacetylation

Other proteins that function as modifiers of chromatin structure are histone acetyl transferases (HATs) and histone deacetylases (HDACs). These proteins modify chromatin structure by adding or removing acetyl groups from histones, respectively (Tian et al., 2005). Acetylated histones are characteristic of transcriptionally active genes, whereas hypoacetylated histones are typical of transcriptionally repressed or silent genes. There are two general ideas about how histone acetylation might facilitate transcription (reviewed in Kuo, 1998; Carozza et al., 2003). One theory predicts that addition of acetyl groups neutralizes the positive charge of histones, thus weakening the association with the negatively charged DNA. The other, not mutually exclusive, idea is that acetylated lysines on histone tails serve as recognition sites for specific factors involved in modulating gene expression (much like H3K4me2 is a recognition site for proteins with PHD domains). Support for the latter idea comes from a recent study of genome-wide changes in gene expression in an Arabidiopsis histone deacetylase mutant, which showed that histone acetylation and deacetylation are promoter-dependent and locus-specific (Tian et al., 2005).

Interconnections

A number of studies point to 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; Fig. 1.1). This model was supported by additional experiments examining the global effects on transcription of mutating a histone deacetylase (Tian et al., 2005).

Figure. 1.1. A model for gene silencing and activation



According to this model (modified from Lawrence, 2004; Soppe, 2002), DNA methylation and repressive histone modifications form a self-reinforcing silencing cycle. Histone deacetylation / acetylation and DNA methylation / demethylation serving as the main control points for switching between repressed and active gene expression states.

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, histone acetyltransferases, deactylases, DNA and histone methyltransferases and chromatin remodeling proteins in the SWI/SNF and ISWI ATPase families have all been shown to be parts of larger protein complexes (*e.g.*, Jones et al., 1998; Wade et al., 1998; Carozza et al., 2003).

Plant HAT and HDAC gene families

HATs and HDACs are divided into numerous families based on sequence and functional similarity, as well as substrate specificity (Table 1.1). We analyzed members of four HAT classes and three HDAC classes.

HAT families

CREB Binding Protein (CBP) Family. CBP proteins serve as coactivators for transcription, but the histone acetylation domain of this group differs from the GNAT and MYST protein families (Pandey et al., 2002). This group of proteins was originally believed to be unique to the animal kingdom, but has been recently discovered in plants as well. In plants, CBP protein genes are given the symbol *hac*. Unlike other groups of HATs, these proteins do not exert their function by binding to nucleosomal DNA; rather, they are recruited to promoter regions where they bind to the transcription factor CREB (Roth et al., 2001). CBP proteins are responsible for regulating genes required for cell cycle control, differentiation, and apoptosis (Carozza et al., 2003).

Table 1.1. Plant histone acetyl transferase and histone deacetylase gene classes

Gene Name[ab]	Protein homology group
Histone acetyl transferase (HAT) genes	
hac[b]	CREB-binding protein transcriptional co-activators
haf	TAF II-250 subunti of TFIID transcription initiation factor complex
hag[b]	GNAT superfamily
ham[b]	MYST superfamily
hxa[b]	ADA2 transcriptional adaptor; part of yeast ADA and SAGA complexes
Histone deacetylase (HDAC) genes	
hcp	Sin3 complex component, possilbe SAP18 homolog
hda[b]	RPD3/HDA1 superfamily
hdt[b]	HD2-type
snt	Sin3 complex component, possilbe Sin3 homolog
srt[b]	SIR2-like, NAD+-dependent

[a] Gene name designations as proposed at www.ChromDB.org.
[b] Gene families analyzed in this study.

GNAT family. The first protein from this family, *Gcn5*, was discovered in yeast, but structurally and functionally similar proteins of this group have been found in all five kingdoms, with representatives from protozoans, algae, archaebacteria, and plants (Pandey et al., 2002). Plant HATs from this family are given the name *hag*. Hallmark features of GNAT proteins include: a bromodomain for the recognition and binding of acetylated histones, an acetyl-transferase domain important for sequestering acetyl-CoA,

an *ADA2* interaction domain, and a domain for the binding of nucleosomes (Pandey et al., 2002).

MYST family. Proteins from this family were originally classified in a joint GNAT/MYST superfamily, but the only motif shared between the two classes is the highly conserved acetyl-CoA binding region common to all histone acetyl-transferases (Pandey et al., 2002). Plant representatives from this family are given the name *ham*. Many proteins from this family possess chromodomains that recognize and bind to methylated histone tails, which could serve as an explanation as to why methylation of H3K4 leads to transcriptional activation, as opposed to silencing (Li et al., 2006).

ADA2 transcriptional adaptors. *Ada2*, the founding member of this family, was first isolated from yeast, but homologs of this gene exist in plants. The plant genes are designated *hxa*. Members of this family have intrinsic histone acetyltransferase activity, but are often a part of large, multi-protein HAT complexes that aid transcriptional activation (Balasubramanian et al., 2001). *Gcn5*, a member of the GNAT superfamily found in yeast, can acetylate naked histones but not nucleosomes to a significant degree. The presence of *Ada2* and other genes from this family increase HAT activity, enabling *Gcn5* to acetylate nucleosomal histones (Balasubramanian et al., 2001) .

HDAC families

RPD3/HDA1 Family. The founding member of this family, *Rpd3*, was discovered in yeast as well, but homologs of this gene exist in plants, animals, and bacteria. Plant HDACs from this family are given the name *hda*. Histone deacetylases from this family are often a part of a co-repressor complex with other proteins such as *Sin3* (Pandey et al., 2002). This repressor protein complex is recruited to promoter regions by other repressor

proteins and nuclear receptors. Despite *Rpd3/Hda*'s inhibitory effects, these histone deacetylases are crucial in morphological development. In plants, for example, proteins from this superfamily are important for meristem formation, flower architecture, and development from the vegetative to reproductive phase (Wu et al., 2000).

HD2 Family. HDACs in this family are proteins that are regulated by phosphorylation pathways, but what is truly unique about the proteins in this group is that they are only found in plants, with the first member being purified from maize chromatin (Zhou et al., 2004). HD2 proteins were originally thought to be responsible for ribosomal DNA chromatin organization because these enzymes are located in the nucleolus, but gene silencing assays have determined that this family of HDACs are crucial for normal embryogenesis and embryo development (Zhou et al., 2004). HD2 proteins in plants are given the symbol *hdt*.

SIR2 Family. The hallmark feature of these enzymes is that they are regulated by increased NAD+ levels; for every lysine that is deacetylated by an SIR2 protein, one molecule of NAD+ is hydrolyzed. Silent information regulator (SIR) proteins are a family of histone deacetylases that are found in a wide variety of organisms, including fungi, animals, bacteria, and plants (Pandey et al., 2002). SIR2 proteins (sirtuins) are crucial for transcriptional inactivation in yeast, but in other organisms these HDACs are responsible for numerous biological processes such as heterochromatin formation, metabolism, and apoptosis (Buck et al., 2004). SIR2 HDACs in plants are named *srt*.

Anthocyanin synthesis and *Pl1-Blotched*

The focus of this thesis research is on chromatin regulated modulations in expression of a maize gene, *Pl1-Blotched*, which regulates synthesis of purple

anthocyanin pigments. In maize, anthocyanins are synthesized in the embryo, the aleurone layer of the endosperm and the vegetative and floral organs, such as the leaf and anther (Coe et al., 1988). There are four regulatory genes that are responsible for anthocyanins: *red1* (*r1*)*, booster1 (b1), colorless1* (*c1*), and *purple plant1* (*pl1)*. Each is necessary for the transcription of enzymatic genes in the anthocyanin pathway. These regulators encode transcription factors that are grouped into two families, *c1/pl1* and *r1/b1,* because each family encodes proteins that are functionally equivalent. Anthocyanin synthesis in any part of the plant requires the interaction of a member of the *r1/b1* family with a member of the *c1/pl1* family (Coe et al., 1988). Anthocyanin synthesis in the kernels, for example, requires functional alleles of *r1* and *c1*, whereas pigment production in the body of the plant requires the presence of functional *b1*and *pl1* alleles.

The *Pl1-Blotched* allele of *pl1* is epigenetically regulated. *Pl1-Blotched*, causes a variegated pattern of pigmentation characterized by pigmented cells arranged in clusters, and within these sectors the degree of anthocyanin expression varies. This pattern of expression is dramatically different from the uniform pigmentation that is caused by the expression of another *pl1* allele, *Pl1-Rhoades*. The degree of pigmentation in plants that express this allele is proportional to the level of *pl1* mRNA (Cocciolone and Cone, 1993). Cells that are weakly pigmented have less *pl1* mRNA than cells that are heavily pigmented, and tissue from *Pl1-Blotched* plants has considerably less *pl1* mRNA than *Pl1-Rhoades* tissue. These contrasts in mRNA levels between the two alleles are not caused by changes in DNA sequence, but by differences in DNA methylation and chromatin structure, as assessed by DNaseI digestion (Hoekenga et al., 2000). *Pl1-*

Blotched genomic DNA typically possesses an altered pattern of DNA methylation and more compact chromatin structure than the fully expressed *Pl1-Rhoades* allele (Cocciolone and Cone, 1993).

Expression of *Pl1-Blotched* in chromatin-gene mutants

To understand how the chromatin structure of *Pl1-Blotched* is regulated, we have taken advantage of a collection of lines carrying inverted repeat (IR) trangenes that target reduction of chromatin gene expression by RNA interference (RNAi; McGinnis et al., 2005; McGinnis et al., 2007). Among the transgenes tested were a group targeting HAT and HDAC genes. If the expression state of *Pl1-Blotched* is regulated by histone acetylation levels, then mutations in HAT and HDAC genes are predicted to have opposite effects on the *Pl1-Blotched* phenotype. Because HATs normally activate gene expression by opening chromatin structure, HAT mutations should lead to more closed chromatin structure and reduced pigmentation of *Pl1-Blotched*. By contrast, mutating HDACs, which normally repress gene expression by closing chromatin, should lead to more open chromatin and increased pigmentation of *Pl1-Blotched*. Phenotypic assays of *Pl1-Blotched* plants carrying some of the transgenes followed these predictions. Transgenes that targeted HAT genes led to lower than normal pigment levels in *Pl1-Blotched* plants, whereas a transgene that targeted a HDAC gene led to higher than normal pigmentation. These observations led to the hypothesis that histone acetylation plays a role in regulating *Pl1-Blotched* expression.

Thesis Overview

This research was aimed at exploring the idea that histone acetylation regulates changes in *Pl1-Blotched* expression. Chapter 2 presents the results of a survey of the

effect of a large number of IR transgenes targeting HAT and HDAC genes on *Pl1-Blotched* pigmentation. To understand how HAT and HDAC genes influence expression of anthocyanin regulators, additional experiments were conducted to address the genetic basis for the pigmentation differences observed in some of the transgenic lines. Chapter 3 presents the results of the second set of experiments. Chapter 4 presents a summary and considerations for future study.

Chapter 2

Survey of pigmentation in *Pl1-Blotched* plants carrying IR transgenes targeting HAT and HDAC genes

Preliminary data indicated that reduced expression of some genes involved in acetylating and deacetylating histones led to changes in pigmentation in *Pl1-Blotched* expression. We tested additional lines to ask if other HAT and HDAC genes would show similar effects. In all, we analyzed 40 IR transgenes that together target five genes representing four classes of HATs and seven genes representing three of the four HDAC classes. For all transgenes, we examined multiple IR transgenes, and for some IR transgenes that showed marked phenotypes in an initial assay, analysis was repeated in one or more subsequent seasons. These repeated measures allowed us to assess how reproducible the transgene-dependent phenotypes were.

MATERIALS AND METHODS

Maize stocks

The *Pl1-Blotched* stock was originally obtained from the Maize Genetics Stock Center at the University of Illinois, Urbana-Champaign. Phenotypes, as well as DNA sequences, have been previously described in detail (Cocciolone and Cone, 1993; Hoekenga et al., 2000).

Transgenic *Pl1-Blotched* lines

To obtain *Pl1-Blotched* transgenic plants, we crossed the *Pl1-Blotched* line with stocks that were hemizygous for IR transgenes. Presence of the transgene was followed by testing individual plants for resistance to the herbicide Basta (glufosinate), which is conferred by the *bar* gene carried in the transgene construct (McGinnis et al., 2005).

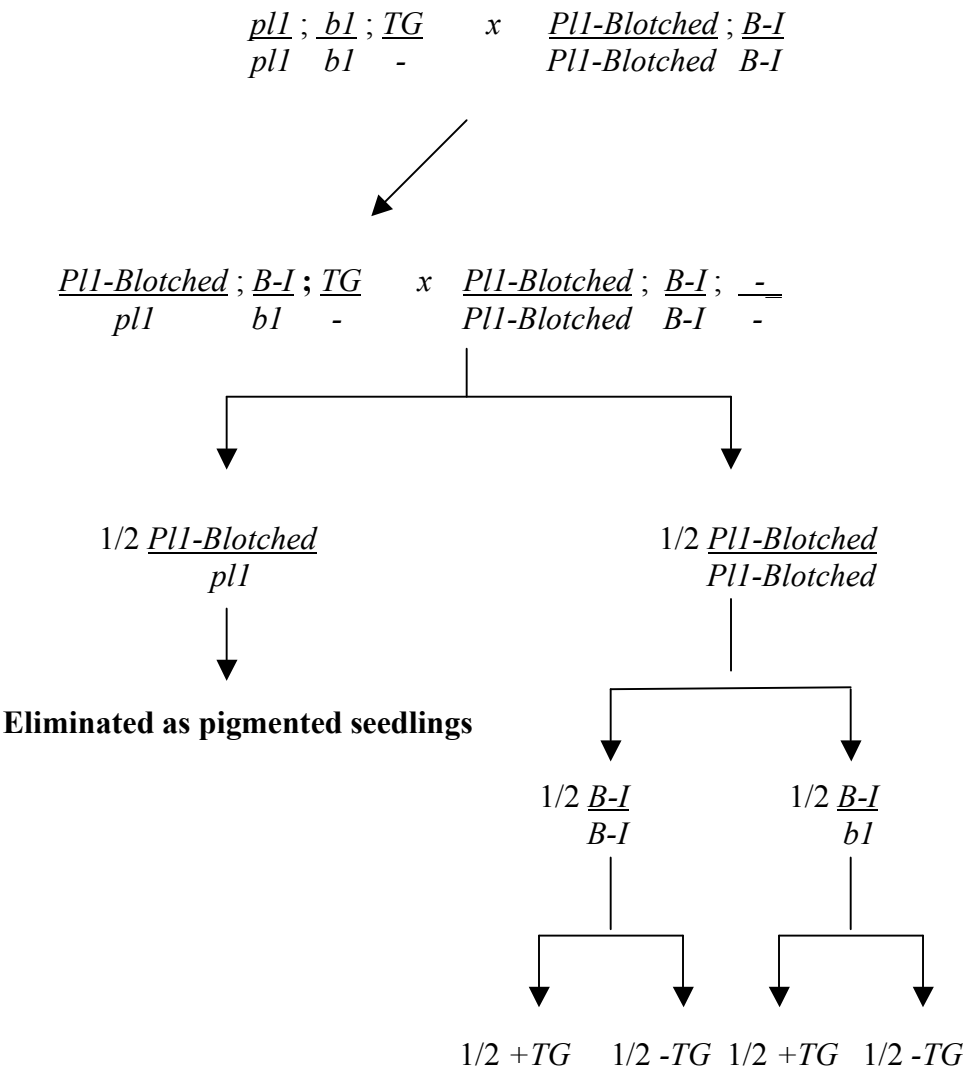
Basta-resistant F1 plants were backcrossed to the *Pl1-Blotched* line to obtain *Pl1-Blotched* homozygotes (Fig.2.1).

As a result of the backcross, roughly half of the progeny were heterozygous for the *pl1* allele from the transgenic parent. This allele leads to a light-dependent sun-red phenotype, which masks the *Pl1-Blotched* phenotype. Thus, these plants were eliminated at the seedling stage by uprooting. The remaining *Pl1-Blotched* homozygotes were grown to maturity.

***b1* genotyping**

The backcross progeny segregated for a non-functional allele of *b1*. Because the dominant *B-I* allele shows a dosage effect on anthocyanin pigmentation (Cocciolone and Cone, 1993), the *b1* genotype of each plant was identified, so that pigment levels could be compared separately among *B-I* homozygotes and *B-I* heterozygotes. For genotyping, a hole puncher was used to collect two punches from a leaf of each plant. The punches were placed in a 96-well PCR plate. DNA was extracted by adding 100 µL of 100 mM NaOH, 2% Tween 20 to each well. The plates were covered with thermofoil or mats and heated in a PCR machine for 10 minutes at 95˚C. Then, 100 µL of 100mM Tris-HCl, 2mM EDTA was added, and the contents were mixed by pipetting slowly. A 100 µL-aliquot of each sample was transferred to a new PCR plate and stored at -20˚C. For genotyping, 2 µL of a 1:10 dilution of the DNA was used as template in PCR. Because the two *b1* alleles segregating in these populations cannot be distinguished easily using primers for the *b1* gene, genotyping was carried out using a neighboring marker, *bnlg1064*, which maps approximately 5 cM from *b1* (primer sequences available from http://MaizeGDB.org).

Fig. 2.1. Construction of transgenic *Pl1-Blotched* lines.



TG = transgene

PCR was performed in a 20 µL reaction consisting of: 10 µL of 10X Red Taq (Sigma; 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 4 mM MgCl, 0.4 mM each dNTP, and 0.03 U/µL Taq Polymerase), 3 µL of autoclaved water, 2 µL of 1/10 diluted genomic DNA, 2 µL of 1% BSA (10mg/mL), 2 µL of 10% PVP, and 1 µL of bnlg1064 primer (10 uM). Amplification conditions were: initial denaturation at 95˚C for 1 minute, annealing at 65˚C for 1 minute, extending at 72C for 1 minute 30 seconds, followed by 11 cycles of 95˚C for 1 minute, 65˚C for 1 minute to allow annealing, and extension at 72˚C for 1 minute 30 seconds. This was followed by 35 cycles of 75 ˚C for 1 minute for denaturing, 55˚C for 1 minute to allow annealing, extension at 72˚C for 1 minute, 30 secconds, and a final extension step at 72˚C for 10 minutes. After PCR, the samples were fractionated on agarose gels. Plants were classified as homozygous if one band (*b1* genotype=1)was detected and heterozygous (*b1* genotype=2) if two bands were detected.

To distinguish transgenic from non-transgenic plants, individuals were tested for Basta (glufosinate) resistance by marking and painting a section of one leaf with a 1:40 dilution of the herbicide Finale, which contains glufosinate as the active ingredient. After three days, the painted leaves were inspected. Basta-resistant plants, which showed no evidence of necrosis, were tagged.

Anthocyanin extraction and spectrometry

For anthocyanin extraction, the leaf sheath attached to the second node below the top ear was harvested from each plant approximately two weeks after anthesis by cutting at the base of the sheath. Sheaths were labeled and stored in plastic bags at -20˚C or -80˚C. For sampling, two hole punches were collected at the base of the sheath just to one side of the vertical midline. The punches were placed into a deep well plate and 500µL

of 3% HCl in 95% EtOH was added to each well. The plate was then covered and incubated at room temperature in the dark for 24 hours. The following day, the plate was gently vortexed, and 75 µL of extract was added to 75 µL of water in a microtiter plate. To three wells, 75 µL of 3% HCl in 95% EtOH was added to serve as blanks. The absorbance of each sample was read on a plate reader (Tecan Sunrise Spectrophotometer) using a wavelength of 520 nm. For samples with readings above 1, the sample was diluted and read again; the absorbance for the diluted sample was multiplied by the dilution factor to obtain the final anthocyanin value.

Data Analysis

Means and standard deviationd were calculated for anthocyanin measurements. Individuals with anthocyanin values above or below two standard deviations from the mean were removed as outliers. Then means were re-calculated.

RESULTS

Transgenic lines containing inverted repeat (IR) segments of HAT or HDAC gene were produced as previously described (McGinnis et al., 2005; McGinnis et al., 2007). Expression of IR in these lines is intended to down-regulate the target chromatin gene by RNA interference (RNAi). Lines are designated with a unique number composed of the four-digit construct number followed by a three-digit number indicating the transformation from which the line was derived. Effectiveness of each transgene was measured using a reverse transcription PCR (RT-PCR) assay to asses reduction of target gene mRNA. For example, line 3826.011 is the eleventh transgenic event transformed

derived from transformation with construct pMCG3826. This line is distinct from 3826.026 which was derived from a separate transformation of the same construct.

For each IR transgene, we backcrossed the transgenes into a *Pl1-Blotched* background and selected homozygous *Pl1-Blotched* plants for pigment measurements (Fig. 2.1). The plants segregate for two *b1* alleles: a non-functional allele from the transgenic parent and a functional *B-I* allele from the *Pl1-Blotched* parent. *B-I* has a dosage effect on pigmentation; thus, within each family, we analyzed pigment levels separately for *B-I/B-I* homozygotes and *B-I/b1* heterozygotes. Mean anthocyanin levels were calculated for transgenic (herbicide-resistant; R) and non-transgenic (herbicide-sensitive; S) plants within each *b1* category. Then an R:S pigment ratio was obtained by dividing the mean pigment value for transgenic plants by the mean pigment value for their non-transgenic sibs. An R:S ratio equal to 1 would indicate no effect of the transgene on pigmentation, whereas R:S ratios above 1 or less than 1 would suggest that the targeted gene plays a role in regulating *Pl1-Blotched* or some other anthocyanin gene.

The cumulative results of all pigment assays are presented in Appendix 1. Subsets, which present the gene by gene results, are presented in the following sections.

Effects of transgenic IR transgenes targeting *hat* genes on pigmentation of *Pl1-Blotched* plants

hac101

The *hac101* gene is a member of the gene family encoding CREB-binding proteins (CBPs). As a group, the CBP HATs are large proteins with multiple functional domains. Only recently have they been described in plants (Pandey et al., 2002). There are five genes of this class in Arabidopsis and four in rice (www.ChromDB.org). In

maize, sequence information for this gene class is limited, but five genes have been identified (www.ChromDB.org). The best characterized gene, *hac101*, is the only one in this class that has been targeted for knockdown by RNAi. We examined the effect of two IR transgenes for *hac101* on pigmentation of *Pl1-Blotched* plants (Table 2.1).

- 3826.002. Target gene expression for this IR transgene, as measured by RT-PCR in seedlings, was variable, *e.g.*, some plants had good reduction of the target gene, but others did not (McGinnis et al., 2007). The effect of this transgene on pigmentation of *Pl1-Blotched* plants was assayed in one field season. The pigment ratio in transgenic (Basta-resistant; R) *vs*. non-transgenic (Basta-sensitive; S) sibs was 0.9 in *B-I* homozygotes and 0.56 in *B-I* heterozygotes.
- 3826.011. Expression of the target *hac101* gene in plants with this transgene was weak. The effect of this transgene on pigmentation was assayed in three seasons. The R:S pigment ratio ranged from 0.56 - 0.90.

Table 2.1: Effect of *hac101* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
3826.002	variable	W2006	746	1	10	0.83	0.17	10	0.92	0.21	0.90
				2	10	0.17	0.05	8	0.30	0.11	0.56
3826.011	weak	S2004	582	2	20	1.46	0.37	20	1.97	0.25	0.74
		S2005	559, 564	1	33	4.13	0.56	24	5.91	0.94	0.70
				2	6	1.46	0.36	18	2.11	0.33	0.69
		S2006	2249	1	15	0.69	0.13	14	1.21	0.16	0.57
				2	20	0.24	0.03	18	0.34	0.04	0.71

[a] Target gene expression measured by RT-PCR, relative to *glyceraldehye phosphate dehydrogenase C* mRNA (McGinnis et al., 2007). Categories: reduced, target gene mRNA < 30% of wild-type; weak, target gene mRNA 30-90% of wild-type; not reduced, target gene mRNA same as wild-type; nd, target gene mRNA level not determined.

The fact that both IR transgenes yielded R:S ratios less than 1 is consistent with the idea that *hac101* is normally involved in transcriptionally activating *Pl1-Blotched*. Reduced expression of *hac101* by the IR transgenes would lead to less activation of *Pl1-Blotched* and thereby less pigmentation in transgenic plants *vs*. in non-transgenic sibs.

hag101

The *hag101* gene codes for a protein in the GNAT class of HATs. It has sequence similarity to the yeast *Gcn5* gene, which encodes the catalytic subunit of the ADA and SAGA histone acetyltransferase complexes. The *hag* genes in plants encode small proteins with two or three functional domains. There are three *hag* genes in Arabidopsis, three in rice and four in maize (www.ChromDB.org). IR transgenic lines have been made for two of the maize *hag* genes. Four IR transgenes for *hag101* were analyzed (Table 2.2).

- 4681.006. The effect of this transgene on pigmentation of *Pl1-Blotched* plants was assayed in two field seasons. R:S pigment ratios varied; *b1* heterozygotes had ratios around 1, whereas the *b1* homozygotes had ratios less than 1. This trend was seen in both years.
- 4681.012. This IR transgene was also assayed in two field seasons. Sample sizes were good in all categories, but the R:S ratios varied.
- 4581.015. R:S ratios differed by two-fold for this IR transgene in the single season it was monitored.
- 4681.022. For this IR transgene, the R:S ratios differed slightly by *b1* genotype, but the patterns were similar in the two years.

Together, these data do not suggest a clear effect of *hag101* on pigmentation of *Pl1-Blotched*.

Table 2.2: Effect of *hag101* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
4681.006	nd	S2005	569	1	4	1.57	0.58	15	3.36	0.82	0.47
				2	8	1.16	0.08	6	0.99	0.16	1.17
		S2006	2285	1	7	0.49	0.16	27	1.04	0.19	0.47
				2	17	0.24	0.03	19	0.22	0.04	1.09
4681.012	nd	W2006	756	1	15	1.02	0.16	15	1.07	0.23	0.95
				2	16	0.40	0.10	13	0.22	0.07	1.82
		S2006	1869	1	13	0.51	0.10	16	0.34	0.03	1.50
				2	13	0.17	0.03	18	0.19	0.04	0.89
4681.015		W2006	766	1	10	1.30	0.22	15	0.94	0.20	1.38
				2	20	0.44	0.09	11	0.73	0.27	0.60
4681.022	nd	W2006	776	1	9	1.48	0.43	8	1.42	0.29	1.04
				2	10	0.89	0.19	18	0.64	0.19	1.39
		S2006	1889	1	9	0.55	0.20	13	0.58	0.12	0.95
				2	12	0.35	0.12	15	0.29	0.05	1.21

hag102

Three events for *hag102* were analyzed for their effect on pigmentation (Table 2.3).

- 4291.007. RT-PCR analysis of *hag102* expression for this IR transgene showed good reduction of target gene expression. The effect of this IR transgene on *Pl1-Blotched* pigmentation was analyzed in three families over two seasons. Five of the six R:S ratios were more than 1.
- 4291.010 and 4291.020. Neither of these transgenes had much effect on target gene expression. We determined the pigmentation effect of both IR transgenes in two seasons; the R:S ratios ranged from 0.40 to 1.28.

The more consistent trend in R:S ratios for 4292.007, compared to the other two *hag102* IR transgenes, may reflect the fact that this transgene effectively reduces target gene expression, whereas the other transgenes do not. The fact that the R:S ratios are greater than 1 for this transgene is not consistent with a direct effect of *hag102* on *Pl1-Blotched*.

Table 2.3: Effect of *hag102* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
4291.007	reduced	W2006	786	1	14	1.31	0.34	6	1.09	0.28	1.20
				2	10	0.58	0.31	10	0.08	0.05	7.22
		S2006	1919	1	19	0.55	0.10	17	0.45	0.11	1.20
				2	10	0.19	0.04	20	0.14	0.02	1.32
			1929	1	13	0.71	0.20	19	0.46	0.07	1.54
				2	17	0.22	0.03	20	0.23	0.02	0.96
4291.010	not reduced	S2005	957	1	8	2.13	0.58	16	4.33	1.00	0.49
				2	8	0.76	0.19	8	0.76	0.13	1.00
		S2006	1899	1	12	0.47	0.07	11	0.40	0.07	1.16
				2	23	0.21	0.01	19	0.20	0.02	1.10
4291.020	not reduced	W2006	796	1	11	0.47	0.13	9	0.84	0.16	0.56
				2	15	0.48	0.10	11	0.38	0.05	1.28
		S2006	1909	1	16	0.50	0.08	13	0.60	0.12	0.84
				2	21	0.19	0.12	11	0.29	0.12	0.66

hxa102

The *hxa102* gene encodes a protein with sequence similarity to the yeast ADA2 protein, which is a component of the SAGA complex and regulates GCN5 histone acetyltransferase activity. The HXA proteins in plants have three functional domains, organized in the same way as in the yeast ADA2 protein; thus, maize HXA is a likely partner of one or more of the HAG acetyltransferases. There are two *hxa* genes in Arabidopsis, one in rice and two in maize (www.ChromDB.org). We analyzed five IR transgenic lines targeting *hxa10*2 (Table 2.4).

- 3544.012. In the first season this transgene was assayed, the R:S ratio was 0.37. In the second season, this trend did not hold, as heterozygotes had a R:S ratio of 3.47.
- 3544.016. This is the only *hxa102* transgene for which target gene expression has been shown to be reduced in seedlings. We analyzed this transgene over two seasons. In 2004, the R:S ratio was less than 1, but in 2005, it was higher than 1.
- 3544.030. In the single assay season, this transgene yielded a R:S ratio less than 1.
- 3544.034. In the single assay season, this transgene yielded a R:S ratio greater than 1.

Overall, these data do not provide clear evidence that *hxa102* regulates *Pl1-Blotched* pigmentation.

Table 2.4: Effect of *hxa102* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
3544.012	not reduced	S2004	512	2	11	1.52	0.29	16	4.17	0.75	0.37
		S2005	634	1	4	0.51	0.17	8	2.29	0.94	0.22
				2	9	2.90	1.10	12	0.84	0.13	3.47
3544.016	reduced	S2004	517	2	5	0.61	0.14	11	1.21	0.34	0.50
		S2005	639	1	8	7.72	2.76	11	4.29	1.60	1.80
				2	12	2.13	1.11	8	0.30	0.06	7.09
3544.027	nd	S2005	927	1	6	4.11	1.58	10	4.77	1.32	0.86
				2	6	0.74	0.19	11	2.40	1.06	0.31
3544.030	nd	S2006	2079	1	14	0.25	0.04	14	0.57	0.09	0.44
				2	11	0.24	0.05	14	0.32	0.03	0.75
3544.034	not reduced	S2005	644	1	7	4.97	2.21	6	3.41	1.92	1.45
				2	13	1.85	0.65	8	0.54	0.07	3.42

ham101

The *ham101* gene belongs to the family coding for the MYST class of HATs. In yeast, the HAM proteins are catalytic subunits of multi-protein complexes that regulate

transcriptional silencing. In plants, the HAM proteins are small, with one conserved functional domain. Arabidopsis has two *ham* genes, rice has one and maize has two (www.ChromDB.org). We analyzed four IR transgenes for the maize *ham101* gene (Table 2.5).

- 4202.001. This IR transgene was analyzed in three seasons. In both seasons, herbicide-resistant plants were less numerous than herbicide sensitive plants. This is the pattern typically seen for unstable transgenes (McGinnis et al., 2007). Thus, the R:S ratios for this IR transgene are not trustworthy.
- 4202.006, 4202.018 and 4202.022. None of these IR transgenes reduced target gene expression in seedlings, as assayed by RT-PCR. Each IR transgene was assayed in a single season. R:S ratios ranged from 0.64 to 0.97.

Transgenic IR transgenes for this gene tended to lead to R:S ratios less than 1, but the effect was modest.

Table 2.5: Effect of *ham101* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
4202.001	nd	S2004	477	2	8	0.92	0.25	61	1.40	0.14	0.66
		S2005	574, 579, 584, 589	1	11	4.08	1.72	42	2.23	0.47	1.83
				2	5	0.62	0.12	34	0.71	0.10	0.88
4202.006	not reduced	S2006	1969	1	11	0.61	0.11	15	0.83	0.19	0.74
				2	14	0.25	0.02	11	0.28	0.04	0.88
4202.018	not reduced	S2006	1979	1	12	0.60	0.14	17	0.90	0.14	0.67
				2	11	0.25	0.03	17	0.26	0.03	0.97
4202.022	not reduced	S2006	1989	2	8	0.16	0.02	26	0.25	0.03	0.64

Effects of HDAC IR transgenes on pigmentation of *Pl1-Blotched* plants

hda101

The *hda101* gene is a member of a large family of genes encoding RPD3-type HDACs. In yeast, these proteins play roles in regulating trancription and silencing of many genes. In plants, these proteins have a single large conserved functional domain. Arabidopsis has twelve *hda* genes, rice has 14 and maize has 10 (www.ChromDB.org). We analyzed the effects of four maize *hda* genes. For *hda101*, three IR transgenes were assayed for their effect on pigmentation in *Pl1-Blotched* plants (Table 2.6).

- 3751.015. This transgene does not reduce expression of the target gene in seedlings. However, in our assays of husk pigmentation, this transgene led to more pigment in transgenic *vs*. nontransgenic sibs.
- 3751.019. In two seasons, this transgene led to R:S ratios around 1 in *b1* homozygotes, but higher ratios (4.03 and 1.47) in *b1* heterozygotes.
- 3751.027. This IR transgene led to R:S ratios over 1 in both *b1* genotypes in the single assay season.

Table 2.6: Effect of *hda101* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
3751.015	not reduced	S2005	594	1	9	6.74	1.38	17	2.66	0.66	2.54
				2	8	1.23	0.41	12	0.84	0.12	1.46
3751.019	nd	W2006			11	1.51	0.30	25	1.40	0.28	1.07
					10	0.59	0.17	7	0.15	0.03	4.03
		S2006	1999	1	10	0.61	0.12	12	0.64	0.14	0.96
				2	17	0.30	0.04	9	0.20	0.01	1.48
3751.027	not reduced	S2005	942	1	11	2.87	0.60	11	2.02	0.53	1.42
				2	6	0.59	0.11	7	0.43	0.11	1.38

Taken together, 3751.015 and 3751.027 showed consistently higher pigmentation in transgenic plants, relative to non-transgenic sibs. This is the pattern predicted for a HDAC that directly regulates *Pl1-Blotched* expression.

hda102

Four IR transgenes for this gene were analyzed (Table 2.7).

- 4571.002. This transgene reduces target gene expression in seedlings. Its effect on pigmentation in *Pl1-Blotched* plants was to reduce pigment production in transgenic plants by ~30 % relative to non-transgenic sibs.
- 4571.004. In both seasons that this transgene was assayed, *b1* heterozygotes had higher R:S ratios than *b1* homozygotes, and in all cases, the R:S ratios were greater than 1.
- 4571.020. This transgene reduces target gene expression in seedlings. Sampling of a small number of plants containing this transgene revealed higher pigmentation in transgenic plants.
- 4571.042. This transgene also is reported to reduce target gene expression, but its effect on *Pl1-Blotched* pigmentation was mixed. R:S ratios ranged from 0.75 to 1.40.

The varied effects of *hda102* transgenic IR transgenes on pigmentation do not lead to a clear idea of how this gene might regulate *Pl1-Blotched*.

Table 2.7: Effect of *hda102* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
4571.002	reduced	S2006	2267	1	16	0.38	0.05	20	0.53	0.08	0.71
				2	16	0.18	0.02	18	0.26	0.05	0.70
4571.004	nd	W2006	866	1	12	3.16	0.43	14	2.26	0.42	1.40
				2	17	2.46	0.59	15	0.65	0.13	3.78
		S2006	2019	1	10	0.54	0.09	11	0.39	0.11	1.40
				2	5	0.66	0.16	11	0.31	0.05	2.11
4571.020	reduced	S2005	614	2	4	2.09	1.82	11	0.67	0.11	3.13
4571.042	reduced	S2005	619	1	8	2.74	1.10	10	3.63	1.35	0.75
				2	5	3.63	2.54	4	3.09	2.23	1.18
		S2006	2276	1	21	0.96	0.20	14	0.68	0.16	1.40
				2	12	0.35	0.05	16	0.31	0.06	1.13

hda109

Two IR transgenes for this gene were analyzed, each in two seasons (Table 2.8).

- 4162.006. This transgene reduces target gene expression in seedlings, according to RT-PCR assays. In all of the pigment assays, R:S ratios were less than 1 and for three of the assays, the pigment levels in transgenic plants was about half the levels in non-transgenic sibs.
- 4162.009. The pigmentation pattern seen with this even showed low R:S ratios for three of the four measurements.

Taken together, these data suggest that *hda109* may regulate pigmentation in *Pl1-Blotched* plants. However, the fact that the R:S ratios are less than 1 means that the effect is not likely to be due to direct regulation of *Pl1-Blotched.*

Table 2.8: Effect of *hda109* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
4162.006	reduced	W2006	836	1	11	0.60	0.12	11	1.45	0.27	0.41
				2	15	0.42	0.08	18	0.81	0.15	0.52
		S2006	2029	1	13	0.35	0.06	21	0.79	0.13	0.44
				2	13	0.20	0.02	16	0.24	0.02	0.83
4162.009	reduced	W2006	846	1	12	1.27	0.24	7	1.63	0.54	0.78
				2	10	0.22	0.07	11	0.50	0.17	0.45
		S2006	2039	1	15	0.59	0.12	15	1.13	0.19	0.52
				2	15	0.22	0.02	19	0.21	0.01	1.03

hda110

Two IR transgenes were analyzed for this gene in a single season each (Table 2.9).

3534.011. For this transgene, in both measurements, R:S ratios were higher than 1.

3534.019. The two measurements for this transgene were not in agreement. In *b1* homozygotes, the R:S ratio was less than 1, but in *b1* heterozygotes, the R:S ratio was slightly greater than 1.

These data do not support a role of *hda110* in controlling pigmentation of *Pl1-Blotched* plants.

Table 2.9: Effect of *hda110* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
3534.011	not reduced	W2006	876	1	15	1.49	0.26	9	1.26	0.37	1.18
				2	18	0.61	0.14	21	0.43	0.08	1.39
3534.019	reduced	S2005	609	1	8	4.04	1.61	13	5.89	1.44	0.69
				2	8	1.00	0.41	13	0.93	0.20	1.07

srt101

The *srt101* gene is in the sirtuin class. The founding member of this group is yeast *SIR2*. The five sirtuins in yeast play roles in telomere maintenance and silencing. In Arabidopsis, two sirtuin genes have been identified; there are two genes in rice, but only one has been identified in maize (www.ChromDB.org). We analyzed the pigmentation effects of five IR transgenes (Table 2.10).

- 3571.002. This transgene shows weak expression of the target gene in seedlings. Its effect on pigmentation varied with *b1* genotype.
- 3571.011 and 3571.014. These transgenes show reduced *srt101* expression in seedlings. Pigmentation effects varied with R:S ratios ranging from 0.54 to 2.51.
- 3571.013 and 3571.030. Effects of these transgenes on target gene expression has not been determined. IR transgene 3571.014 led to R:S ratios higher than 1, but 3571.030 did not show this same trend.

The data for the *srt101* transgenes do not support the idea that this gene regulates pigmentation.

Table 2.10: Effect of *srt101* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
3571.002	weak	S2005	932	1	10	3.82	0.78	8	6.24	1.65	0.61
				2	10	1.01	0.17	8	0.83	0.09	1.22
3571.011	reduced	S2004	502	2	13	2.80	0.58	10	1.21	0.22	2.32
		S2005	649	1	8	6.84	2.43	13	6.64	1.83	1.03
				2	7	3.75	1.22	7	1.49	0.52	2.51
		S2006	2089	1	7	0.51	0.17	12	0.95	0.39	0.54
				2	12	0.19	0.02	25	0.24	0.03	0.80
3571.013	nd	S2006	2099	1	7	0.83	0.14	15	0.65	0.11	1.28
				2	9	0.34	0.06	18	0.25	0.03	1.38
3571.014	reduced	S2005	654	1	8	4.46	1.35	11	4.75	1.81	0.94
				2	9	1.90	0.90	11	2.81	0.97	0.68
3571.030	nd	S2005	937	1	9	3.70	0.83	8	3.95	1.03	0.94
				2	10	1.02	0.20	9	1.44	0.45	0.71

hdt101

The *hdt101* gene is a member of a plant-specific group of histone deacetylases. Arabidopsis has four *hdt* genes, rice has two and maize has four (www.ChromDB.org). We analyzed IR transgenes for two of the maize genes (Table 2.11).

- 3955.001 and 3955.046. Both of these transgenes reduce target gene expression in seedlings. Assay of their effect on pigmentation in husks varied, with no clear trend evident.
- 3955.026. The effect of this transgene on target gene expression has not been determined. Like the other transgenes, it showed varied effects on pigmentation.

These data do not provide evidence that *hdt101* regulates pigmentation.

Table 2.11: Effect of *hdt101* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
3955.001	reduced	S2004	472	2	13	1.17	0.17	15	0.70	0.10	1.66
		S2005	599	1	13	3.33	0.97	9	1.30	0.29	2.56
				2	6	3.39	0.95	5	0.75	0.19	4.48
		S2005	624	1	10	1.71	0.52	12	2.83	0.97	0.60
				2	6	0.90	0.23	10	2.42	0.71	0.37
		S2006	2258	1	9	0.97	0.21	18	0.83	0.16	1.17
				2	15	0.28	0.04	22	0.33	0.03	0.86
3955.005	nd	S2005	947	2	11	1.12	0.16	7	1.60	0.42	0.70
3955.026	nd	W2006	886	1	7	0.62	0.10	9	1.24	0.14	0.50
				2	10	0.52	0.12	15	0.49	0.07	1.06
		S2006	2059	1	9	0.36	0.08	15	0.28	0.05	1.32
				2	11	0.25	0.04	20	0.50	0.11	0.51
3955.046	reduced	S2006	2069	1	18	0.41	0.05	16	0.47	0.07	0.88
				2	17	0.23	0.02	24	0.25	0.03	0.92

hdt103

Two IR transgenes for this gene were analyzed (Table 2.12).

3361.001. This transgene reduces target gene expression in seedlings. Over three seasons, this IR transgene showed consistently high R:S ratios.

3361.005. This transgene shows weak expression of the target gene in seedlings. In our hands, this transgene was very unstable and did not produce resistant plants in any year other than 2005. In 2005, sample size was small, such that no meaningful conclusion can be drawn for this IR transgene.

The consistent data for 3361.001 suggest that *hdt103* may be important for regulating pigmentation, and the high R:S ratios are what one would predict if this gene has a direct effect on *Pl1-Blotched*.

Table 2.12: Effect of *hdt103* IR transgenes on pigmentation

IR transgene	Target Expression[a]	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
					#	Mean	SE	#	Mean	SE	
3361.001	reduced	S2003	728	1	22	1.74	0.28	31	1.10	0.14	1.58
		S2005	1008, 1012, 1013	1	13	6.17	1.33	9	3.15	0.94	1.96
				2	7	2.84	1.23	6	1.32	0.44	2.15
		S2006	2231	1	16	1.49	0.29	16	0.70	0.17	2.14
				2	27	0.60	0.14	35	0.38	0.05	1.59
3361.005	weak	S2005	922	1	3	1.74	0.78	4	1.25	0.25	1.39

Assays of IR transgenes over multiple seasons

Twenty IR transgenes were assayed over two or three seasons (Table 2.13). Most assays were performed on plants grown in the summer in Columbia, MO (S2003, S2004, S2005 and S2006), but some were done on plants grown in Juana Diaz, Puerto Rico (W2006). Because environmental conditions differed from year to year and in different locations, assaying across multiple seasons allowed us to assess how repeatable the phenotypes were in different environments. Eleven transgenes had mean R:S ratios that varied from the transgene mean by less than 20% (designated with asterisk in Table 2.13); five of these varied by less than 10%. The remaining transgenes showed higher variation.

This analysis also permitted an examination of phenotypes for multiple IR transgenes for the same IR construct. All three transgenes for *hag101* (construct 4681) and both IR transgenes for *hda109* (construct 4162) showed low variation across seasons.

Table 2.13. Comparison of R:S means by season and IR transgene

Gene	IR transgene	previous RT-PCR	Assay Season	Season R:S Mean[a]	IR transgene R:S Mean[b]	Difference[c]
hac101	3826.011*	weak	S2004	0.74	0.68	0.09
			S2005	0.70		0.02
			S2006	0.64		-0.06
hag101	4681.006*	nd	S2005	0.82	0.80	0.03
			S2006	0.78		-0.03
	4681.012*	nd	W2006	1.41	1.31	0.08
			S2006	1.21		-0.08
	4681.022*	nd	W2006	1.22	1.14	0.07
			S2006	1.07		-0.07
hag102	4291.007	reduced	W2006	4.21	2.24	0.88
			S2006	1.26		-0.44
			S2006	1.25		-0.44
	4291.010	not reduced	S2005	0.75	0.94	-0.21
			S2006	1.13		0.21
	4291.020*	not reduced	W2006	0.92	0.84	0.10
			S2006	0.75		-0.10
ham101	4202.001	nd	S2004	0.66	1.12	-0.41
			S2005	1.36		0.21
hda101	3751.019		W2006	2.55	1.89	0.35
			S2006	1.22		-0.35
hda102	4571.004*	nd	W2006	2.59	2.17	0.19
			S2006	1.76		-0.19
	4571.020	reduced	S2005	3.13	1.51	1.07
			S2006	0.71		-0.53
	4571.042*	reduced	S2005	0.97	1.12	-0.13
			S2006	1.27		0.13
hda109	4162.006*	reduced	W2006	0.47	0.55	-0.15
			S2006	0.64		0.15
	4162.009*	reduced	W2006	0.62	0.70	-0.12
			S2006	0.78		0.12
hdt101	3955.001	reduced	S2004	1.66	1.67	-0.01
			S2005	3.52		1.10
			S2005	0.49		-0.71
			S2006	1.02		-0.39
	3955.026*	nd	W2006	0.78	0.85	-0.08
			S2006	0.92		0.08
hdt103	3361.001*	reduced	S2003	1.58	1.88	-0.16
			S2005	2.06		0.09
			S2006	1.87		-0.01
hxa102	3544.012	not reduced	S2004	0.37	1.35	-0.73
			S2005	1.85		0.36
	3544.016	reduced	S2004	0.5	3.13	-0.84
			S2005	4.45		0.42

Table 2.13, continued

Gene	IR transgene	previous RT-PCR	Assay Season	Season R:S Meana	IR transgene R:S Meanb	Differencec
srt101	3571.011	reduced	S2004	2.32	1.44	0.61
			S2005	1.77		0.23
			S2006	0.67		-0.53

[a] Season mean is the average of R:S ratios across *b1* genotypes.
[b] IR transgene mean is the average of R:S ratios across *b1* genotypes and seasons.
[c] Difference was calculated as: (Season R:S Mean - IR transgene R:S Mean) / IR transgene R:S Mean.
* IR transgenes with <20% difference in Season *vs*. IR transgene mean.

DISCUSSION

We surveyed the effects of 40 IR transgenes on pigmentation of *Pl1-Blotched* plants. Eight IR transgenes, targeting five genes, had pigmentation phenotypes that were reproducible across *b1* genotypes and over multiple seasons and produced R:S ratios that varied from 1 by more than 30%. Two of the genes encode HATs and three encode HDACs.

The HAT IR transgenes targeted genes belonging to two classes--CBP and GNAT. HAT activity is typically associated with increased gene expression (Pandey et al., 2002). Thus, mutation in a HAT gene that directly influenced *Pl1-Blotched* would show less expression in transgenic plants, relative to non-transgenic sibs, *i.e*., an R:S ratio less than 1. This was the trend seen with IR transgenes 3826.002 and 3826.011, which target the CBP-type HAT gene, *hac101*. Together, these results suggest that *Pl1-Blotched* expression is directly regulated by *hac101*. However, for IR transgene 4219.007 targeting the GNAT-like *hag102* gene, the R:S ratio was greater than 1. This finding could be explained if *hag102* regulates a repressor of *Pl1-Blotched*, such that inactivation of this negative regulator would result in increased expression of *Pl1-Blotched*, and thus more pigmentation in transgenic progeny, relative to non-transgenic sibs.

The HDAC IR transgenes also showed disparate effects on pigmentation. HDAC activity is usually associated with gene silencing (Wade et al., 1998). Thus knocking down expression of HDAC genes involved in directly down-regulating *Pl1-Blotched* transcription should lead to increased pigmentation in transgenic *vs*. non-transgenic plants. This trend was seen for one transgene targeting *hdt103* (3361.001) and two of the transgenes targeting *hda101* (3751.015 and 3751.027); all three transgenes led to R:S ratios greater than 1. A third *hda101* IR transgene (3751.019), had R:S ratios greater than 1 in the *b1* heterozygotes. These observations are consistent with the idea that *hdt103* and *hda101* have a role in directly regulating *Pl1-Blotched*. By contrast, for two *hda109* IR transgenes (4162.006 and 4162.009), the R:S ratios were less than 1, hinting that regulation by this HDAC is not through a direct effect on *Pl1-Blotched*.

Measurement of pigmentation phenotypes for the same IR transgene over multiple seasons showed variability for some transgenes and not for others. The factors that would lead some IR transgenes to show stable phenotypes over multiple seasons and others to show variability are not yet clear. Transgenes that showed low variation in R:S ratios over multiple seasons were not necessarily the same transgenes that had R:S ratios with large deviations from 1. For example, two of the *hda102* transgenes (Table 2.7) had low seasonal variation, but one led to a high R:S ratio, whereas the other had a R:S ratio around 1.

For all of the transgenes, more than one IR transgene were analyzed, which offered us the opportunity to see whether a transgene, when inserted in different chromosomal contexts, would have the same effect on pigmentation. Our prediction was that the effects would vary, as not all IR transgenes for a single construct were equally

effective at reducing target gene expression. The data show that not all IR transgenes for the same construct produce the same phenotype. However, this variation is not readily correlated with whether or not the transgene induces strong reduction of target gene expression. For some transgenes, like the 4291 transgenes targeting *hag102*, there was a positive correlation between the effect on R:S ratio and the transgene effect on target gene expression. IR transgene 4291.007 had a strong effect on pigmentation and reduced target gene expression, whereas transgenes 4291.010 and 4291.020 that did not have a strong phenotype also did not have reduced target gene expression. For other transgenes, however, like the two 3826 IR transgenes targeting *hac101* and the two 4162 IR transgenes targeting *hda109*, both transgenes for a single construct had similar effects on R:S ratio. For the *hac101* transgenes, target gene expression was not very effectively reduced, whereas for the *hda109* transgenes, the transgene strongly reduced target expression. Yet other transgenes showed a third pattern; for example, for the three IR transgenes targeting *hda101*, the two with consistently high R:S ratios did not have reduced target gene expression. It is important to point out that target gene expression for the chromatin project IR transgenes was usually measured in seedling tissue, not in husks, which is the tissue we sampled. Thus, to more accurately correlate target gene expression with pigmentation phenotype, target gene expression should be measured in husks. Chapter 3 presents results of such an experiment.

Chapter 3

Correlation of gene expression and pigment levels in *Pl1-Blotched* plants with HAT and HDAC transgenes

The results of the survey of the effects of HAT and HDAC IR transgenes on *Pl1-Blotched* showed pigment differences in transgenic *vs*. non-transgenic sibs for eight transgenes. These results suggested that histone acetylation might influence pigmentation by acting on *Pl1-Blotched* expression, but there are other possible interpretations. For example, one unknown variable that could influence pigmentation is the effectiveness of the transgenes. Some of the implicated HAT and HDAC genes were analyzed previously for target gene expression levels, but not all transgenes are equally effective at reducing their gene targets (McGinnis et al., 2007). Previous expression analyses were done in seedlings, not in the mature adult tissues where we measured pigmentation. Another possibility is that the chromatin modifiers modulate expression of some other gene, such as *b1* or other unknown genes, which are segregating in the plant populations analyzed. The *b1* gene is a known regulator of anthocyanin synthesis, and some alleles of *b1* are subject to epigenetic regulation (Coe et al., 1988; Chandler and Stam, 2004).

To explore some of the genetic factors that are likely to contribute to the perturbation of pigmentation seen in *Pl1-Blotched* plants with HAT and HDAC transgenes, we measured transcript levels for target genes, *Pl1-Blotched* and *b1* in mature tissue. For this analysis, we selected thirteen families--some that showed marked alterations in pigmenation in previous seasons and, as controls, some that did not. The

goals for this experiment were to address two questions: Do anthocyanin pigment levels reflect differences in target HAT or HDAC gene expression? What effect do reduced target gene levels have on *Pl1-Blotched* and *B-I* expression?

MATERIALS AND METHODS

RNA extraction and Reverse Transcription-PCR

Total RNA was isolated from husks that had been harvested, frozen with liquid nitrogen, and stored at -80˚C. For RNA extraction, 0.5 g of frozen husk tissue was ground to a fine powder in a 15mL polypropylene centrifuge tube using a pre-chilled glass rod. To each tube, 500µL of NTES buffer (100 mM NaCl, 10mM Tris, pH 7.5, 1 mM EDTA, and 1% SDS) was added and the mixture was vortexed for 30 seconds. The tubes were incubated at room temperature for 10 minutes in a shaker, then 500µL phenol:chloroform:iso-amyl alcohol (100:100:1) was added. The samples were vortexed for 30 seconds, then incubated at room temperature for 10 minutes with shaking. The contents were poured into a 1.5 mL microfuge tube, and spun in a microfuge for 5 minutes. The aqueous phase was transferred to a new tube, and the organic phase was back extracted with 250 µL of NTES buffer. The aqueous phases were pooled. Isopropanol (250 µL) and 250 µL of RNA precipitation solution (1.2M NaCl, 0.8M sodium citrate) were added. The samples were mix by vortexing, incubated for 10 minutes at room temperature, and spun for 10 minutes in a microfuge at 4˚C. The supernatant was removed, the pellet was washed in 70% EtOH, dried briefly, and suspended in 50ul water.

Concentration of RNA was determined by measuring absorbance at 260 nm. Then the RNA samples were treated with deoxyribonuclease I (DNaseI) to remove genomic DNA. Reaction conditions for DNAse I treatment were: RNA (30 µg/mL); DNase I buffer (100 mM Tris-HCl, pH 7.5, 25mM $MgCl_2$, 1 mM $CaCl_2$); DNaseI (1 U/µL, Fermentas) in a volume of 300 µL. The samples were incubated at 37˚C for 30 minutes, then phenol extracted and precipitated with ethanol. The pellet was resuspended in 15 µL DEPC-treated water and its concentration was measured by reading absorbance at 260 nm.

For reverse transcription, 10-15 µg of RNA in 18.5 µL of DEPC-treated water was combined with 1 µL of oligodT primer (0.5 µg/µL; Promega) and incubated at 70˚C for 10 minutes. The samples were cooled on ice, then the following reagents (Promega) were added: 6 µL of 5X reverse transcriptase buffer (50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM MgCl2 and 10mM DTT), 3 µL of 5mM dNTPs, 1 µL of MMLV Reverse transcriptase (200 U/µL), and 0.5 µL of RNAsin (40 U/µL) were added to bring the total reaction volume to 30 µL. The tubes were incubated in a 42˚C water bath for 50 minutes, then placed in a 70˚C bath for 10minutes to terminate the reaction. Water (70 µL) was added to each tube and the samples were phenol-extracted and ethanol precipitated. Pellets were suspended in 20 µL water.)

Expression levels for *Pl1-Blotched*, *B-I*, and target chromatin genes were determined using a relative quantitative RT-PCR assay (Kerschen 2004). In this assay, first-strand cDNA for the gene of interest is amplified in the same reaction as the cDNA for a control genes, which encodes glyceraldehyde-3-phosphate dehydrogenase C (*GAPC*). *GAPC* is a constitutively expressed gene that plays a role in glycolysis, and its

expression is likely to be consistent across all samples. Because *GAPC* is expressed at higher levels that the genes of interest, amplification of *GAPC* must be reduced by adding inhibitory, non-extensible primers that are related in sequence to the control *GAPC* primers, except that the inhibitor primers are slightly longer and they terminate in a 3' dideoxynucleotide. *GAPC* was amplified using primer pair F4 (5'-CACTGCTACCCAGAAGACTGTTG-3') and R2 (5'-GTATCCCCACTCGTTGTCGTAC-3'), producing 419 bp PCR product. The corresponding amplification inhibitors of *GAPC* were F4' (5'-CATGCCATCACTGCTACCCAGAAGACTGTTG-dideoxyC-3') and R2' (5'-CGGGTGCTGTATCCCCACTCGTTGTCGTAC-dideoxyC3').

For each RNA sample, an inhibitor concentration test was performed using undiluted cDNA template, primers for the gene of interest, *GAPC* primers and a range of concentrations of *GAPC* inhibitor primers (0-55%). The inhibitor concentration that produced similar band intensities between the gene of interest and *GAPC* was used for PCR assays. Primer sequences for the genes tested, sizes of amplified products, and corresponding *GAPC* inhibitor concentrations are shown in Table 4.

PCR was performed in a 20 µL reaction consisting of: 5 µL of undiluted cDNA as template, 10 µL of 10X Red Taq (Sigma; 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 4 mM $MgCl_2$, 0.4 mM each dNTP, and 0.03 U/µL Taq polymerase), 2 µL of *GAPC* control/inhibitor mix (5 µM for control, 0-5 µM for inhibitor), 2 µL of gene-specific primers (5 µM), and 1 µL of water. Amplification conditions were: initial denaturation at 94˚C for 3 minutes, followed by 40 cycles of 94˚C for 30 sec, 52˚-60˚C (depending on the gene-specific primer) for 30 seconds to allow annealing, extension at 72˚C for 30

seconds, and a final extension step at 72˚C for 7 minutes. PCR products were fractionated on agarose gels. Images were captured digitally using an Alpha-Imager 2200, and the bands on the image were quantified using Image Gauge software (Fuji). The intensities of *b1*, *Pl1-Blotched*, target gene, and *GAPC* were measured and ratios of expression (*b1/GAPC*, *Pl1l/GAPC*, and target/*GAPC*) were compared in the basta-resistant and -sensitive classes.

IR sequencing

Families that underwent RNA analysis and RT-PCR were also tested for the presence of the transgene to verify that the HAT/HDAC of interest was being targeted by RNAi. For verification, the inverted repeats of the transgenes were amplified. The process was conducted using total genomic DNA extracted from husk tissue.

For DNA extraction, 0.3 g of frozen tissue was ground to a fine powder in a 15 mL falcon tube using pre-chilled glass rods. To each tube, 600 µL of lysis buffer (1X lysis buffer, 7M urea, 2% sarkosyl, 50mM EDTA) was added and the mixture warmed in the 42˚C bath until thawed. The tubes were incubated at 37˚C for 10 minutes with shaking, followed by the addition of 500 µL of 100:100:1 phenol:chloroform:iso-amyl alcohol and vortexing for 30 seconds. The tubes were shaken again at 37˚C, and the contents were poured into a 1.5 mL microfuge tube and spun for 5 minutes. 500 µL of the supernatant was transferred to a new tube, and 50 µL of 3 M NaAc (pH 5) and 600 µL of isopropanol were added. The tubes were inverted, then spun for 1 minute. The supernatant was removed with a glass pipette, and the pellet was washed with 500 µL of 70% EtOH. The tubes were spun for 30 seconds, and the supernatant was removed. The pellet was resuspended in 100 µL TE and vortexed until completely dissolved.

The IR sequences were amplified using primer pair 7942_180-203 F (5'-TTTTTACAACGTGCACAACAGAAT-3') and 7942_180-203 R (5'-ATCCCCTAGCCACCCAAGAA-3'). PCR was performed in a 20 µL reaction consisting of: 2 µL of 1/10 diluted DNA, 6 µL of autoclaved water, 10 µL of 10X Red Taq (Sigma; 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 4 mM $MgCl_2$, 0.4 mM each dNTP, and 0.03 U/µL Taq polymerase), and 2 µL of primers (5 uM each). PCR conditions were: initial denaturation at 95˚C for 20 minutes, followed by 41 cycles of denaturation at 95˚C for 30 seconds, annealing at 55˚C for 30 seconds, extending at 72˚C for 1 minute, and a final extension step at 72˚C for 10 minutes.

Prior to sequencing, amplified DNA was purified using a commercially available kit (Qiagen) and the absorbency was measured with the Nanodrop spectrophotometer. In a 500 µL microfuge tube, 50 ng of PCR product (in various volumes) was added to 2 µL of F primer (5uM) and the final volume was brought up to 12 µL with autoclaved water. The samples were sent to the DNA Core for sequencing, and were aligned using MegAlign (DNAstar software) to confirm sequence similarity between PCR products and their respective inverted repeats.

Whole leaf sheath anthocyanin extraction

Anthocyanins were extracted from a large rectangular portion of the leaf sheath. A horizontal cut was made at the top of the leaf sheath spanning the points of attachment of the leaf blade to the sheath. Vertical cuts were then made on the left and right borders, forming a rectangular section of the leaf sheath. After each rectangle was measured and its surface area recorded, the rectangle was cut into two-cm strips, which were placed in a 50-mL screw-cap polypropylene tube. For each two cm^2 of tissue, 0.5 mL of 3% HCl in

95% EtOH was added, and the tubes were capped and incubated at room temperature in the dark for 24 hours. The next day, each tube was shaken vigorously and 1 mL of the extract was transferred to a 96 well plate. From there, 75-µL aliquot was removed and added to 75 µL of water in a microtiter plate. To three wells, 75 µL of 3% HCl in 95% EtOH was added 75 µL of water to serve as blanks. The plates were read in a plate reader as described previously.

Statistical analysis

Statistical comparisons were performed using analysis programs from SAS (Cary, NC). Correlations of pigment measurements from hole punch *vs*. whole leaf sheath sampling were made using Spearman's correlation. For regression analyses, raw data were square root-transformed so that they fit a normal distribution, as monitored by increases in the Shapiro-Wilk statistic for each data point. Outliers were removed by two rounds of residual analysis. Regression was conducted using three methods of model selection in PROC REG: R-square selection, backward elimination and maximum improvement.

RESULTS

Target gene expression levels

Target gene expression in husks was measured for the thirteen selected IR transgenes using a RT-PCR assay, in which expression of the target was measured relative to the constitutively expressed *GAPC* gene (Table 3.1). The ratio of mean values for transgenic (R) plants *vs.* non-transgenic (S) plants ranged from 0.01 to 1.19, indicating that some transgenes are very efficient at reducing target gene expression,

whereas others are not effective. For three of the 13 transgenes, the level of target gene expression correlated well with previous estimates of target gene expression in seedlings. Four transgenes had not previously been analyzed. For six of the 13 genes, the level of target gene expression did not correlate well. At least part of the discrepancy is likely due to the fact that previous estimates were not strictly quantitative for all transgenes; instead the level of reduction of target gene expression was estimated from comparisons of band intensities on gel images to images obtained in pilot experiments where quantitation was performed (K. McGinnis, personal communication).

We attempted to correlate R:S pigment ratios with R:S target gene expression levels to ask if transgenes that gave R:S pigment ratios higher or lower than 1 were more likely to show good reduction in expression of their targets. However, there was no significant trend.

This prompted us to reconsider our method for sampling leaf sheath material for pigment extraction. Our method of taking two hole punches located in the same place on every leaf sheath could be biased, in that heavily pigmented portions of a leaf sheath might not be sampled. Thus, we re-sampled the sheaths of the plants carying the 13 IR transgenes, extracting pigment from a large rectangle of tissue, which encompassed a much larger proportion of the sheath. Our reasoning was that pigment extracted from this larger sample should be more representative of the total amount of pigment in the entire sheath.

Table 3.1. Target gene expression in husks and pigmentation in leaf sheaths for HAT and HDAC transgenes

Gene	IR transgene	Family	# R	# S	Mean Target / *GAPC* expression			Target expression		Mean Pigmentation [a]					
										Hole Punch			Whole Sheath		
					R	S	R:S ratio	Seed-ling	Husk	R	S	R:S ratio	R	S	R:S ratio
hac101	3826.001	2249	12	12	0.97	1.43	0.68	weak	weak	0.91	1.41	0.65	0.52	0.75	0.69
hag101	4681.012	1869	11	15	1.08	1.45	0.74	nd	weak	0.74	0.4	1.85	0.35	0.25	1.40
hxa102	3544.003	2079	11	12	0.81	0.87	0.93	nd	not reduced	0.45	0.59	0.76	0.43	0.65	0.66
ham101	4202.006 [b]	1969	11	14	0.77	0.99	0.78	not reduced	weak	0.61	1.19	0.51	0.33	0.48	0.69
hda101	3751.019	1999	7	10	0.14	0.61	0.23	nd	reduced	0.69	0.7	0.99	0.50	0.45	1.11
hda102	4571.002 [b]	2267	15	16	0.91	1.27	0.72	reduced	weak	0.4	0.56	0.71	0.27	0.35	0.77
hda102	4571.042 [b]	2276	17	15	0.52	1.12	0.46	reduced	weak	1.28	0.85	1.51	0.93	0.34	2.74
hda109	4162.006 [b]	2029	12	21	0.64	0.94	0.68	reduced	weak	0.33	1.1	0.30	0.38	0.44	0.86
hda109	4162.009 [b]	2039	16	10	0.45	0.72	0.63	reduced	weak	0.7	1.14	0.61	0.54	0.58	0.93
hdt101	3955.001	2258	10	12	0.17	0.5	0.34	reduced	reduced	1.17	1.33	0.88	0.68	0.56	1.21
hdt101	3955.026	2059	7	15	0.66	0.66	1.00	nd	not reduced	0.42	0.35	1.20	0.34	0.38	0.89
hdt101	3955.046 [b]	2069	15	16	1.15	0.97	1.19	reduced	not reduced	0.44	0.47	0.94	0.61	0.47	1.30
hdt103	3361.001	2231/33	12	15	0.01	1.23	0.01	reduced	reduced	1.53	0.73	2.10	0.69	0.53	1.30

[a] Pigmentation in leaf sheaths sampled by hole punch. Values are different from values presented in Chapter 2, because not all plants were included in this analysis.

[b] Transgenes for which mean target gene R:S ratios for husks do not agree with previous target gene expression assessments in seedling.

Statistical analysis was used to ask how the two phenotypic measures compare to one another (Table 3.2). Correlation coefficients ranged from a low of -0.292 to a high of 0.815. For four families--4681.012, 4202.006, 3955.026 and 3361.001--the correlations were not statistically significant ($p > 0.05$), but for nine families, the correlation was significant ($p < 0.05$).

Table 3.2. Correlation of hole punch and whole-sheath anthocyanin values

Gene	IR transgene	Family	Spearman's Coefficient	P-value
hac101	3826.011	2249	0.592	0.008
hag101	4681.012	1869	0.104	0.636
hxa102	3544.003	2079	0.617	0.006
ham101	4202.006	1969	0.114	0.622
hda101	3751.019	1999	0.796	0.001
hda102	4571.002	2267	0.529	0.007
hda102	4571.042	2276	0.815	<.0001
hda109	4162.006	2029	0.574	0.002
hda109	4162.009	2039	0.472	0.026
hdt101	3955.001	2258	0.654	0.002
hdt101	3955.026	2059	-0.292	0.240
hdt101	3955.046	2069	0.694	<.0001
hdt103	3361.001	2231/33	0.247	0.130

The effects of target gene, *B-I*, and *Pl1-Blotched* on pigment (hole punch)

The effects of target gene, *B-I*, and *Pl1-Blotched* on hole punch pigment were analyzed statistically using regression. Three regression methods were used; all provided substantially the same outcomes. The results of R-square selection, in which variables are added to the regression model at random, are presented in Table 3.3. R-square values for a 3-variable model of target + *B-I* + *Pl1-Blotched* ranged from 0.041 to 0.837. For the IR transgenic line (3751.019; *hda101*) with the highest R-square value, target gene expression alone accounted for 10% of the variance in phenotype and the best two-

variable model, explaining 77% of the variance, included *B-I* and target; *Pl1-Blotched* makes only a modest additional contribution to the 3-variable model. *B-I* plus target also accounted for most of the variance for several other families: 4202.006 (*ham101*); 4571.002 (*hda102*); 4162.009 (*hda109*); 3955.001 (*hdt101*); 3955.026 (*hdt101*). However, for several transgenes, *Pl1-Blotched* plus target explained the majority of the variance: 3826.001 (*hac101*); 3955.046 (*hdt101*); 3361.001 (*hdt103*). For two transgenes, 4571.042 (*hda102*) and 4162.006 (*hda109*), two 2-variable models explained high levels of variance: *Pl1-Blotched* plus target and *Pl1-Blotched* plus *B-I.* For two transgenes, 4681.012 (*hag101*) and 3544.003 (*hxa102*), the 3-variable model accounted for less than 10% of the phenotypic variance.

The effects of target gene, *B-I*, and *Pl1-Blotched* on pigment (leaf sheath)

Regression models were constructed to look at the effect of target gene, *B-I* and *Pl1-Blotched* on pigmentation as measured by extracting pigments from a large portion of the leaf sheath (Table 3.4). Only four families had 3-variable models explaining over 10% of the variance in pigmentation. The largest effect was seen again for transgene 3751.019 (*hda101*), with the largest 2-variable contributions made by *B-I* and target gene. The transgene with the next largest effect (37%) was 4571.042 (*hda102*); in this case *Pl1-Blotched* was the largest single contributor to phenotypic variance, whereas the 2-variable models gave almost equal weight to *Pl1-Blotched* plus target and *Pl1-Blotched* plus *B-I.* This same pattern was seen for transgene 4202.006 (*ham101*), although the total variance explained was only 19%. For these two transgenes, the data are consistent with the idea that the target genes regulate *Pl1-Blotched*, which interacts with its partner *B-I* to control pigmentation. For transgene 4162.009 (*hda109*)--for which the 3-variable model

Table 3.3. The effects of *target gene*, *B-I*, and *Pl1-Blotched* on pigment (hole punch)

Gene	Transgene	Family	N	Model: 1 variable			Model: 2 variable			Model: 3 variable
				target	B	Pl	B + target	Pl + target	Pl + B	
hac101	3826.001	2249	16	0.190	0.035	0.006	0.192	0.298	0.048	0.339
hag101	4681.012	1869	17	0.033	0.013	0.006	0.086	0.044	0.017	0.093
hxa102	3544.003	2079	15	0.010	0.002	0.020	0.011	0.036	0.020	0.041
ham101	4202.006	1969	19	0.007	0.140	0.000	0.219	0.007	0.163	0.222
hda101	3751.019	1999	10	0.109	0.039	0.058	0.775	0.182	0.090	0.837
hda102	4571.002	2267	25	0.019	0.090	0.002	0.186	0.021	0.091	0.186
hda102	4571.042	2276	18	0.090	0.094	0.246	0.169	0.269	0.280	0.302
hda109	4162.006	2029	23	0.083	0.164	0.218	0.198	0.290	0.283	0.325
hda109	4162.009	2039	15	0.073	0.345	0.083	0.346	0.116	0.350	0.350
hdt101	3955.001	2258	20	0.002	0.075	0.120	0.161	0.135	0.130	0.212
hdt101	3955.026	2059	15	0.015	0.178	0.030	0.275	0.053	0.183	0.284
hdt101	3955.046	2069	23	0.034	0.002	0.066	0.034	0.124	0.071	0.125
hdt103	3361.001	2231/33	35	0.005	0.057	0.137	0.061	0.137	0.212	0.212

Numbers represent R-square values for possible 1-, 2-, and 3-variable models.

Shaded cells represent highest values in 1-, 2-, and 3-variable models.

accounts for 13% of the phenotypic variance--the major contributors were *Pl1-Blotched* and *B-I*; variation in target gene expression did not contribute significantly to variance for this transgene.

DISCUSSION

Analysis of gene expression in families of *Pl1-Blotched* plants carrying IR transgenes targeting HAT and HDAC genes revealed two probable genetic interactions important for regulating pigmentation. The first, exhibited by transgene 3751.019 (*hda101*) was the very large contributrion of *B-I* and the target gene, *hda101*, to variation in anthocyanin pigmentation in both hole punch and whole sheath extractions. The magnitude of this effect may have been overestimated for this family, though; although the family included 17 individuals (7 resistant and 10 sensitive), only nine of the individuals were retained in the dataset after outliers were removed; thus the sample size for the regression analysis was small for this family. Nevertheless, the observation in six other families that a model including *B-I* plus target gene explains most of the phenotypic variation in hole punch anthocyanin levels bolsters the idea that *B-I* is being regulated at the level of chromatin. Expression of *B-I* is known to be regulated in part by an upstream enhancer element that is subject to alterations in its chromatin state (Stam et al., 2002); thus, it is possible that this enhancer is the target of regulation by *hda101* and/or one of the other chromatin genes.

Table 3.4. **The effects of *target gene*, *B-I*, and *Pl1-Blotched* on pigment (leaf sheath)**

Gene	Transgene	Family	N	Model: 1 variable			Model: 2 variable			Model: 3 variable
				target	B	Pl	B + target	Pl + target	Pl + B	target + B + Pl
hac101	3826.001	2249	15	0.000	0.035	0.017	0.036	0.041	0.042	0.048
hag101	4681.012	1869	19	0.002	0.003	0.047	0.007	0.048	0.056	0.061
hxa102	3544.003	2079	16	0.019	0.060	0.063	0.084	0.065	0.080	0.089
ham101	4202.006	1969	19	0.000	0.002	0.139	0.002	0.178	0.175	0.194
hda101	3751.019	1999	9	0.053	0.054	0.104	0.610	0.162	0.147	0.679
hda102	4571.002	2267	26	0.008	0.043	0.027	0.043	0.035	0.071	0.071
hda102	4571.042	2276	21	0.144	0.047	0.249	0.192	0.329	0.290	0.371
hda109	4162.006	2029	23	0.000	0.001	0.117	0.001	0.118	0.130	0.135
hda109	4162.009	2039	14	0.001	0.001	0.012	0.001	0.020	0.018	0.022
hdt101	3955.001	2258	22	0.027	0.001	0.014	0.050	0.068	0.029	0.073
hdt101	3955.026	2059	15	0.004	0.026	0.037	0.039	0.037	0.077	0.082
hdt101	3955.046	2069	22	0.001	0.011	0.035	0.011	0.036	0.040	0.042
hdt103	3361.001	2231/33	33	0.023	0.014	0.026	0.034	0.046	0.034	0.053

Numbers represent R-square values for possible 1-, 2-, and 3-variable models.

Shaded cells represent highest values in 1-, 2-, and 3-variable model.

The second genetic interaction explaining a portion of the variation in pigmentation in both hole punch and whole sheath extracts is a model that includes *Pl1-Blotched* either in combination with the target or in combination with *B-I*. This pattern was seen for 4202.006 (*ham101*), 4162.006 (*hda109*), and for one of the *hda102* transgenes, 4571.042. In each case, variation in *Pl1-Blotched* was the best single-factor contributor to phenotypic variation, suggesting that the primary effect of the transgenes is on expression of *Pl1-Blotched*. If this is true, then it should be possible to demonstrate differences in histone acetylation of *Pl1-Blotched* chromatin in plants carrying these transgenes.

With the exception of 3751.019 (*hda101*) , the regression models explain only a small amount of the variation in anthocyanin pigmentation. The remaining variation is likely due to the interaction of other genes and the environment. The plants assayed were from backcross populations, thus considerable heterozygosity remains. Continued introgression of the most promising transgenes into the *Pl1-Blotched* background might reduce variation due to other alleles. Introgressed lines could then be grown in different locations to dissect the environmental contribution to phenotypic variance.

Chapter 4

Summary and Future Directions

In this study, the goal was to ask if histone acetylation is involved in regulating *Pl1-Blotched* expression. The experimental strategy was to assay anthocyanin pigmentation in *Pl1-Blotched* plants carrying IR transgenes designed to reduce expression of target HAT or HDAC genes by RNAi. Pigmentation differences between transgenic and non-transgenic sibling plants were detected for eight transgenes. Four of these were included in the thirteen genes chosen for further analysis. Table 4.1 summarizes some of the data for these thirteen transgenes.

Table 4.1. Summary of HAT and HDAC transgene effects on pigment, target gene expression, and regression models

Gene	Transgene	Mean R:S			R-square for 3-variable model	
		Pigment		Target/ GAPC		
		Hole punch	Whole sheath		Hole punch	Whole sheath
hac101	3826.011*	0.65	0.69	0.68	0.339	0.048
hag101	4681.012	1.85	1.40	0.74	0.093	0.061
hxa102	3544.003	0.76	0.66	0.93	0.041	0.089
ham101	4202.006	0.51	0.69	0.78	0.222	0.194
hda101	3751.019	0.99	1.11	0.23	0.837	0.679
hda102	4571.002	0.71	0.77	0.72	0.186	0.071
hda102	4571.042	1.51	2.74	0.46	0.302	0.371
hda109	4162.006*	0.30	0.86	0.68	0.325	0.135
hda109	4162.009*	0.61	0.93	0.63	0.350	0.022
hdt101	3955.001	0.88	1.21	0.34	0.212	0.073
hdt101	3955.026	1.20	0.89	1.00	0.284	0.082
hdt101	3955.046	0.94	1.30	1.19	0.125	0.042
hdt103	3361.001*	2.10	1.30	0.01	0.212	0.053

* Transgenes judged as promising in the Chapter 2 survey.

Of the four genes analyzed in both the survey and the detailed study, none explained a large proportion of the phenotypic variance in both regression models.

Transgene 4162.006 (*hda109*) explained 32% of the variance in pigment in hole punch extracts, but only 13% of the variance in whole sheath extracts. The other three transgenes had R-square values in the 21-35% range for hole punch pigment, but very low values for whole sheath pigment. This discrepancy was not due to lack of correlation between the two pigment measures, as correlations were good for two of the three (Table 3.2). In fact, hole punch pigment measurements seem to be a good screening method for identifying transgenes that are promising candidates for regulating *Pl1-Blotched*.

Three other transgenes had high R-square values for both hole punch and whole sheath pigmentation. Transgenes 3751.019 (*hda101*) and 4571.042 (*hda102*) were analyzed previously, but did not have have a consistent effect on *Pl1-Blotched* pigmentation. 3751.019 was analyzed in two seasons, but had disparate R:S ratios in *b1* homozygotes *vs*. heterozygotes. 4571.042 had a modest effect on R:S ratio in the two seasons it was assayed, as did 4202.006 (*ham101*) in a single season's assays.

Analysis of target gene expression revealed a range of efficiencies for transgene silencing of target genes. The most effective transgene was 3361.001 (*hdt103*), which reduced target expression over 90%. This transgene did not yield a credible R-square model for whole sheath pigmentation, despite repeatably high R:S pigment ratios for hole punch extracts over three seasons. The reasons for this discrepancy are unclear.

Considerations for future study

This study identified four genes that are potential regulators of *Pl1-Blotched* and / or *B-I*. Follow-up studies should include analysis of larger populations to verify the effects observed here. Molecular analysis of histone modification patterns of *Pl1-*

Blotched in transgenic plants could help confirm these candidates. In addition, because changes in histone modification can lead to changes in DNA methylation, the methylation status of *Pl1-Blotched* could also be analyzed in plants with the transgenes. Finally, the four transgenes identified as potential candidates in the survey, but not subjected to detailed analysis in this project, should be studied further.

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Appendix 1: Survey of pigment phenotypes in *Pl1-Blotched* plants carrying HAT and HDAC transgenes

Entries are arranged in alphabetical order by gene name. *b1*: 1, homozygous *B-I/B-I*; 2, heterozygous *B-I/b1*. S.E.: standard error of the mean.

Gene	IR Transgene	Previous RT-PCR	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
						#R	mean	S.E.	#S	mean	S.E.	
hac101	3826.002	variable	W2006	746	1	10	0.83	0.17	10	0.92	0.21	0.90
					2	10	0.17	0.05	8	0.30	0.11	0.56
	3826.011	weak	S2004	582	2	20	1.46	0.37	20	1.97	0.25	0.74
			S2005	559, 564	1	33	4.13	0.56	24	5.91	0.94	0.70
					2	6	1.46	0.36	18	2.11	0.33	0.69
			S2006	2249	1	15	0.69	0.13	14	1.21	0.16	0.57
					2	20	0.24	0.03	18	0.34	0.04	0.71
hag101	4681.006	nd	S2005	569	1	4	1.57	0.58	15	3.36	0.82	0.47
					2	8	1.16	0.08	6	0.99	0.16	1.16
			S2006	2285	1	7	0.49	0.16	27	1.04	0.19	0.47
					2	17	0.24	0.03	19	0.22	0.04	1.08
	4681.012	nd	W2006	756	1	15	1.02	0.16	15	1.07	0.23	0.96
					2	16	0.40	0.10	13	0.22	0.07	1.86
			S2006	1869	1	13	0.51	0.10	16	0.34	0.03	1.50
					2	13	0.17	0.03	18	0.19	0.04	0.91
	4681.015	nd	W2006	766	1	10	1.30	0.22	15	0.94	0.20	1.38
					2	20	0.44	0.09	11	0.73	0.27	0.60
	4681.022	nd	W2006	776	1	9	1.48	0.43	8	1.42	0.29	1.04
					2	10	0.89	0.19	18	0.64	0.19	1.39
			S2006	1889	1	9	0.55	0.20	13	0.58	0.12	0.94
					2	12	0.35	0.12	15	0.29	0.05	1.19

Gene	IR Transgene	Previous RT-PCR	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
						#R	mean	S.E.	#S	mean	S.E.	
hag102	4291.020	not reduced	S2006	1909	1	16	0.50	0.08	13	0.60	0.12	0.84
					2	21	0.19	0.12	11	0.29	0.12	0.66
	4291.007	reduced	S2006	1919	1	19	0.55	0.10	17	0.45	0.11	1.20
					2	10	0.19	0.04	20	0.14	0.02	1.32
			W2006	786	1	14	1.31	0.34	6	1.09	0.28	1.20
					2	10	0.58	0.31	10	0.08	0.05	7.22
			S2006	1929	1	13	0.71	0.20	19	0.46	0.07	1.54
					2	17	0.22	0.03	20	0.23	0.02	0.96
	4291.010	not reduced	S2005	957	1	8	2.13	0.58	16	4.33	1.00	0.49
					2	8	0.76	0.19	8	0.76	0.13	1.00
			S2006	1899	1	12	0.47	0.07	11	0.40	0.07	1.16
					2	23	0.21	0.01	19	0.20	0.02	1.10
			W2006	796	1	11	0.47	0.13	9	0.84	0.16	0.56
					2	15	0.48	0.10	11	0.38	0.05	1.28
ham101	4202.001	nd	S2004	477	2	8	0.92	0.25	61	1.40	0.14	0.66
			S2005	574, 579, 584, 589	1	11	4.08	1.72	42	2.23	0.47	1.83
					2	5	0.62	0.12	34	0.71	0.10	0.88
	4202.006	not reduced	S2006	1969	1	11	0.61	0.11	15	0.83	0.19	0.74
					2	14	0.25	0.02	11	0.28	0.04	0.88
	4202.018	not reduced	S2006	1979	1	12	0.60	0.14	17	0.90	0.14	0.67
					2	11	0.25	0.03	17	0.26	0.03	0.97
	4202.022	not reduced	S2006	1989	2	8	0.16	0.02	26	0.25	0.03	0.64

Gene	IR Transgene	Previous RT-PCR	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
						#R	mean	S.E.	#S	mean	S.E.	
hda101	3751.015	not reduced	S2005	594	1	9	6.74	1.38	17	2.66	0.66	2.54
					2	8	1.23	0.41	12	0.84	0.12	1.46
	3751.019	nd	W2006	856	1	11	1.51	0.30	25	1.40	0.28	1.07
					2	10	0.59	0.17	7	0.15	0.03	4.03
			S2006	1999	1	10	0.61	0.12	12	0.64	0.14	0.96
					2	17	0.30	0.04	9	0.20	0.01	1.48
	3751.027	not reduced	S2005	942	1	11	2.87	0.60	11	2.02	0.53	1.42
					2	6	0.59	0.11	7	0.43	0.11	1.38
hda102	4571.002	reduced	S2006	2267	1	16	0.38	0.05	20	0.53	0.08	0.71
					2	16	0.18	0.02	18	0.26	0.05	0.70
	4571.004	nd	W2006	866	1	12	3.16	0.43	14	2.26	0.42	1.40
					2	17	2.46	0.59	15	0.65	0.13	3.78
			S2006	2019	1	10	0.54	0.09	11	0.39	0.11	1.40
					2	5	0.66	0.16	11	0.31	0.05	2.11
	4571.020	reduced	S2005	614	2	4	2.09	1.82	11	0.67	0.11	3.13
			S2005	619	1	8	2.74	1.10	10	3.63	1.35	0.75
					2	5	3.63	2.54	4	3.09	2.23	1.18
			S2006	2276	1	21	0.96	0.20	14	0.68	0.16	1.40
					2	12	0.35	0.05	16	0.31	0.06	1.13
hda109	4162.006	reduced	W2006	836	1	11	0.60	0.12	11	1.45	0.27	0.41
					2	15	0.42	0.08	18	0.81	0.15	0.52
			S2006	2029	1	13	0.35	0.06	21	0.79	0.13	0.44
					2	13	0.20	0.02	16	0.24	0.02	0.83
	4162.009	reduced	W2006	846	1	12	1.27	0.24	7	1.63	0.54	0.78
					2	10	0.22	0.07	11	0.50	0.17	0.45
			S2006	2039	1	15	0.59	0.12	15	1.13	0.19	0.52
					2	15	0.22	0.02	19	0.21	0.01	1.03

Gene	IR Transgene	Previous RT-PCR	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
						#R	mean	S.E.	#S	mean	S.E.	
hda110	3534.011	not reduced	W2006	876	1	15	1.49	0.26	9	1.26	0.37	1.18
					2	18	0.61	0.14	21	0.43	0.08	1.39
	3534.019	reduced	S2005	609	1	8	4.04	1.61	13	5.89	1.44	0.69
					2	8	1.00	0.41	13	0.93	0.20	1.07
hdt101	3955.001	reduced	S2004	472	2	13	1.17	0.17	15	0.70	0.10	1.66
			S2005	599	1	13	3.33	0.97	9	1.30	0.29	2.56
					2	6	3.39	0.95	5	0.75	0.19	4.48
			S2005	624	1	10	1.71	0.52	12	2.83	0.97	0.60
					2	6	0.90	0.23	10	2.42	0.71	0.37
			S2006	2258	1	9	0.97	0.21	18	0.83	0.16	1.17
					2	15	0.28	0.04	22	0.33	0.03	0.86
	3955.005	nd	S2005	947	2	v	1.12	0.16	7	1.60	0.42	0.70
	3955.026	nd	W2006	886	1	7	0.62	0.10	9	1.24	0.14	0.50
					2	10	0.52	0.12	15	0.49	0.07	1.06
			S2006	2059	1	9	0.36	0.08	15	0.28	0.05	1.32
					2	11	0.25	0.04	20	0.50	0.11	0.51
	3955.046	reduced	S2006	2069	1	18	0.41	0.05	16	0.47	0.07	0.88
					2	17	0.23	0.02	24	0.25	0.03	0.92
hdt103	3361.001	reduced	S2003	728	1	22	1.74	0.28	31	1.10	0.14	1.58
			S2006	2231	1	16	1.49	0.29	16	0.70	0.17	2.14
					2	27	0.60	0.14	35	0.38	0.05	1.59
			S2005	1008, 1012, 1013	1	13	6.17	1.33	9	3.15	0.94	1.96
					2	7	2.84	1.23	6	1.32	0.44	2.15
	3361.005	weak	S2005	922	1	3	1.74	0.78	4	1.25	0.25	1.39

Gene	IR Transgene	Previous RT-PCR	Assay Season	Family	*b1*	Resistant Plants			Sensitive Plants			R:S ratio
						#R	mean	S.E.	#S	mean	S.E.	
hxa102	3544.012	not reduced	S2004	512	2	11	1.52	0.29	16	4.17	0.75	0.37
			S2005	634	1	4	0.51	0.17	8	2.29	0.94	0.22
					2	9	2.90	1.10	12	0.84	0.13	3.47
	3544.016	reduced	S2004	517	2	5	0.61	0.14	11	1.21	0.34	0.50
			S2005	639	1	8	7.72	2.76	11	4.29	1.60	1.80
					2	12	2.13	1.11	8	0.30	0.06	7.09
	3544.027	nd	S2005	927	1	6	4.11	1.58	10	4.77	1.32	0.86
					2	6	0.74	0.19	11	2.40	1.06	0.31
	3544.030	nd	S2006	2079	1	14	0.25	0.04	14	0.57	0.09	0.44
					2	11	0.24	0.05	14	0.32	0.03	0.75
	3544.034	not reduced	S2005	644	1	7	4.97	2.21	6	3.41	1.92	1.45
					2	13	1.85	0.65	8	0.54	0.07	3.42
srt101	3571.002	weak	S2005	932	1	10	3.82	0.78	8	6.24	1.65	0.61
					2	10	1.01	0.17	8	0.83	0.09	1.22
	3571.011	reduced	S2004	502	2	13	2.80	0.58	10	1.21	0.22	2.32
			S2005	649	1	8	6.84	2.43	13	6.64	1.83	1.03
					2	7	3.75	1.22	7	1.49	0.52	2.51
			S2006	2089	1	7	0.51	0.17	12	0.95	0.39	0.54
					2	12	0.19	0.02	25	0.24	0.03	0.80
	3571.013	nd	S2006	2099	1	7	0.83	0.14	15	0.65	0.11	1.28
					2	9	0.34	0.06	18	0.25	0.03	1.38
	3571.014	reduced	S2005	654	1	8	4.46	1.35	11	4.75	1.81	0.94
					2	9	1.90	0.90	11	2.81	0.97	0.68
	3571.030	nd	S2005	937	1	9	3.70	0.83	8	3.95	1.03	0.94
					2	10	1.02	0.20	9	1.44	0.45	0.71

Appendix 2. PCR Primers and Inhibitor Concentrations used for RT-PCR

Primer	Target Gene	Sequence (5' to 3')	cDNA length (bp)	Genomic DNA length (bp)	Annealing Temperature	GAPC inhibitor conc (%)
MCG580A	*hac101*	CCATACAAATCGAAGGCCATTCTC	374	561	53.2˚C	35
MCG580B		ATCAGGTTCTGGTACCAGCTTC				
MCG342C	*ham101*	CATGAAGCGCAAAGAACAGC	402	1845	53.6˚C	32.5
MCG342D		TCCGGTGTCCCAACTTTACC				
MCG209D	*hda101*	AGGATGGTGGCAGGGTTG	501	631	55.5˚C	27.5
MCG209E		ACACGCAATGAATCACCCTTT				
MCG582A	*hdt101*	GGTCGGGGGTCAAGAAGTGA	396	880	54.9˚C	37.5
MCG582B		TCCTTCGCAGGAACCTTTGC				
455..474 F	*hda109*	TGCTGTTCTTGCAGCTGGTT	493	1794	52.6˚C	37.5
947..928 R		CCCCGCTGAAACCAGAATTA				
MCG378C	*hda102*	TGCCAAATCTATGCGGGAGG	707	816	51.9˚C	42.5
MCG378D		ACTTTCAATGTATAATCTGGAGC				
MCG253C	*hxa102*	GGCTAAAGTGCAGGGTGAAAG	527	2278	53.9˚C	35
MCG253D		TTGTAAGATCCTTCTCCAAGGG				
MCG587A	*hdt103*	TGCTCTTGGGGAATCGAAGA	563	816	55.1˚C	30
MCG587B		TCAGCACGGAACTTTCAGCA				
512..528 F	*hag101*	CGTACAGCGCCCGTGAG	470	1520	54.5˚C	40
981..958 R		GTACCCTTGCCATCTTTCTTTGTC				
pl_Bh_f_20070313	*Pl1-Blotched*	CTAGCTAGCTGGACACCGAGAG	181	292	60.0˚C	50
pl_Bh_r_20070313		CACCGACGCAAACCGGCTT				
MCG617E	*B-I*	ATTGCTGAAGAAAGCGTTGG	316	387	57.1˚C	15
MCG617F		GTTGGAGCCCACACAGACTT				