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TRANSGENERATIONAL INHERITANCE OF EPIGENETIC REGULATION BY *UNSTABLE FACTOR FOR ORANGE1* IN MAIZE

A Thesis in

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by

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ABSTRACT

Epigenetics is the study of heritable changes in gene expression or cellular phenotype that are attributable to mechanisms other than changes in DNA sequence. In plants, epigenetic regulation plays a key role in various biological processes such as paramutation, genomic imprinting, and gene silencing. Although numerous studies have been done to elucidate the underlying mechanism behind epigenetic regulation, many questions are yet to be answered. In this study, the maize *pericarp color1* (*p1*) gene is used as a reporter to study the nature of epigenetic inheritance of stable tissue-specific gene expression. An allele of *p1*, *P1-wr* was used as a phenotypic marker to investigate the transgenerational inheritance of *Unstable factor for orange1* (*Ufo1-1*)-induced changes in maize. *Ufo1-1* activates *p1* expression and thus phlobaphenes are ectopically accumulated throughout the plant body indicating loss of tissue-specificity of *p1* expression. Previous study has shown that *Ufo1-1*-induced pigmentation phenotypes are only observed in a subset of *P1-wr; Ufo1-1* plants. Interestingly, within this subset, pigmentation level is highly variable. Also, it has been shown that this increased pigmentation is associated with changes of DNA methylation pattern in the *P1-wr* distal enhancer and intron sequences. Moreover, the increased pigmentation phenotypes in the backcross population are accompanied by progressive loss of *P1-wr* methylation from one generation to the next. Thus, the objective of this study was to investigate the inheritance of *Ufo1-1* through genotyping using linked markers. This information was then compared to the *Ufo1-1*-induced phenotypes to verify the epigenetic regulation of *P1-wr* by *Ufo1-1*. The second objective of this study was to establish a qRT-PCR based assay to investigate DNA methylation level at *p1* in different genotypes of *P1-wr; Ufo1-1* plants. The relative methylation levels provided better understanding of the correlation between the range of pericarp pigmentation in *P1-wr; Ufo1-1* plants and their respective DNA methylation states at *p1*.

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Chapter 1

Introduction

Regulation of gene expression in multi-cellular organisms is intricate because of the complexity of their cell specializations. Multiple levels of regulation are employed by eukaryotic organisms to temporally and spatially express their genes during different developmental stages. These regulations take place at different levels such as transcriptional initiation, RNA processing, translational initiation and post-translational modifications. Another unique mechanism of regulation of gene expression is epigenetics. Literally, epigenetics means "outside conventional genetics" [see review (JAENISCH and BIRD 2003)]. Although studies on epigenetic regulation have begun as early as in the 1950s, many additional intriguing questions remain. Herein, we have used the well-characterized maize flavonoid biosynthetic pathway to study epigenetic inheritance over several generations. A novel epigenetic modifier is used to investigate this non-Mendelian phenomenon of inheritance of gene silencing or gene expression. Understanding of the epigenetic mediated inheritance of gene expression in maize can be applied in other organisms as well.

Epigenetics

The term was originally coined by Waddington in 1942 to explain causal interactions between genes of a genotype and their phenotypic effects (WADDINGTON 1942). Today, epigenetics is widely defined as the study of heritable changes in gene expression without a change in DNA sequence [see review (WOLFFE and MATZKE 1999)]. Over the past decades, many studies on epigenetics have been done

on different organisms ranging from unicellular fungi to mammals; for examples, in yeast [see review (KLAR *et al.* 2007)], fruit fly [see review (MOEHRLE and PARO 1994)], mice (HARPER 2005; MORGAN *et al.* 1999) and plants (BECKER *et al.* 2011; LI *et al.* 2008). The roles of epigenetics in regulation of gene expression have been demonstrated in various biological processes such as paramutation [see review (CHANDLER 2007; HOLLICK 2010)], genomic imprinting [see review (MORA-GARCIA and GOODRICH 2000; SCOTT and SPIELMAN 2004)], X chromosome inactivation [see review (JAENISCH and BIRD 2003)], transgene silencing (MATZKE *et al.* 2000), and cancer biology [see review (EGGER *et al.* 2004; HERCEG and USHIJIMA 2010)]. Epigenetics regulates gene expression through stable repression of genes in specific cell types during different developmental stages. DNA methylation, chromatin modifications and small RNAs are known mechanisms that mediate epigenetic regulation (LI *et al.* 2008). These suppressive mechanisms control gene repression or even inactivation and thus generate tissue- specific expression patterns. In the following paragraphs, epigenetic mechanisms are described in details.

DNA methylation

DNA methylation (presence of a $-CH_3$ (methyl) group on cytosine) plays a pivotal role in epigenetic regulation of gene expression in animals and plants [see review (BIRD 2002; GOLL and BESTOR 2005)]. In mammals, DNA methylation solely occurs at cytosine bases in the symmetrical CG context at a very high frequency; roughly around 70-80% of CG dinucleotides of the genome are methylated. The unmethylated CG dinucleotides are mostly located near promoter regions of genes and are known as CpG islands (CGIs) (YAMADA *et al.* 2004). In contrast, plant DNA methylation occurs at cytosine bases in both the symmetrical, CG, CHG and asymmetrical CHH context (in which H= A, T or C) [see review (HENDERSON and JACOBSEN 2007)]. Recent work has highlighted 5-methylcytosine as a bona fide epigenetic mark that is involved in transcriptional silencing and actively participates in maintaining

genome stability and normal development [see review (GRANT-DOWNTON and DICKINSON 2005; GRANT-DOWNTON and DICKINSON 2006)]. DNA methylation in plants is mostly found over repetitive DNA structures and transposable elements (TEs). Recent evidence has shown that perturbation of the DNA methylation pattern might result in transcriptional reactivation and enhanced mobilization of TEs [see review (TEIXEIRA and COLOT 2010)]. Establishment, maintenance and modification of DNA methylation has been extensively studied in model plant, *Arabidopsis thaliana*. These genetic and molecular studies have since revealed the presence of RNA -mediated *de novo* DNA methylation, which is known as RNA – directed DNA methylation (RdDM) (WASSENEGGER *et al.* 1994). Many components involved in this RdDM pathway have been characterized. These components include RNA interference (RNAi) machinery (Dicer and Argonaute families), plant –specific RNA Polymerases (Pol IV and Pol V), putative chromatin-remodeling factors and other newly discovered proteins [see review (LAW and JACOBSEN 2010)]. Small interfering RNAs (SiRNAs) that are produced through complex RdDM machinery form a silencing complex and recruit the downstream modifying proteins to mediate *de novo* methylation at their target loci. In plants, *Domains Rearranged Methyltransferase 2* (DRM2), a homologue of *DNA methyltransferase 3* (DNMT3), catalyzes the *de novo* cytosine methylation in all sequence contexts. Although only DRM2 is responsible for the establishment of cytosine methylation, the maintenance of DNA methylation requires three different methyltransferases depending upon the sequence contexts. The symmetric CG and CHG methylation are maintained by DNA Methyltransferase1 (MET1) and Chromo methylatransferase3 (CMT3) respectively. On the other hand, the asymmetric CHH methylation is sustained through constant *de novo* methylation by DRM2 and RdDM. Nevertheless, at some loci, the CHH methylation is maintained by both CMT3 and DRM2. However, little is known on the pathways controlling this maintenance of DNA methylation. Although DNA methylation is a stable epigenetic mark, in some cases, reduction or loss of methylation is observed during development (SEKHON and CHOPRA 2009). Nonetheless, the mechanism of modification or removal of DNA methylation is not well

characterized. In plants, DNA glycosylase activity is responsible for active demethylation. *Demeter* (*DME*) and *Repressor of Silencing1* (*ROS1*) are the genes encoding DNA glycosylases in *Arabidopsis*. These glycosylases identify and actively erase the methylated cytosines in all sequence contexts. Hitherto, the mechanisms of action of these DNA glycosylases are not well understood.

Chromatin modification

Chromatin is the state in which chromosomal DNA is packaged with histone proteins inside the cell. In eukaryotes, the fundamental unit of chromatin is nucleosome core particles (NCPs) that are composed of a histone octamer complex made up of dimer each of the histones (H2A, H2B, H3 and H4) and enfolded by an approximately 147-bp DNA [see review (ERIC and DANNY 2009)]. A linker histone, H1, completes the chromatosome by binding to inter-nucleosomal linker DNA and consequently further condenses the chromatin. Chromatin can be divided into two types; heterochromatin and euchromatin. Heterochromatin is a highly condensed form of chromatin with limited access for transcriptional processes to occur and thus is usually transcriptionally silent. Secondly, euchromatin, a lightly packed chromatin, is easily accessible for transcriptional processes and hence is transcriptionally active. Therefore, the dynamics of chromatin structure can serve as a molecular switch for either the activation or inactivation of gene expression. Chromatin structure can be altered by the modification of either the DNA or histone proteins through DNA methylation, chromatin remodeling and histone modification. Chromatin remodeling involves alteration of nucleosome occupancy through displacement or removal of histone proteins from the nucleosome complexes. This ATP – driven interaction is a highly energy-intensive process because the remodeling enzymes need to disturb hundreds of contacts between DNA and histones [see review (GANGARAJU and BARTHOLOMEW 2007)]. Histone post-translational modifications occur on their N-terminal “tails” and there are at least eight different types of modifications including

acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerization [see review (KOUZARIDES 2007)]. These modifications are added onto distinct amino acids and histone variants (STRAHL and ALLIS 2000). In general, methylation of histone 3 lysine 9 (H3K9) or histone 3 lysine 27 (H3K27) are associated with silenced regions. In contrast, acetylation of histone 3 and histone 4 lead to gene activation since acetylation can modify the net positive charge of a nucleosome and thus loosen the histone and DNA complex (LIU *et al.* 2010).

Maize flavonoid biosynthetic pathway: an excellent system to study epigenetics

Research over the past few decades has shed light on epigenetic mechanisms. Yet despite what has been accomplished, many questions remain largely unanswered. The prevalence of epigenetic modifications in the plant kingdom allows plants to be excellent systems to study epigenetics. However, little has been done to identify or isolate the epigenetic modifications because their effects tend to be lost over generations. Herein, we have used the flavonoid biosynthetic pathway in maize to study epigenetic inheritance. This well-characterized biosynthetic pathway leads to the biosynthesis of two major maize flavonoid pigments; purple or red anthocyanins and brick red phlobaphenes (Figure 1-1). Anthocyanins are derived from flavan-3, 4-diol and can be produced in almost all tissues in maize. Meanwhile, phlobaphenes are derived from polymerization of flavan-4-ols, (apiferol and luteoforol) and found predominantly in the maize floral organs such as kernel pericarp (outermost layer of ovary wall) and cob glumes (palea and lemma, floral bracts subtending the kernel) (COE *et al.* 1988; STYLES and CESKA 1977). Both pathways share some common precursors like chalcones and flavanones. However, the two pathways are regulated independently. Anthocyanin accumulation is regulated by transcription factors *C1* + *R1* in the aluerone layer of the endosperm and *Pl1* + *B1* in the vegetative plant tissues, whereas phlobaphenes accumulation is regulated by *pericarp color1* (*p1)* (STYLES and CESKA 1977). The maize *p1*

gene encodes an R2R3 *Myb*-like transcription factor that activates transcription of phlobaphene structural genes, including *c2,* (*colorless2;* chalcone synthase), *chi,* (*chalcone isomerase;* chalcone isomerase) and *a1 ,* (*anthocyaninless1;* dihydro flavonol reductase) (GROTEWOLD *et al.* 1994).

***pericarp color1 (p1*)**: **a reporter gene to study epigenetic regulation**

The presence of more than 100 natural alleles of *p1* contributes to a high degree of phenotypic diversity (COCCIOLONE *et al.* 2001; STYLES and BRINK 1969). Distinct *p1* alleles are distinguished by their pigmentation patterns in pericarp and cob glumes. For instance, *P1-wr* specifies *w*hite pericarp and *r*ed cob glumes, and *P1-rr* specifies *r*ed pericarp and *r*ed cob glumes. Likewise, *P1-rw* designates *r*ed pericarp and *w*hite cob glumes, whereas *P1-ww* characterizes *w*hite pericarp and *w*hite cob glumes. The phenotypic differences between *P1-rr* and *P1-wr* are really intriguing because these two alleles have 99.9% DNA sequence similarity in their coding, proximal promoter and upstream promoter regions (CHOPRA *et al.* 1998). One striking difference between these two alleles is their gene structure; *P1-rr* allele contains only one copy, whereas *P1-wr* allele carries six copies of a 12.6 kb gene unit containing the coding and regulatory regions (CHOPRA *et al.* 1998). Despite the high sequence similarity between *P1-wr* and *P1-rr,* the transcript level in colorless *P1-wr* pericarp is reduced to 30% of that in *P1-rr* pericarp (CHOPRA *et al.* 1996). Further functional analysis of transgenic plants carrying promoter and coding region of *P1-wr* and *P1-rr*, and their respective DNA methylation patterns suggested that the unique pigmentation pattern of the *P1-wr* may be epigenetically regulated (COCCIOLONE *et al.* 2001). Epialleles of the *p1* gene such as *P1-rr', P1-wr** and *P1-pr*^{Tp} have also been shown to be epigenetically regulated through epigenetic marks including DNA methylation and histone modifications (GOETTEL and MESSING 2013; RHEE *et al.* 2010; SEKHON *et al.* 2012).

Unstable factor for orange1 (Ufo1)*: an epigenetic modifier of *pericarp color1

A spontaneous, dominant mutant designated as *Unstable factor for orange1 (Ufo1)* has been characterized to induce ectopic phlobaphene accumulation in plants carrying *P1-wr* allele (CHOPRA *et al.* 2003; STYLES 1982; STYLES *et al.* 1987). *Ufo1-1* modifies the *P1-wr* expression resulting in red pigmentation in kernel pericarp and cob glumes and thus *P1-wr; Ufo1-1* ears resemble *P1-rr* phenotypically (Figure 1-2). Restoration of phlobaphene pigmentation in *P1-wr; Ufo1-1* plants is not only limited to pericarp and cob glumes, but also clearly visible in other organs such as silks, husk, tassel glumes and leaf sheath (Figure 1-3). Importantly, *P1-wr* is hypermethylated compared to *P1-rr* (CHOPRA *et al.* 1998) and interestingly, the DNA methylation level in *P1-wr* sequence is reduced in the presence of *Ufo1-1* (CHOPRA *et al.* 2003). Therefore, the *Ufo1-1*-induced gain of pericarp pigmentation has been presumptively associated with the reduction of DNA methylation level of *P1-wr*. *Ufo1-1* shows poor penetrance (only around 30% of F_1 progeny of *P1-wr*/*P1-wr*; *ufo1/ufo1* and *P1-ww/P1-ww; Ufo1-1/Ufo1-1* cross showed gain of pericarp pigmentation) and low expressivity (the extent of pigmentation is highly variable)(CHOPRA *et al.* 2003). However, the penetrance and expressivity are improved after several generations of backcrossing with the *P1-wr* as a recurrent parent (SEKHON and CHOPRA 2009). Furthermore, in the presence of *Ufo1-1* mutation, *P1-wr* transcripts in pericarp are three-fold higher than that compared to the wild- type *P1-wr* (CHOPRA *et al.* 2003). This suggests that wild-type *ufo1* might be involved in tissue-specific silencing of *P1-wr* presumably by DNA methylation, the most common epigenetic mark.

Recent works have also demonstrated the role of wild-type *ufo1* in gene silencing at other *p1* alleles including spontaneous epialleles, *P1-wr** and *P1-pr*^{TP} (SEKHON *et al.* 2007; SEKHON *et al.* 2012), tissue culture-induced epiallele, *p1-ww:DP* (RHEE *et al.* 2010), paramutagenic allele, *P1-rr'* (SEKHON *et al.* 2012) and silenced epiallele, *P1-pr* (GOETTEL and MESSING 2013). Recent evidences also show that histone

modification (loss of H3K9me2 mark from the enhancer region of *p1* gene) also plays a role in maintaining epigenetic silencing of *p1* (SEKHON et al. 2012).

Transgenerational epigenetic inheritance: Investigating the inheritance of *Ufo1-1* and its effects

Epigenetics has now become an established discipline since the 1970s and progress in this field has led to a better understanding of the underlying molecular mechanisms in epigenetic regulation of gene expression during plant development as well as in response to environmental stress [see review (DAXINGER and WHITELAW 2010; JABLONKA and RAZ 2009)]. The boosting of knowledge on this aspect has sparked more interest in transgenerational epigenetic inheritance (TEI) over the recent years. TEI is the inheritance of expression states across generations and thus phenotypic traits are not determined by the DNA sequence alone [see review (PASZKOWSKI and GROSSNIKLAUS 2011)]. In animal systems, epigenetic marks such as DNA methylation and histone modifications are erased and reset in primordial germ cells (PGCs) and in the zygote to ensure establishment of cellular totipotency of the early embryo [see review (DAXINGER and WHITELAW 2010; HAUSER *et al.* 2011)]. This transgenerational resetting of epigenetic "default-state" is less well understood in plants, but it has been well documented in mammalian systems [see review (FENG *et al.* 2010; JULLIEN and BERGER 2010)]. The presence of naturally occurring epialleles that are stably inherited over several generations indicates that this inter-generational reprogramming is apparently incomplete. A classical example of natural epialleles involves a symmetry change in phenotype of *Linaria vulgaris* flowers. The phenotypic change from bilateral to radial symmetry correlates with the degree of DNA methylation in the upstream promoter region of the *Lcyc* gene; reversions to bilateral flowers symmetry correlate with hypomethylation at the locus (CUBAS *et al.* 1999).

Recent studies on natural and environmentally/stress-induced epialleles show some correlations between the new heritable traits and persistent change in DNA methylation level (MANNING *et al.* 2006; STEWARD *et al.* 2002). In plants, such cases were reported in snapdragon (HASHIDA *et al.* 2003; HASHIDA *et al.* 2006), dandelion (VERHOEVEN *et al.* 2010) and *Arabidopsis* (SOPPE *et al.* 2000). Epigenetic memory of stress has been constantly studied because of its importance in short- or long- term adaptation to environmental influences. The *Ufo1-1* mutation disrupts stable inheritance of *P1-wr* tissue-specific expression indicating that the wild type *ufo1* is responsible for maintaining the *P1-wr* expression over generations. Intriguingly, activation and maintenance of *Ufo1-1*-induced plant phenotypes do not require external environmental stresses. Following this rationale, the *Ufo1-1* mutant is a perfect tool to study transgenerational epigenetic inheritance. This study on *Ufo1-1* provides basic understanding of the mechanisms needed for a stable inheritance of gene expression. Stable inheritance of introduced traits or genes is the key for successful breeding strategies. Therefore, in a broader perspective, *ufo1* like epigenetic modifiers could be used to enhance the efficiency of breeding programs; to resist periodic breakdown of the new cultivars/ hybrids, and control expression of specific agronomic traits.

Objectives

The *Ufo1-1*-induced pericarp pigmentation is unstable and this property also depends on its genetic background. In the previous experiments where *Ufo1-1* was introgressed into the inbred line 4Co63 (genotype *p1-ww c1 r-r*) and crossed to *P1-wr* (W23 inbred), incomplete penetrance and poor expressivity of the *Ufo1-1*-induced phenotypes were observed. Additionally, extended exposure to the *Ufo1-1* mutation by several backcrossings has led to a gradual increase in penetrance and expressivity of *Ufo1-1*-induced phenotypes. These improvements in expressivity were associated with a progressive loss of DNA methylation of *P1-wr* gene sequence over multiple generations. These observations raise a

set of interesting questions. If expressivity of *Ufo1-1* mutation is background dependent, then what is the nature of this interaction? Are the *Ufo1-1* allele and its effects stably inherited over several generations? What are the epigenetic mechanisms imposed by wild type *ufo1* that maintain silencing of *p1* gene over generations? Are these epigenetic marks heritable? In an effort to answer the aforementioned questions and to learn more about the nature of *Ufo1-1* inheritance, I proposed the following hypothesis.

Hypothesis: Given the fact that *Ufo1-1* shows incomplete penetrance and variable expressivity in different maize genetic backgrounds, I hypothesize that *Ufo1-1* is inherited as a single genetic factor and that its penetrance is affected by the extent of silencing at *P1-wr* in the previous generation. Hence, uniformly dark expressing ears will give rise to highly penetrant families, whereas silent ears will give rise to poorly penetrant families. Also, the penetrance and expressivity of *Ufo1-1* may be correlated to the presence of other loci that affect expression of *Ufo1-1*. To test this hypothesis, following experiments were performed:

1. Investigating genotypic and phenotypic inheritance of *Ufo1-1* from crosses between (1) *Ufo1-1* stock (in its original background) and *P1-wr* [B73] and (2) introgressed *Ufo1-1* (in *P1-ww* [4Co63] background) and *P1-wr* [W23]
2. Developing qRT-PCR based assay to compare DNA methylation level at *p1* in different genotypes of *P1-wr; Ufo1-1* plants (i.e. homozygous *Ufo1-1* or heterozygous *Ufo1-1*) showing either *Ufo1-1* expresser or non–expresser phenotypes

The genotypic and phenotypic data served to address the hypothesis on the transgenerational epigenetic inheritance of *Ufo1-1*. Meanwhile, the DNA methylation results helped in explaining the non-concordance between genotypic and phenotypic data. The relative methylation levels provided better understanding of the range of pericarp pigmentations in *P1-wr; Ufo1-1* plants.

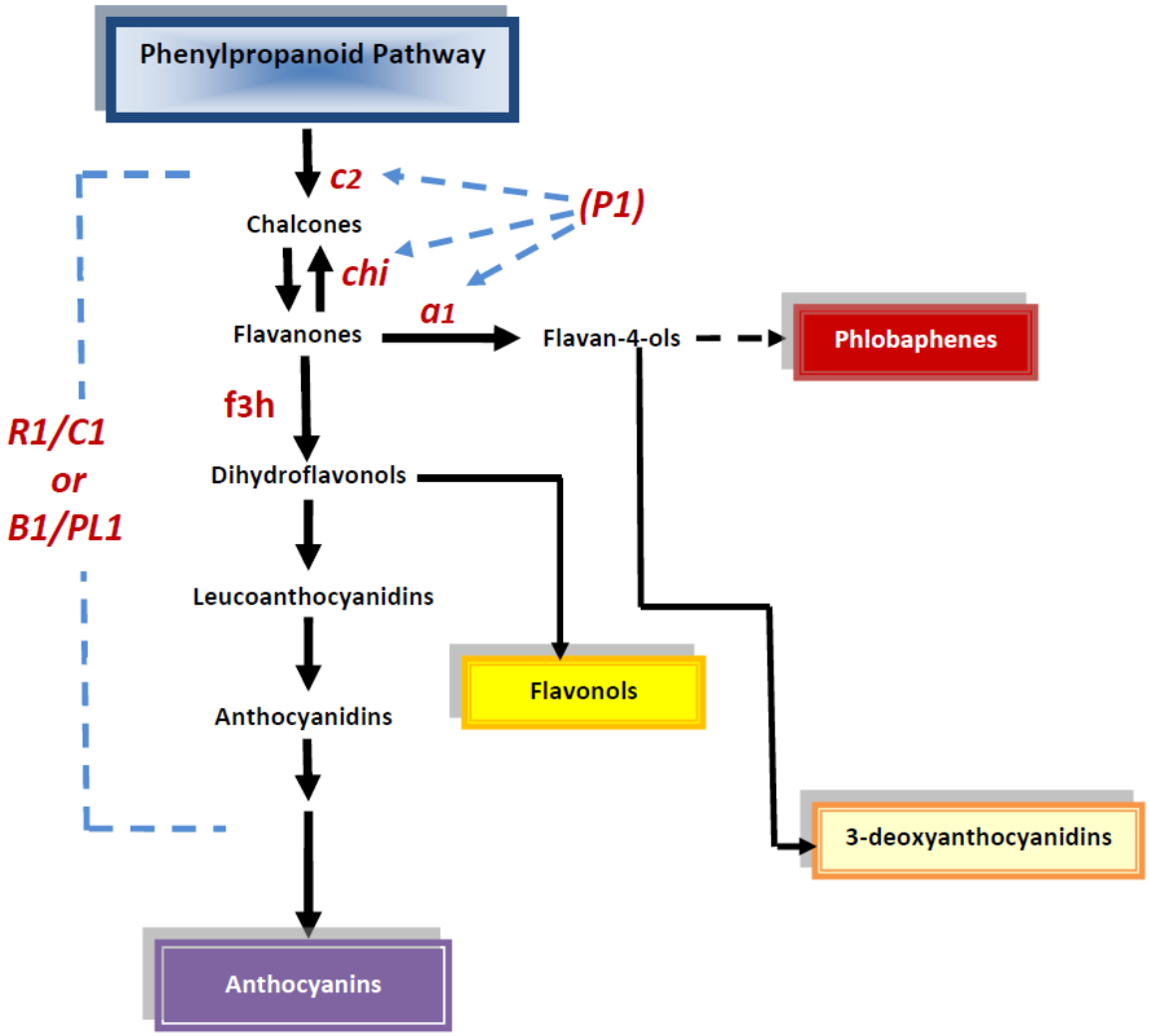

Figure 1-1: Flavonoid biosynthetic pathway in maize. Structural genes shown are: *colorless2, c2* (chalcone synthase); *chalcone isomerase, chi* (chalcone isomerase); *anthocyaninless1, a1* (dihydroflavonol reductase); *flavanone 3-hydroxylase*, *f3h* (flavanone 3-hydroxylase). Regulatory genes shown are*: pericarp color1* (P1), *red1* (R1), *purple plant1* (PL1), *booster1* (B1) *and colorless1* (C1).The P1, PL1 and C1 are MYB transcription factors, whereas B1 and R1 are bHLH domain proteins.

Figure 1-2: Ear phenotypes of *P1-rr, P1-wr* alleles and *P1-wr* with *Ufo1-1* mutation

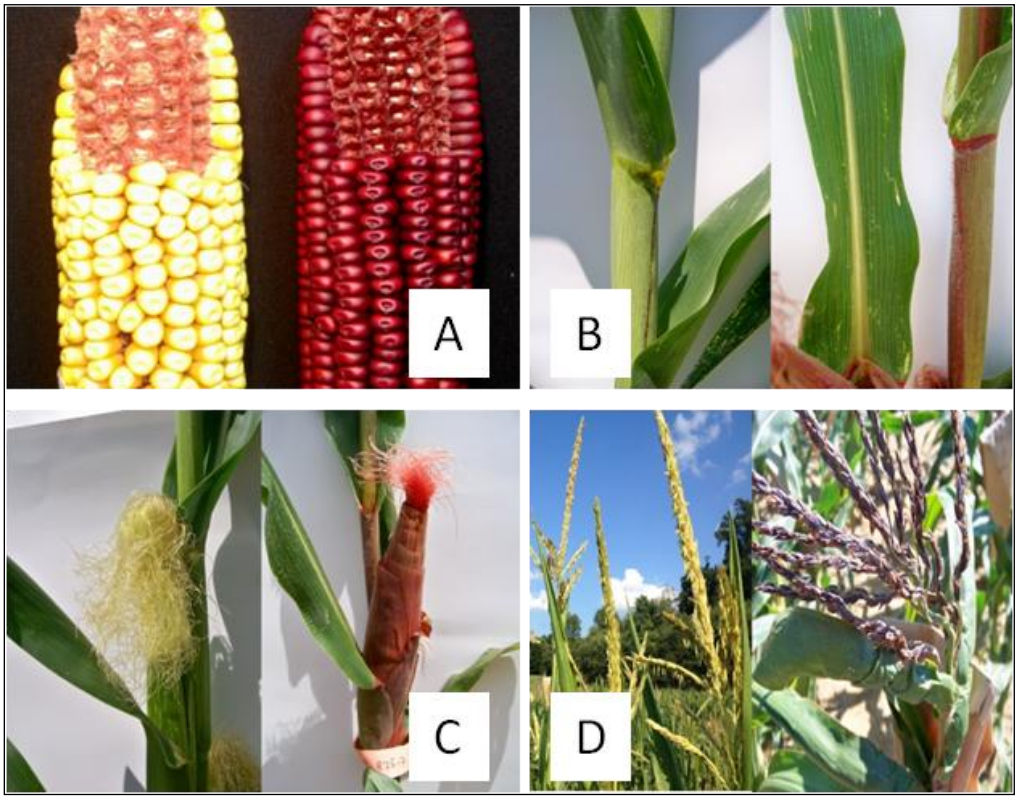

Figure 1-3: *Ufo1-1*-induced phenotypes in *P1-wr; Ufo1-1* plants. Left panel represents the wild type, whereas the right panel represents the *Ufo1-1* mutant plants. Ear (A), leaf sheath (B), silk and husk (C) and tassel glumes (D).

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Chapter 2

Transgenerational inheritance of *Ufo1-1* and its phenotypic effects

Abstract

Gene expression in plants and other organisms is faithfully inherited following Mendel's laws. Recent studies have highlighted epigenetics as a player in gene silencing through mechanisms such as DNA methylation and histone modifications. Herein, we have utilized pigmentation in maize as a marker to study epigenetic inheritance. The maize *pericarp color1 (p1)* gene is being used as a reporter to investigate epigenetic inheritance of a dominant mutant, *Unstable factor for orange1 (Ufo1)*. In the presence of *Ufo1-1* mutation, *p1* expression is hyperactivated and thus phlobaphenes ectopically accumulate throughout the plant body. A previous study has shown that *Ufo1-1*-induced phenotypes were only observed in a subset of *P1-wr; Ufo1-1* plants and these phenotypes were highly variable. Also, it has been shown that this increased pigmentation is associated with changes of DNA methylation pattern in the *P1-wr* distal enhancer and intron sequences. To understand the mechanism of stable inheritance of gene expression, we studied genotypic and phenotypic inheritance of *Ufo1-1*. In this study, we investigated how *Ufo1-1* and its effects are inherited over generations. We studied large plant populations and utilized PCR based genotyping using *Ufo1-1*-linked markers. Phenotyping for *Ufo1-1*-induced effects was performed based on phlobaphenes accumulation in pericarp. Results show that *Ufo1-1* is stably inherited over generations. However, its phenotypic effects violate Mendelian inheritance in which the expression of the reporter *p1* allele is silenced in advanced generations.

Introduction

Epigenetics, generally defined as heritable changes in gene expression that are not attributable to any alteration in DNA sequence, plays an important role in gene regulation. DNA methylation, chromatin modification, and RNA based silencing are well documented epigenetic mechanisms that are responsible for transcriptional gene silencing. Recent evidence shows that these mechanisms are linked and interact with each other to regulate gene expression. For example, in *Arabidopsis*, DNA methylation and methylation of specific histone tail residues are highly inter-dependent; Histone3 Lysine9 dimethylation (H3K9me2) regulates CHROMO-METHYLTRANSFERASE3 (CMT3)-catalyzed DNA methylation in the CHG sequence context (JACKSON *et al.* 2002), meanwhile, METHYLTRANSFERASE1 (MET1)-catalyzed CG DNA methylation directs H3K9 methylation (MATHIEU *et al.* 2005; TARIQ *et al.* 2003). Moreover, in maize, maintenance of CHH methylation of silenced allele *Pl-Rh* is regulated by a SNF2 chromatin remodeling protein encoded by *required to maintain repression1* (*rmr1*) gene (HALE *et al.* 2007). Additionally, RNA based silencing has also been shown to control cytosine methylation [see review (MATHIEU and BENDER 2004)] and chromatin architecture [see review (BERNSTEIN and ALLIS 2005)]. Studies have revealed that in *Arabidopsis*, cytosine methylation of the retrotransposon element *AtSN1* is impacted by the production of small RNAs (HAMILTON *et al.* 2002; ZILBERMAN *et al.* 2003). Furthermore, small interfering RNAs (siRNAs) have also been shown to play a key factor in regulating heterochromatic silencing [see review (LIPPMAN and MARTIENSSEN 2004)] and maintenance of telomeric DNA methylation (VRBSKY *et al.* 2010). Together, all the aforementioned studies have proven that DNA methylation, chromatin modification, and RNA based silencing are intertwined and collectively result in epigenetic regulation of gene expression.

Epigenetic gene regulation has been observed in many organisms and maize is one of the model systems for epigenetic studies. Numerous biological events including paramutation (WOODHOUSE *et al.*

2006), imprinting (ZHANG *et al.* 2011), transgene silencing (MCGINNIS *et al.* 2006), epimutation (DAS and MESSING 1994) and transposon inactivation (BARKAN and MARTIENSSEN 1991) have been associated with epigenetic regulation in maize. The maize flavonoid biosynthetic pathway provides an exceptional system to study the fundamental mechanisms of epigenetic gene regulation as well as the inheritance of epigenetic traits. This biosynthetic pathway has been extensively characterized genetically and molecularly. Two major classes of maize flavonoid compounds are anthocyanins and phlobaphenes. The accumulation of brick red phlobaphene pigment is regulated by the *p1* gene and these pigments are predominantly present in floral organs. The *p1* gene encodes an R2R3 *Myb* transcription factor and possesses more than 100 natural alleles plus epialleles (COE *et al.* 1988; STYLES and CESKA 1989). These multiple alleles of *p1* can be distinguished based on their expression patterns in pericarp (outer layer of the ovary wall) and cob glumes (palea and lemma). A two-letter suffix is used to designate these distinct *p1* alleles. For example, *P1-wr* produces *w*hite pericarp and *r*ed cob glumes, whereas *P1-rr* specifies *r*ed pericarp and *r*ed cob glumes (BRINK and STYLES 1966) .

Distinct expression patterns of *P1-rr* and *P1-wr* in which the *P1-wr* allele is unable to generate any pigmentation in pericarp are very fascinating because these two alleles share over 99% sequence similarity in their coding and regulatory regions. However, *P1-wr* and *P1-rr* differ significantly in terms of their gene structure; *P1-rr* is a single-copy allele, whereas *P1-wr* carries more than six copies of a 12.6 kb gene unit that are arranged in a head-to-tail tandem repeat complex (CHOPRA *et al.* 1998; SIDORENKO *et al.* 2000). Further analysis on pericarp specific silencing of *P1-wr* has revealed that this multicopy allele is epigenetically regulated resulting in gene silencing. This repeat-induced gene silencing (RIGS) is a common phenomenon in plants where expression of repeated sequences may be inactivated, including both endogenous genes and transgenes (ASSAAD *et al.* 1993; YE and SIGNER 1996). The *P1-wr* transcript level in colorless pericarp is reduced to 30% of that in *P1-rr* pericarp (CHOPRA *et al.* 1996). Moreover, further studies have revealed that the multicopy *P1-wr* is hypermethylated in the promoter region and

coding region in contrast to *P1-rr* (CHOPRA *et al.* 1998). Therefore, the reduction of *P1-wr* transcripts and the silenced *P1-wr* expression in pericarp were hypothesized to be regulated by DNA methylation (CHOPRA *et al.* 1998). In addition, functional analyses of transgenic plants carrying promoter and coding regions of *P1-wr* and *P1-rr* have shown that the *P1-wr* phenotype is attributed to enhanced cytosine methylation of the *P::P* transgene (COCCIOLONE *et al.* 2001). These transgenic experiments (COCCIOLONE *et al.* 2001) and recent study on DNA methylation patterns of *P1-wr* (SEKHON and CHOPRA 2009) have suggested that the unique pigmentation pattern of *P1-wr* is epigenetically regulated through DNA methylation.

The *Unstable factor for orange1* (*Ufo1*) is a spontaneous mutation which was first identified by Dr. Charles Burnham in the 1960s and has been further characterized to induce ectopic accumulation of phlobaphenes in *P1-wr* plants (STYLES and CESKA 1987; STYLES 1982). *Ufo1* is a dominant mutation because F_1 plants obtained from a *P1-wr/P1-wr; ufo1/ufo1 x P1-ww/P1-ww; Ufo1-1/Ufo1-1* cross show gain of pigmentation in pericarp and cob glumes, resembling the phenotype of *P1-rr* allele; RR (red pericarp/red cob glumes). However, incomplete penetrance, where not every individual with the genotype expresses the corresponding phenotypes, is observed in the *Ufo1-1*-induced phenotypes. Only approximately 30% of F_1 progeny plants of *P1-wr* X *Ufo1-1* cross show enhanced pericarp pigmentation. In addition, the *Ufo1-1*-induced gain of pericarp pigmentations exhibit poor expressivity with a range of pericarp pigmentation (Figure 2-1). Interestingly, the gain of pericarp pigmentation is meiotically unstable; a subset of the progeny revert their phenotypes back to the *P1-wr* expression pattern (CHOPRA *et al.* 2003). However, recent analysis shows that this incomplete penetrance and low expressivity are gradually improved after multiple generations of backcrossing with the recurrent parent, *P1-wr*. This improvement of penetrance and expressivity is tightly linked to progressive loss of DNA methylation of *P1-wr* (SEKHON and CHOPRA 2009). Thus, this study was aimed to investigate how *Ufo1-1* and its effects are inherited over several generations. To understand the incomplete penetrance of *Ufo1-1*, the *P1-wr*

and *Ufo1-1* interaction was followed over several generations in both the backcross and self-pollinated populations. Additionally, the original stock of the *Ufo1-1* mutant was used in this study to answer the question of background dependency of *Ufo1-1* expressivity. In the previous study (SEKHON and CHOPRA 2009), the introgression of *Ufo1-1* into an inbred line 4Co63 might have introduced a genetic background harboring possible epistatic genes or genomic modifiers.

The incomplete penetrance could result from the lack of the genomic modifiers in the background, or the presence of epistatic genes, and possibly caused by the environment (GRIFFITHS *et al.* 2010). Continuous efforts in Chopra's lab to fine map the *Ufo1-1* has yielded to the discovery of several *Ufo1-1* tightly linked markers including simple sequence repeat (SSR) and cleaved amplified polymorphic sequences (CAPS) markers. Transgenerational inheritance of *Ufo1-1* was examined through genotyping using these linked markers. The genotyping result may explain the poor penetrance of *Ufo1-1,* which was observed phenotypically. In addition, segregation of *Ufo1-1* plants from the self-pollinated populations will determine if the *Ufo1-1* is stably inherited following the Mendel's law of segregation. Previously, we have distinguished the *Ufo1-1* mutation in *P1-wr* plants through phenotypic observation of accumulation of phlobaphenes pigments in the floral organs such as pericarp, leaf, sheath, husks and tassel glumes. Through the use of phenotypic selection *P1-wr*; *Ufo1-1* expressers were selected and advanced to next generation. Recent discovery of *Ufo1-1* tightly linked markers now allow us to collect genotypic data and relate that to the phenotypic data. Furthermore, the genotyping results will also determine in segregating plants, if *Ufo1-1* is required to maintain the reactivation of *P1-wr* expression. This study may provide direct evidence for the correlation between the epigenetic inheritance of *Ufo1-1* and its phenotypic effects in multiple generations.

Materials and Methods

Genetic stocks

The original *Ufo1-1* (*P1-wr/P1-wr; Ufo1-1/Ufo1-1*) stock and the introgressed *Ufo1-1* mutation (*p1-ww/p1-ww; Ufo1-1/Ufo1-1*) were used in this study. This original *Ufo1-1* stock was in the *P1-wr* background and acquired from Dr. Derek Styles, University of Victoria (Victoria, BC, Canada). The *Ufo1-1* mutation was introgressed into an inbred line 4Co63 (genotype *p1-ww c1 r-r*, National Seed Storage Laboratory, Fort Collins, CO) as previously described (CHOPRA *et al.* 2003). The B73 and W23 inbred lines carrying a *P1-wr* allele were obtained from Maize Genetics Cooperation Stock Center (Urbana, IL). The field study consisting of all the generations in both backcross and self-pollinated populations were carried out at the Russell E. Larson Agricultural Research farm in Rock Springs, PA from May 2011 to October 2012.

Genetic crosses

Crossing schemes used to develop the backcross and self-pollinated populations are shown in Figure 2-2 and Figure 2-3, respectively. Three previously developed populations from Chopra lab were used in genotyping and phenotyping experiments. Population I is the B73 backcross population from the crosses between B73 (*P1-wr/P1-wr; ufo1/ufo1*) and original *Ufo1-1* using B73 as a recurrent parent (Figure 2-2A). For developing this B73 backcross population, F_1 plants (*P1-wr/P1-wr; Ufo1-1/ufo1*) with strong *Ufo1-1*-induced phenotypes were backcross with B73 to generate BC_1F_1 ears. Strong *Ufo1-1* expressers (uniform dark orange pericarp pigmentation) in BC_1F_1 ears were grown and were subjected to subsequent cycles of backcrossing and selection to develop later generations. Two sets of experiments were performed on this population. First, BC_1F_1 to BC_3F_1 generations were planted in

summer 2011. The second set was carried out during summer 2012 with larger sample sizes of BC_2F_1 and BC_4F_1.

Population II is a backcross population from the crosses between W23 inbred lines (*P1-wr/P1-wr; ufo1/ufo1*) and the introgressed *Ufo1-1* mutation (Figure 2-2B). The W23 backcross population was developed by backcrossing the F_1 plants (*P1-wr/P1-ww; Ufo1-1/ufo1*) with W23 as a recurrent parent and the BC_1F_1 ears demonstrating intense *Ufo1-1*-induced pericarp pigmentation were chosen for next backcross cycles. BC_6F_1 and $BC_{10}F_1$ of W23 backcross population were used in 2012 of this study.

Population III is a self-pollinated population of the crosses between the B73 (*P1-wr/P1-wr; ufo1/ufo1*) and the original *Ufo1-1* stock (Figure 2-3). For developing the self-pollinated population, F_1 plants (*P1-wr/P1-wr; Ufo1-1/ufo1*) with strong *Ufo1-1*-induced phenotypes were selected for self-pollination to obtain F_2 generation. The F_2 plants grown from these selected ears were subjected to subsequent cycles of self-pollination and selection to develop later generations. Two sets of experiments were done on these self-pollinated populations. The first set was carried out during summer 2011 and six self-pollinated generations (F_1 to F_6) were studied. The second set was planted in summer 2012; only advance generations (F_5 to F_7) were analyzed but larger sample sizes were employed.

High-throughput DNA extraction

Genotyping was performed on leaf genomic DNA extracted using a high-throughput UREA DNA extraction method. Briefly, two leaf discs collected by a paper hole puncher were ground in a 96-well plate containing 400 µl of extraction buffers (NaCl 0.4 M, urea 8.9 M and 62.5 mM Tris of pH 8.0). The grinding was done using Geno/grinder 2000 (SpexCertiPrep, Matuchen, NJ, USA) with a 3.2 mm stainless

steel bead at 1000 rpm for 4 minutes. 100 µl of n-laurylsarcosine (150 mM) was added to the ground tissues and incubated at 65°C for 10 minutes. The plate was centrifuged at 3000 rpm for 10 minutes. The crude DNA in the supernatant was transferred into another 96-deep well plate containing 4.4 M NH_4OAC: isopropanol (10:1) mix and was allowed to precipitate at -20°C for a minimum of an hour. The plate was spun down and the pellet was rinsed with 200 µl of 70% EtOH. The pellet was dried before it was re-suspended in 50 µl of ddH_2O.

SSR and CAPS markers and PCR amplification for genotyping of *Ufo1-1*

PCR reactions were carried out in 96-well plates using GoTaq Green Master Mix (Promega, Madison, WI) in a total reaction volume of 20 µl. Annealing temperature is dependent on the melting temperature (T_m) of specific SSR or CAPS primer used. The primers used for genotyping are listed in Supplemental Table S2-1. The PCR conditions for SSR31 were as follows: 94°C for 4 minutes, 35 cycles of 94°C (45 seconds), annealing temperature of 59°C (45 seconds), 72°C (45 seconds) and final extension at 72°C for 7 minutes. The PCR conditions for CAPS 5 were: 94°C for 4 minutes, 35 cycles of PCR amplification and each cycle consisted of 94°C (30 seconds), annealing temperature of 54°C (30 seconds), 72°C (30 seconds), and final extension at 72°C for 7 minutes.

The PCR products of CAPS5 were first digested with *Eco*RI for 2 hours at 37°C before separating on a 2% agarose gel at a constant voltage of 70 Volts. The PCR products of SSR31 (~121 bp) were directly loaded on to a 4% SFR agarose gel and run with constant voltage of 80 Volts. After electrophoresis, genotype of each sample was determined by distinguishing homozygous (*Ufo1-1/Ufo1-1*), heterozygous (*Ufo1-1/ufo1*) and wild type (*ufo1/ufo1*) bands (Figure 2-4). Genotyping in 2011 was done using SSR31 marker and CAPS5 was used for genotyping in 2012 study.

Phenotyping of *Ufo1-1*-induced effects

Analysis of flavan-4ols and phlobaphenes pigments

Ear pigmentation phenotypes were determined at reproductive stage, R4 which was approximately 17 weeks after planting (WAP) and were divided into two categories; red pericarp/red cob (RR) and white pericarp/red cob (WR). Additional colorimetric test using modified acid-methanol assay was done on phenotypically challenging plants showing some anthocyanins interferences (GROTEWOLD *et al.* 1998). Briefly, approximately 0.025 g pericarp tissues were extracted in cold methanol overnight at 4°C. Cold concentrated sulfuric acid was added and then the extracts were analyzed for absorbance using UV Mini 1240 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). The absorbance value at 564 nm (λ_{max}) was used to detect the presence of flavan-4-ols, the precursor of phlobaphenes pigments. On the contrary, the presence of flavan-3,4, diols, the precursor of anthocyanins could be detected at the λ_{max} of 533nm (GROTEWOLD *et al.* 1998).

Plant height

Plant height was measured at final vegetative stage, VT (**v**egetative stage with fully emerged **t**assel). The plant height was measured from the soil surface to the ligules of the uppermost leaf on each plant. Measurements were taken from each plant in self-pollinated and B73 backcross populations grown in summer 2011.

Statistical analysis

The genotypic and phenotypic segregation of *Ufo1-1* were analyzed using a Chi-square (X^2) goodness of fit test in SAS 9.3 Software (SAS Institute Inc., Cary, NC, USA). P is the value from the X^2 distribution for the statistics and the appropriate degrees of freedom. The statistical analysis was

conducted at 95% confidence level and a P- value less than 0.05 was considered statistically significant. A test for heterogeneity was run to ensure pooling of data was acceptable. One way analysis of variance (ANOVA) was performed on plant height data. The normality of data was checked beforehand and non-normal data were transformed using natural log and re-analyzed. The plant height data were analyzed using PROC GLM and adjusted by the Tukey method in SAS 9.3 Software (SAS Institute Inc., Cary, NC, USA). In some cases when normalization of data using natural log was not successful, PROC NPAR1WAY was used and means were compared using either Kruskal-Wallis or Van der Waerden Scores methods at $P < 0.05$.

Results

Ufo1-1 is stably inherited following Mendel's law of segregation

Transgenerational inheritance of *Ufo1-1* was followed in several generations of three populations. In the 2011 study, the inheritance of *Ufo1-1* was analyzed in B73 backcross (Population I) and self-pollinated (Population III) populations. Population I consisted of three generations of backcrosses with the B73 inbred line as the recurrent parent. Population III was composed of six filial generations; F_1 through F_6. Due to limited amount of seed availability, the population size used in this study was relatively small. Each generation was comprised of progenies from two closely related families. For instance, both the families in BC_2F_1 generation were developed from Family 2 of BC_1F_1. Genotyping of *Ufo1-1* locus was done using *Ufo1-1*-linked marker, SSR31. Since genotyping information for the progenitors was not available in 2011 study, the expected segregation ratio was calculated based on the assumption of faithful Mendelian inheritance of *Ufo1-1*. In other words, segregating families are expected to segregate in a 1:2:1 ratio of homozygous (*Ufo1-1/Ufo1-1*), heterozygous (*Ufo1-1*/*ufo1*) and wild type (*ufo1/ufo1*). In all inheritance tables in this chapter (Table 2-1 through Table 2-12), tested null

hypotheses are indicated with superscript letters and statistically significant P-values are marked with an asterisk ('*'). Inheritance of *Ufo1-1* for each family in every generation for both backcross and self-pollinated populations is reported in Table 2-1. Although the expected frequency of one of the categories in F_5 generation was found to be less than 5, a Chi-square goodness-of-fit test is still valid to be used as explained by Jerrold H. Zar (ZAR 2010). Of all the tested families, three families from population III violated the expected segregation ratios (Table2-1). Out of these three families, two of the F_3 families and one of the F_6 family did not follow Mendel's law of segregation (P <0.05). To increase statistical power, Chi-square tests were also performed on data pooled from all families in each generation (Table 2-2). The data were pooled only if the families were proven to be homogenous using Test for Homogeneity (ZAR 2010). As expected, pooled F_3 data was significant (X^2= 15.15; P <0.001) which strongly indicated that the progenies in this generation did not segregate in 1:2:1 manner. Interestingly, pooled F_5 data also disobeyed the expected inheritance ratio (X^2= 7.46; P <0.05) (Table 2-2). However, these rejections of null hypotheses could be due to small sample size used in this analysis. For example, pooled F_5 data consisted of only 39 plants. Furthermore, polymorphism of the SSR31 bands was slightly hard to visualize on the SFR gel. Therefore, based on the result of 2011 inheritance study, a second *Ufo1-1* study with larger sample size was conducted in 2012.

The 2012 *Ufo1-1* inheritance study was composed of three population sets; population I, II and III. Population I was comprised of two B73 backcross generations; BC_2F_1 and BC_4F_1. Population II was developed from a cross between the W23 inbred line and the *Ufo1-1* mutation which has been previously introgressed into an inbred line, 4Co63 which carries *P1-ww* alleles. Two advanced generations of population II (BC_6F_1 and $BC_{10}F_1$) were analyzed in this study. In population III, only advanced filial generations, F_5 through F_7, were included. To test if *Ufo1-1* is faithfully inherited following Mendel's first law, segregation of *Ufo1-1* was analyzed in progenies of parents which were previously genotyped as homozygous (*Ufo1-1/ Ufo1-1*), heterozygous (*Ufo1-1/ufo1*) and wild type (*ufo1/ufo1*). The

CAPS5 marker was used to genotype *Ufo1-1* locus since this molecular marker is polymorphic for *Ufo1-1* in both B73 and W23 genetic backgrounds. The genotypic data showed that *Ufo1-1* is stably inherited following Mendelian inheritance in both the backcross populations, population I and II, (Table 2-6) and in the self-pollinated population, population III, (Table 2-7). As expected, Chi-square analyses on pooled data also yielded the same results (Table 2-8). The 2012 genotypic data are more reliable than the 2011 data since larger sample sizes were employed and the parent's genotypes have been previously determined from 2011 study. In addition, CAPS5 polymorphic bands are easy to distinguish as compared to SSR31 bands (Figure 2-4). Therefore, the above results indicated that the *Ufo1-1* is faithfully inherited in accordance with Mendel's genetics.

***Ufo1-1*-induced phenotypic effects violate Mendelian inheritance**

Previous work has shown that *Ufo1-1* induces gain of pericarp pigmentation in *P1-wr* plants (CHOPRA *et al.* 2003) and the inheritance of *Ufo1-1*-induced pigmentation phenotype improves over generations (SEKHON and CHOPRA 2009). However, no direct comparison has been made between the genetic inheritance of *Ufo1-1* and the inheritance of its phenotypic effects. Therefore, we collected phenotypic data from the large plant populations used in the genotyping study. Expected phenotypes are based on hypothetical assumptions that *Ufo1-1*-induced pigmentation also follows Mendelian expectations as observed in the genotypic data and *Ufo1-1* has complete penetrance. Therefore, half of the progeny in the backcross population are expected to carry *Ufo1-1* mutation and thus, exhibit *Ufo1-1*-induced pericarp pigmentation showing RR phenotype. Meanwhile, in filial generations, segregating families are expected to show 3:1 ratio of the RR to the WR phenotypes. In addition, all progenies from a homozygous *Ufo1-1/Ufo1-1* parent are expected to demonstrate the RR phenotype.

In 2011, phenotypic data were collected from population I and population III. In general, most of the families in the backcross population showed 1:1 ratio of the RR vs. WR phenotypes (Table 2-3).

However, Family 2 of BC_3F_1 showed lack of agreement to Mendel's law of segregation (X^2= 11.61; P < 0.001) where most of the progenies (30/38) displayed the WR phenotype. In population III, one segregating family in each filial generation (F_2 through F_6) was significant at P < 0.05 indicating rejection of null hypothesis that the RR and WR phenotypes segregate at 3:1 ratio. Interestingly, of the 31 plants carrying homozygous *Ufo1-1* mutation in Family 2 of F_6 generation, 19 plants retained the WR phenotype implicating silencing of *P1-wr* despite the presence of *Ufo1-1.* The Chi-square goodness-of-fit tests were also performed on pooled data given that the families are homogenous (Table 2-4). The same trend was observed where ear phenotypes skewed to the WR phenotype in pooled F_5 generation (P < 0.001). However, due to small sample size used in this 2011 study, one could argue that the statistical analyses were not powerful enough to make any conclusion on the inheritance of *Ufo1-1*–induced phenotypic effects. Therefore, the inheritance study of the *Ufo1-1*-induced gain of pericarp pigmentation was continued in the following year as larger plant populations were employed in 2012.

The phenotypic study in 2012 was done using three populations; population I, II and III. With known parental genotypes, the progeny phenotypes were expected to follow Mendelian genetics. As expected, all progenies of negative segregants (wild type for *ufo1/ufo1*) showed the WR phenotype in all the generations of the three populations under study. The phenotypic data of population I BC_2F_1 *Ufo1-1/ufo1* progenies showed that the RR and WR classes segregated as 1:1 ratio (Table 2-9). Surprisingly, in population I BC_4F_1 *Ufo1-1/ufo1* progenies, 105 out of 113 plants retained the WR phenotypes leading to a rejection of the null hypothesis of 1:1 ratio for RR and WR classes (X^2=81.56*; P* < 0.0001). The same observation was made in population II BC_6F_1 and $BC_{10}F_1$ progenies, which showed significance at P <0.0001. The phenotypic data on population III (selfed advanced generations (Table 2-10)) showed a similar trend that was observed in 2011 study (see Table 2-3). All the segregating families (*Ufo1-1/ufo1*) in F_5 through F_7 generations showed significant segregation distortion (P < 0.0001) with observed phenotypes skewed to the WR class (Table 2-10). Interestingly, of 161 plants genotyped as homozygous

Ufo1-1 in F_6 generation, half of them exhibited the WR phenotype (Table 2-11). A similar silencing phenomenon was observed in F_7 generation where 72 plants carrying *Ufo1-1* mutation maintained the WR phenotype. These results suggest that *Ufo1-1* effects on gain of pericarp pigmentation are gradually disappearing over generations and thus, silencing at *P1-wr* re-emerged in later generations regardless of the presence of *Ufo1-1* in the genome. This phenomenon was also observed in advanced generation of backcross populations with B73 and W23 genetic backgrounds. In conclusion, *Ufo1-1*-induced pigmentation is not inherited stably and does not follow Mendelian genetics.

Penetrance of *Ufo1-1* is influenced by genetic backgrounds

It has been previously reported that *Ufo1-1* has incomplete penetrance in W23 genetic background (SEKHON and CHOPRA 2009). In this previous study, only 30% of the F_1 progenies showed gain of pericarp pigmentation. However, this incomplete penetrance improves over generations. Interestingly, crosses between *P1-wr* [B73 inbred] and the original *Ufo1-1* stock yielded F_1 progenies with improved penetrance where 80% of the progeny plants showed *Ufo1-1*-gain of pericarp pigmentation (WANG 2012). We tested if the penetrance of *Ufo1-1*-induced phenotypes would also improve in our backcross population in B73 genetic background. Surprisingly, penetrance decreased over generations with the highest expressivity in BC_1F_1 (85.4%). Penetrance was variable in other backcross generations: BC_2F_1 (83.4%; average of 2011 and 2012), BC_3F_1 (75.0%) and BC_4F_1 (14.3%) (Table 2-13). The *Ufo1-1* penetrance in selfed generations also showed a similar trend with a complete penetrance in F_1, followed by variable expression in F_2 (84.1%), F_3 (81.8%), F_4 (77.8%), F_5 (54.3%), F_6 (45.8%), and F_7 (22.0%) (Table 2-13). In contrast, population II originating from *P1-ww* [4Co63] *Ufo1-1* crossed with W23 inbred line showed improved *Ufo1-1* penetrance where BC_6F_1 showed 50.0% penetrance and $BC_{10}F_1$ (69.1%) in agreement to the previous study (SEKHON and CHOPRA 2009). In summary, the penetrance of *Ufo1-1* is influenced by different genetic backgrounds.

***Ufo1-1* causes pleiotropic defects in maize**

The presence of *Ufo1-1* mutation not only results in ectopic accumulation of phlobaphenes pigment throughout the plant body, but also induces pleiotropic defects. Such pleiotropic developmental phenotypes include stunted growth, short plant height, trapped leaf, bent stem, rolled leaf, and weak plant stature (Figure 2-5). Moreover, the *P1-wr; Ufo1-1* plants also produce smaller ears. These *Ufo1-1*-induced pleiotropic defects are often associated with the gain of pericarp pigmentation exhibiting RR ear phenotype. To see if *Ufo1-1* effects are dosage dependent, plant height in different genotypes was measured in backcross (population I) and self-pollinated (population III) populations. In backcross population, *P1-wr/P1-wr; Ufo1-1/ufo1* plants showed reduction in plant height as compared to the *P1-wr/P1-wr; ufo1/ufo1* plants. However, the mean differences were only statistically significant in advanced generation BC_3F_1 (Figure 2-6). Interestingly, all filial generations except F_4 showed that the homozygous *Ufo1-1* plants have the smallest mean plant height, followed by the heterozygous *Ufo1-1* plants, while the wild type *ufo1* plants have the highest mean value. However, these differences were not statistically significant at $\alpha < 0.05$. Interestingly, we also observed a range of variation in plant height in F_1 plants (heterozygous *Ufo1-1/ufo1*; Figure 2-7), indicating that *Ufo1-1* is exhibiting differential expression in different F_1 plants. Strong association was observed between the ear phenotypes and the plant height; plants with the RR phenotype showed statistically significant reduction in height in BC_2F_1 and BC_3F_1 (Figure 2-8). In addition, the RR plants in F_5 generation also exhibited statistically significant reduction in plant height as compared to the WR plants. In general, the RR plants in all filial generations were shorter than the WR plants. The result of plant height may have been affected because some of the *Ufo1-1* plants that exhibited extremely stunted growth phenotype died before maturity and thus were not included in the measurements.

A germination rate study was performed on families of F_5, F_6 and F_7 generations to test how different genotypes (*Ufo1-1/Ufo1-1*, *Ufo1-1/ufo1* and *ufo1/ufo1*) influence seed germination. As

expected, homozygous *Ufo1-1* families showed the lowest germination rate in all three filial generations (Figure 2-9). Surprisingly, the germination rate of homozygous *Ufo1-1* in F_7 generation increased to be closer to that of the wild type *ufo1*. As mentioned earlier, many of the F_7 progeny plants showed loss of pericarp pigmentation suggesting a connection between improved germination rate and loss of *Ufo1-1*-induced pericarp pigmentation.

Discussion

One of the fundamental incentives in the early studies of heredity and variation was to understand the evolutionary process and many of those studies stem from an understanding of Mendel's principles. However, Mendel's laws have been violated in numerous epigenetic phenomena such as paramutation and genomic imprinting where heritable changes in gene expression and phenotypes are attributed to mechanisms such as DNA methylation and histone modifications. Transgenerational inheritance of these epigenetic marks has been of much interest, parallel with the idea of potential crop improvement via stable inheritance of newly acquired phenotypic traits. The *pericarp color1, p1* gene is an excellent model system to study transgenerational epigenetic inheritance. The default epigenetic state of the *P1-wr* (white pericarp/red cob) multicopy complex has been credited to hypermethylation of the *p1* sequence (CHOPRA *et al.* 1998) and is epigenetically regulated (COCCIOLONE *et al.* 2001). The *P1-wr* allele fails to produce pericarp pigmentation and this tissue-specific gene silencing is perturbed in the presence of *Ufo1-1* mutation. *Ufo1-1* induces loss of methylation at the distal enhancer region of the *P1-wr* and thus reactivates *p1* expression resulting in gain of pericarp pigmentation. Herein, we are interested in how this dominant mutant, *Ufo1-1,* and its effects on *P1-wr* are inherited across generations.

Genotyping of *Ufo1-1* locus has been made possible by the discovery of *Ufo1-1*-linked markers, SSR31 and CAPS5. To elucidate the inheritance of *Ufo1-1*, two backcross populations (population I and II with B73 or W23 inbred line as the recurrent parent, respectively), and a self-pollinated population (population III) were subjected for genotyping analysis. Genotypic data were collected for two years in which SSR31 marker was used in 2011 and CAPS5 marker was employed in 2012. To test if the genotypic data fit any of the Mendelian ratios, segregation ratios of *Ufo1-1* were compared to expected ratios using a Chi-square goodness-of fit test. Two key requirements have to be met before implementing the Chi-square test in order to avoid committing Type II error, the failure to reject a null hypothesis that is actually false. First, all expected counts must be ≥ 1, and second, that no more than 20% of the expected counts are < 5 (commonly due to small sample sizes) (STATSOFT 2013). In such cases, the distributions were not well approximated by the Chi-squared distribution and thus, tests such as Fisher's exact test or the binomial test were performed. However, according to Jerrold H. Zar in his fifth edition of Biostatistical Analysis textbook, the Chi-square test is still applicable for situations where number of categories, $k \geq 3$, sample size, $n \geq 10$, and $n^2/k \geq 10$ (ZAR 2010). This special case was applied on data from F_5 generation in 2011 study. Further analysis using Fisher's exact test on the same data set yielded the same result (data not shown). In addition, Yates's correction for continuity were applied whenever the degree of freedom, $v = 1$ to avoid committing Type I error; rejection of a true null hypothesis (QUINN and KEOUGH 2002). In summary, majority of the tested families in 2011 study showed *Ufo1-1* was inherited following Mendelian genetics. Although in some cases, violations of Mendel's law of segregation were observed. However, these deviations were later regarded as errors due to small sample size used in the study and visual difficulties in distinguishing polymorphic bands of SSR31 PCR products. To overcome this issue, we later employed larger sample size of the plant populations in 2012 attempting to increase statistical power of the analysis. The use of CAPS5 marker provided visually easier to distinguish PCR bands and thus, produced more reliable genotyping results. In all the tested

families of 2012, *Ufo1-1* segregated in accordance to Mendel's principle of heredity. Based on these results, we concluded that *Ufo1-1* is faithfully inherited following Mendelian expectation patterns regardless of its genetic backgrounds.

To gain more insights on the extent of *Ufo1-1* effects on the *P1-wr* plants, the inheritance of *Ufo1-1*-induced phenotypes was investigated in all the genotyped populations. Ear phenotypes were scored based on accumulation of brick red phlobaphenes pigment in the pericarp and were divided into two categories; WR (white pericarp/red cob) and RR (red pericarp/red cob). Segregation analysis using Chi-square goodness-of-fit test was performed with an assumption that the phenotypes will segregate based on the previously determined genotypes. Surprisingly, *Ufo1-1*-induced pericarp pigmentations violated basic tenets of Mendelian inheritance. Significantly, the WR phenotypes were over-represented in the segregating families of advanced backcross generations (BC_3F_1, BC_4F_1, BC_6F_1, and $BC_{10}F_1$) and self-pollinated (F_5, F_6, and F_7) populations compared to the predicted ratios. In the backcross generations, the percentage of genetic contribution from the recurrent parent increases proportionally by each generation of backcrossing. Thus, the re-silencing phenomenon of *P1-wr* observed in these advanced generations could be explained by lack of factor (s) or *Ufo1-1* enhancer (s) that possibly carried only in the donor parent and segregated away during backcrossing. In addition, the re-silencing event in later generations of segregating families in the filial population may be due to inbreeding depression. In maize, the effects of inbreeding depression on multiple traits such as grain yield, plant height, ear length, stalk lodging, and etc. have been extensively studied for decades (BENSON and HALLAUER 1994; MEGHJI *et al.* 1984). Supporting this idea is the fact that our result in plant height study showed significant reduction of plant height throughout filial generations, F_1- F_6 indicating inbreeding depression (Figure 2-6). Moreover, there is a correlation between reduction of plant height and gain of pericarp pigmentation as illustrated in Figure 2-8. Short and/or extremely weak plant stature was often accompanied with uniformly dark ear pigmentation. Possible explanation behind this observation is that

both phlobaphenes and lignin biosynthetic pathways require the same substrate, phenylalanine. Thus, increase in phlobaphenes production will cause reduction of lignin synthesis (ROBBINS *et al.* 2013). However, further study such as linear regression analysis should be explored in order to quantitatively analyze the correlation between the plant height and the pericarp pigmentation.

The disagreement between the genotypic inheritance of *Ufo1-1* and the phenotypic inheritance of its induced gain of pericarp pigmentation is clearly observed in filial generations carrying homozygous *Ufo1-1*/*Ufo1-1* (See Table 2-5 and Table 2-12 for direct comparison between genotypic and phenotypic inheritance of *Ufo1-1* in 2011 and 2012 study, respectively). Despite the presence of *Ufo1-1* mutation, the *P1-wr* allele was re-silenced and thus reverted to WR phenotype. This scenario may be attributed to incomplete penetrance or low expressivity of *Ufo1-1*. Interestingly, the penetrance and expressivity of *Ufo1-1* vary in different ears from the same cross. In such cases, ears with poor or lack of expressivity produced progeny with the phenotype skewed towards WR class. In contrast, highly expressed ears yielded progenies with improved penetrance in subsequent generations. Incomplete penetrance is a common occurrence in maize and has been observed in other mutant alleles such as *early phase change* (*epc*)(VEGA et al. 2002), *knotted1* (*kn1*)(SATO *et al.* 1999), and *maternal effect lethal1* (*mel1*) (EVANS and KERMICLE 2001). The mechanisms underlying incomplete penetrance are still unclear. However, genetic-redundancy has been one of the suggested mechanisms of this phenomenon in maize. Regulation of gene silencing has been attributed to interplay of epigenetic mechanisms including DNA methylation, chromatin modification and RNA-based mechanisms [see review (SAZE *et al.* 2012)]. Thus, redundancy of these gene silencing mechanisms may contribute to incomplete penetrance as loss of one of these mechanisms could be partly compensated by the other. Another possible mechanism that could lead to poor penetrance is presence of other modifiers (enhancers/suppressors) in different genetic backgrounds. Such notion was supported by our penetrance analysis of *Ufo1-1*-induced pericarp pigmentation in two different genetic backgrounds; original *Ufo1-1* stock and the introgressed *Ufo1-1*

(*P1-ww* [4Co63]). The F_1 generation of the cross between *P1-wr* [B73] and *P1-wr* [original *Ufo1-1*] has a complete penetrance. However, the number of ears analyzed was too small. Though, in other previous work, the penetrance of *Ufo1-1* in F_1 generation from the same cross was very high, approximately 80% (WANG 2012). On the other hand, the F_1 generation of the cross between *P1-wr* [W23] and *Ufo1-1 P1-ww* [4Co63]) was only 26.6% (SEKHON and CHOPRA 2009). Therefore, there is a possibility that suppressor(s) of *Ufo1-1* is only present in the *P1-ww* [4Co63] *Ufo1-1* stock and thus resulting in the suppression of *Ufo1-1*-induced activation of *P1-wr*. However, the penetrance of *Ufo1-1* from this cross is progressively improved over several generations of backcrossing as this suppressor might have segregated away (this study and (SEKHON and CHOPRA 2009)). Conversely, the *P1-wr* [original *Ufo1-1*] might have an enhancer of *Ufo1-1* which promotes ectopic increase in *p1* expression. Moreover, our study showed that the penetrance of *Ufo1-1* from the cross between *P1-wr* [B73] and *P1-wr* [original *Ufo1-1*] is substantially decreased after several generations of backcrossing. Again, the enhancer (s) of *Ufo1-1* in this genetic background could have segregated away following several backcrossing events.

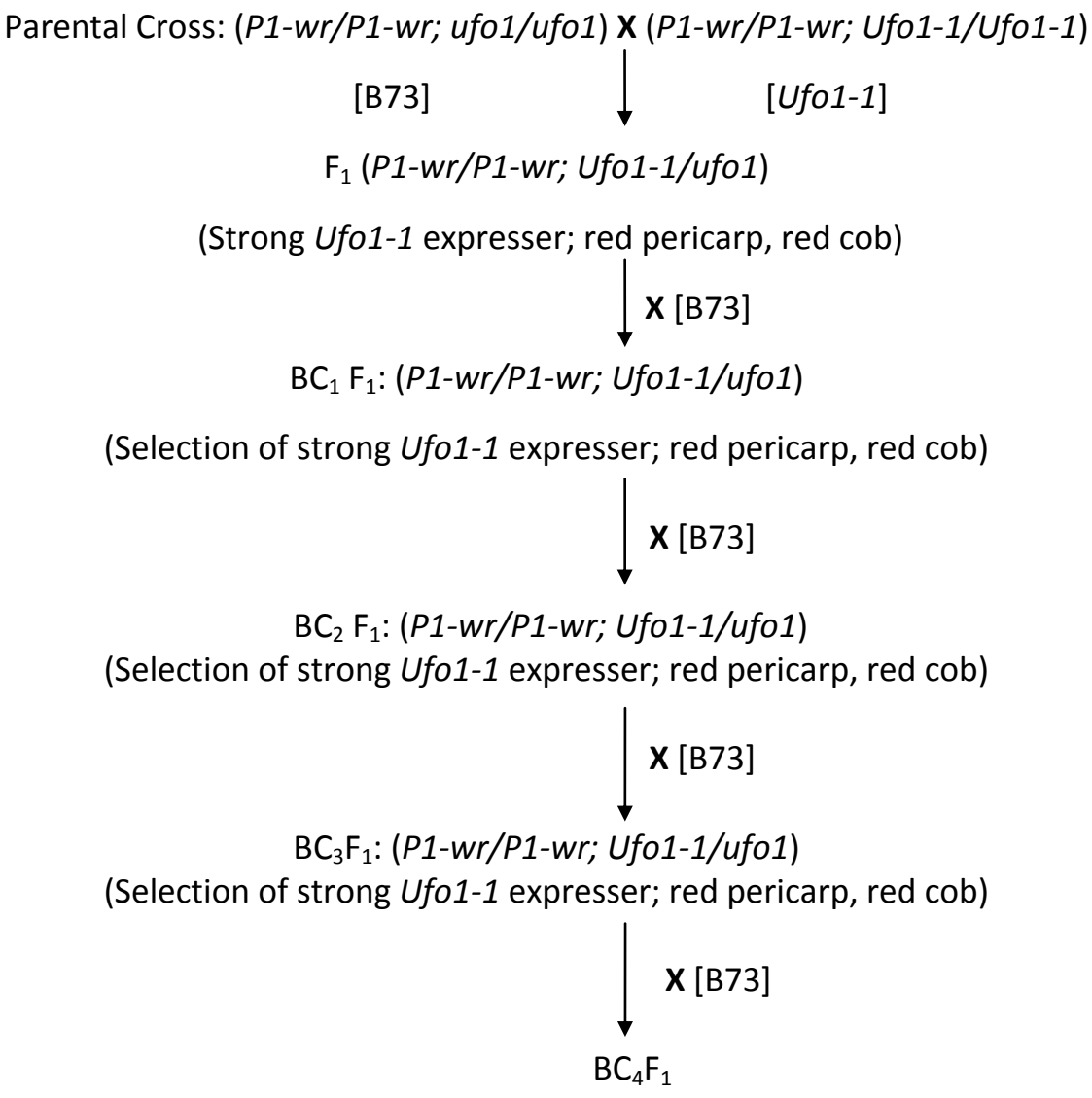
Another observation made from this study was an association between the re-silencing of *P1-wr* and increased germination rates in the advanced generations of the filial population. Based on 2012 phenotypic study, the homozygous *Ufo1-1* families in F_5 showed 100% penetrance (all plants were RR), 50% plants of F_6 exhibited RR phenotypes and 77% of F_7 displayed the WR phenotype indicating an increase in silencing of *P1-wr* over generations. Interestingly, these homozygous *Ufo1-1* families in F_5 have an average germination rate of 81.7%, F_6 (83.2%) and F_7 (92.5%). Hence, these results suggested that loss of *Ufo1-1* function in activating *P1-wr* is coupled with loss of other *Ufo1-1* effects on *P1-wr* plants such as germination. Besides, the homozygous *Ufo1-1* plants of F_7 generation also showed better survival rate than the homozygote in F_6 and F_5 generations (see Supplemental Figure S2-1). Therefore, there is a possibility that *ufo1* acts on multiple loci and thus has global effects on plant growth and development.

In the previous work, *Ufo1-1*-induced gain of pericarp pigmentation in *P1-wr* has been associated with the loss of DNA methylation in the promoter region of *P1-wr*. Thus, it would be of much interest to determine if this is still the case in the silenced (non-expresser) plants carrying homozygous *Ufo1-1* mutation. Further research on the mechanism underlying gene silencing observed in the advanced generations of filial populations was explored in Chapter 3 of this study.

Figure 2-1: Expressivity of gain of pericarp pigmentation in representative F_6, *P1-wr; Ufo1-1* progeny plants

Backcross Populations

A: Population I



B: Population II

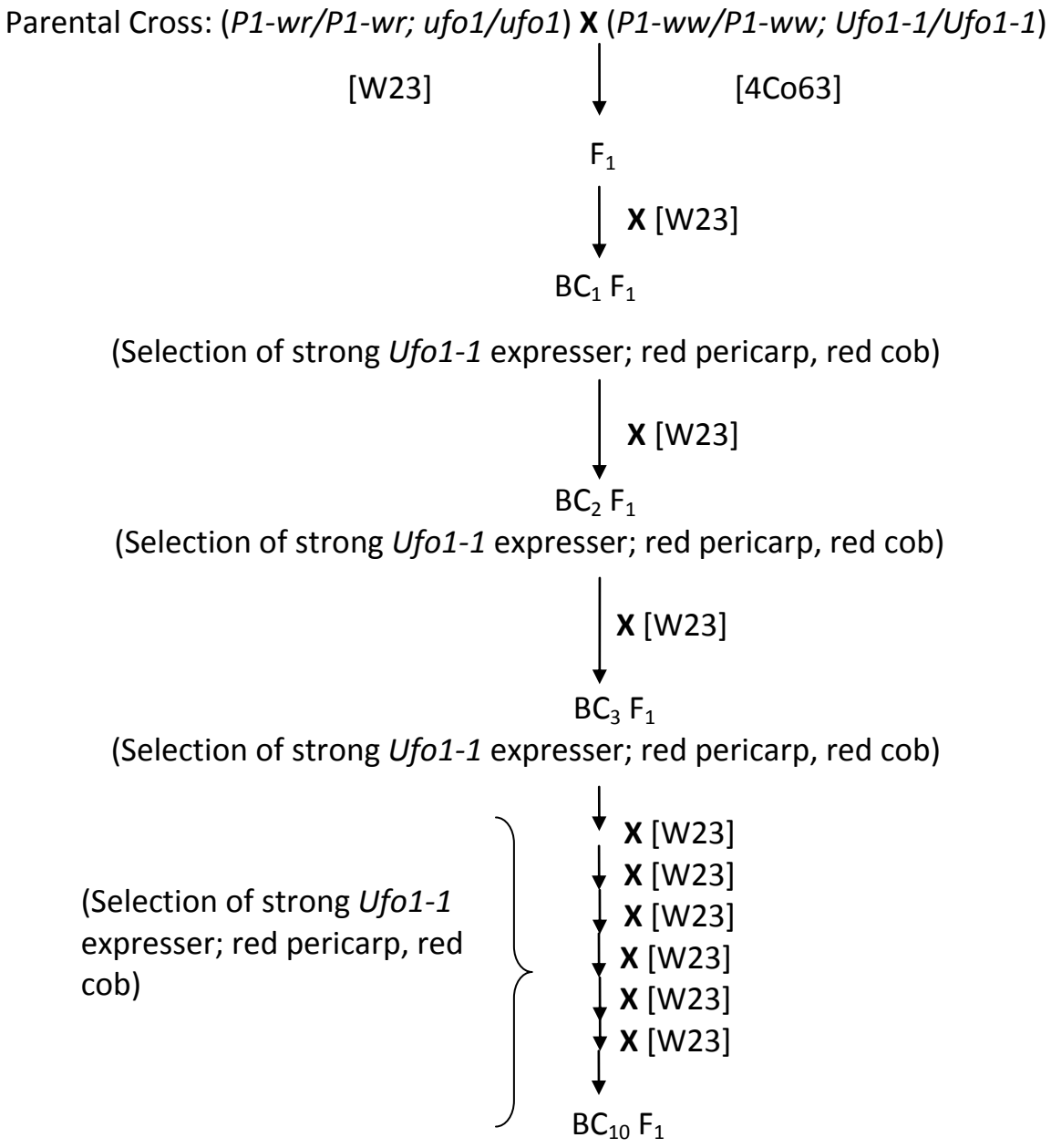


Figure 2-2: A backcross crossing scheme for development of B73 backcross populations (A) and W23 backcross population (B)

Self-Pollinated Population

Population III

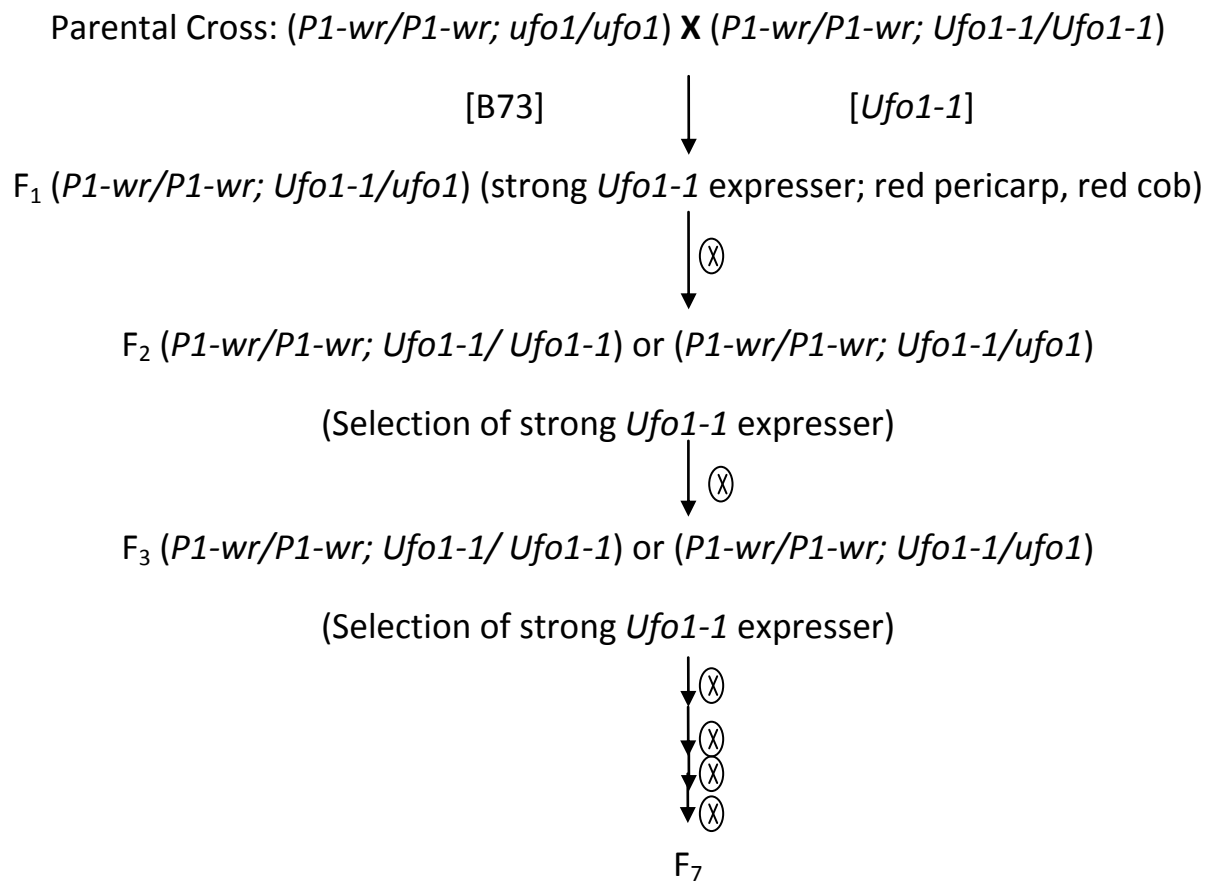


Figure 2-3: A self-pollinated crossing scheme for *Ufo1-1* mutation

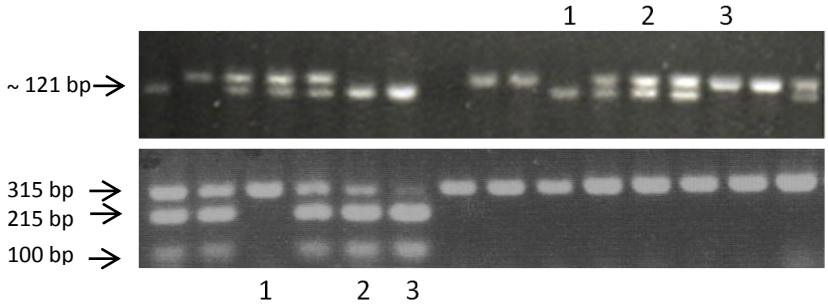


Figure 2-4: Genotyping of the *Ufo1-1* using SSR31 marker separating on a 4% SFR gel (top panel) and CAPS5 separating on a 2% agarose gel (bottom panel). Box 1: *ufo1/ufo1* (wild type *ufo1*); Box 2: *Ufo1-1/ufo1* (heterozygous *Ufo1-1*); Box 3: *Ufo1-1/Ufo1-1* (homozygous *Ufo1-1*). Sizes of bands in base pairs are shown.

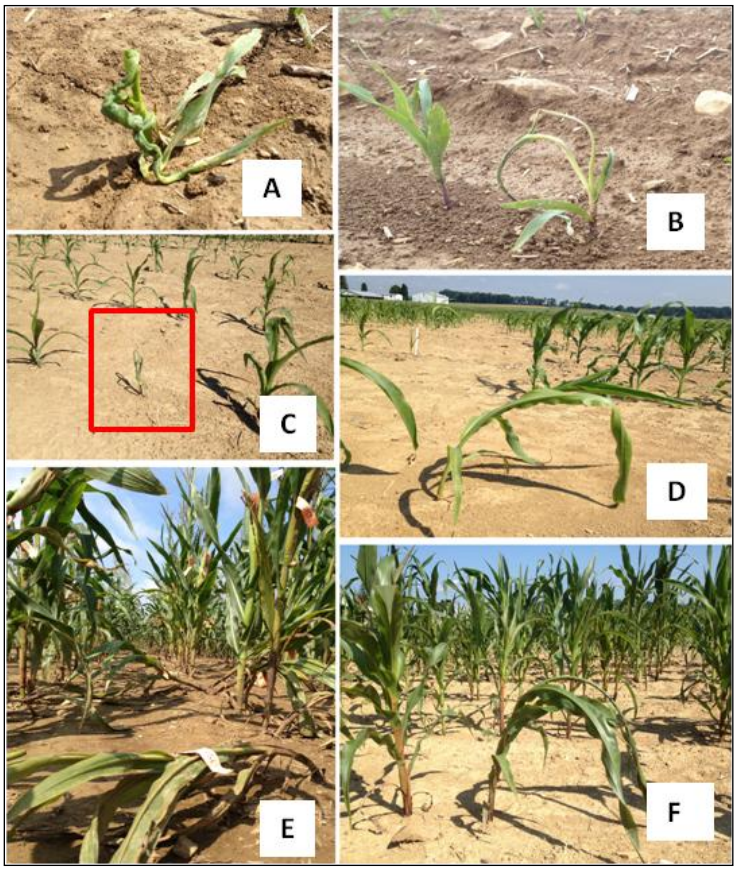


Figure 2-5: *Ufo1-1*-induced pleiotropic effects in *P1-wr* plants. (A) stunted growth, (B) trapped leaf, (C) dwarf plant, (D) bent stem, (E) weak plant stature and (F) rolled leaf.

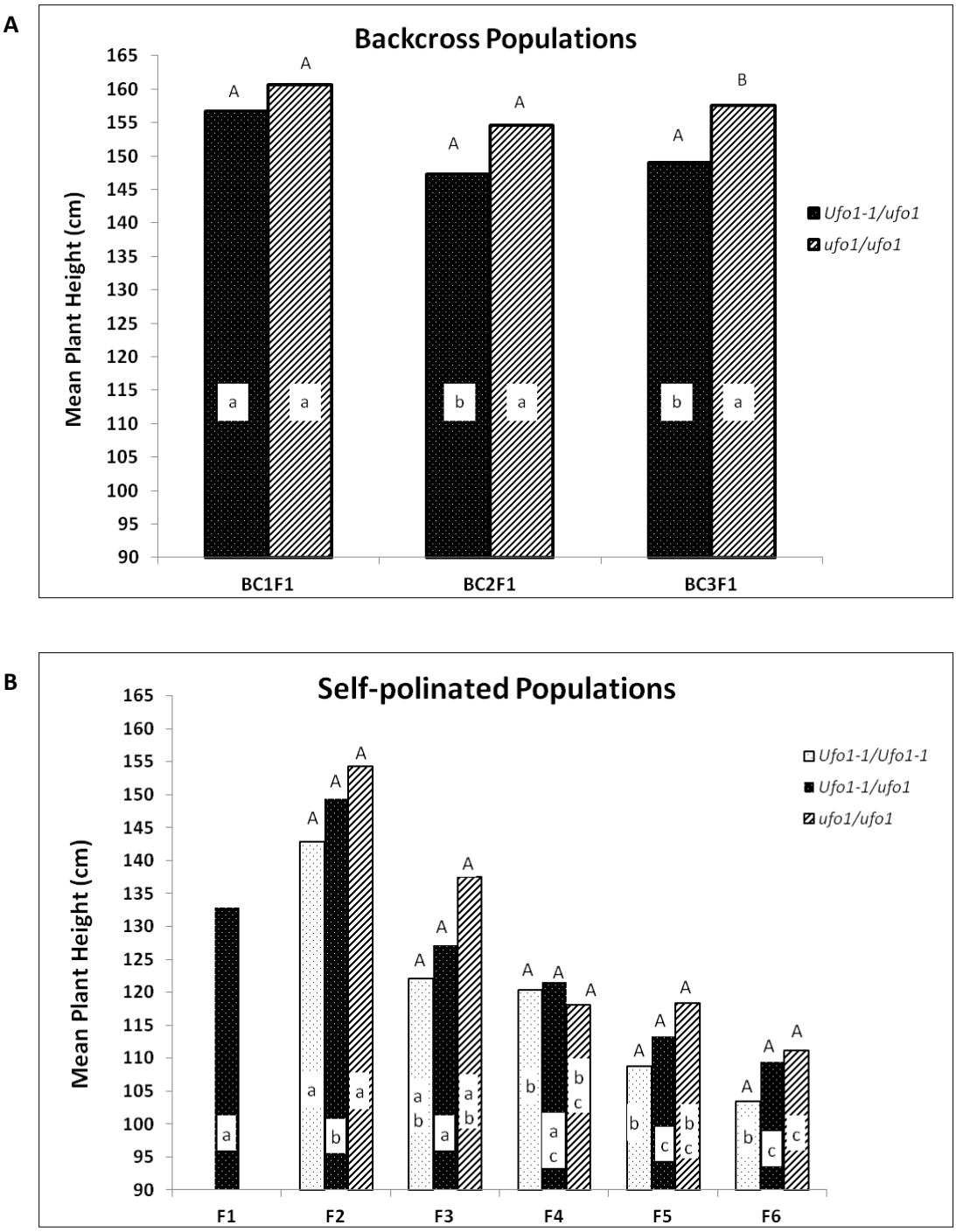


Figure 2-6: Mean plant height by genotypes; homozygous (*Ufo1-1/Ufo1-1*), heterozygous (*Ufo1-1/ufo1*) or wild type (*ufo1/ufo1*) in backcross populations, BC_1F_1 through BC_3F_1 (A) and in filial populations, F_1 through F_6 (B). Significant differences were determined using one-way analysis of variance test. Different capital letters indicate significant differences with α <0.05 within the generation. Different lowercase letters indicate significant differences with α <0.05 for homozygous (*Ufo1-1/ Ufo1-1*), heterozygous (*Ufo1-1/ ufo1*) or wild type (*ufo1/ufo1*) across the generations.

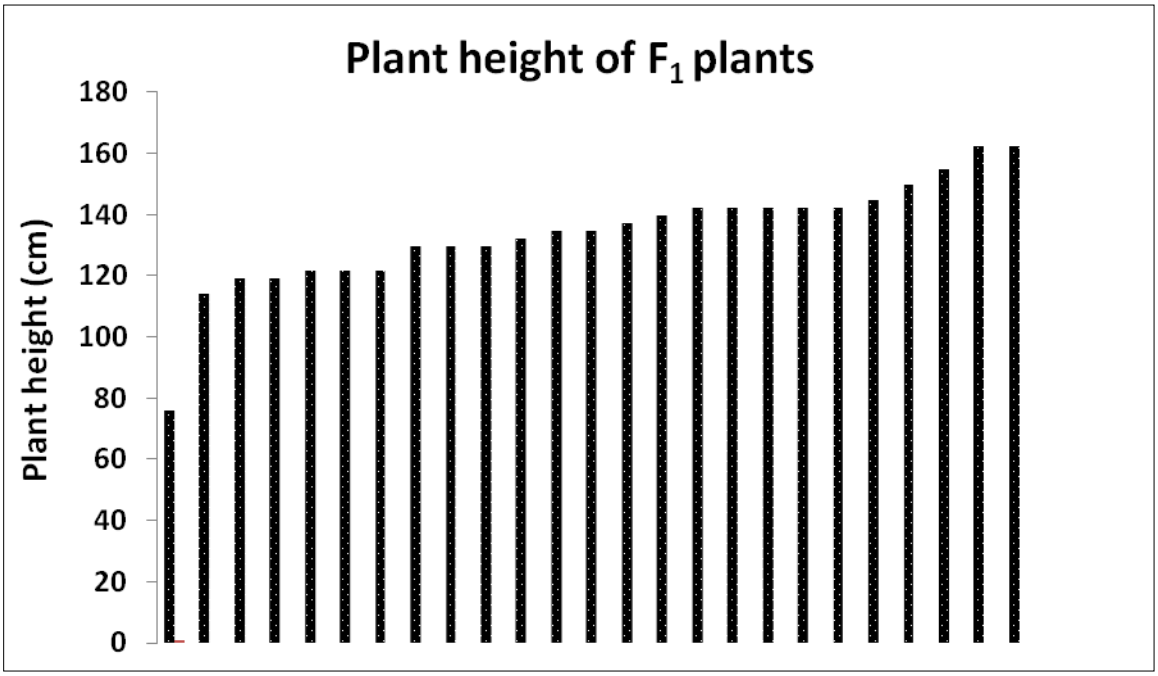


Figure 2-7: Variation in plant height of F_1 plants.

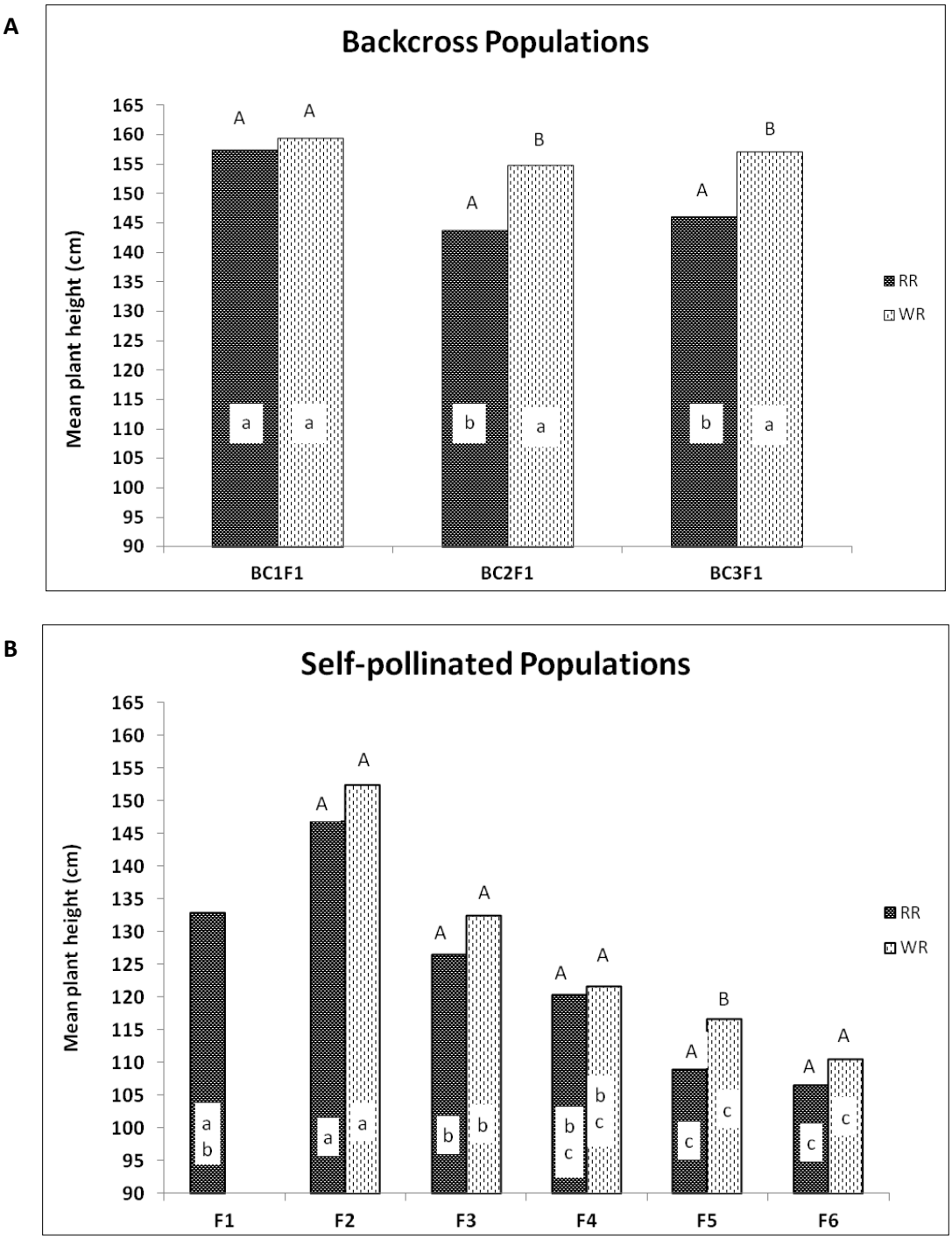


Figure 2-8: Mean plant height by ear pigmentation (RR and WR) in backcross populations, BC_1F_1 through BC_3F_1 (A) and in filial populations, F_1 through F_6 (B). Significant differences were determined using one-way analysis of variance test. Different capital letters indicate significant differences with α <0.05 within the generation. Different lowercase letters indicate significant differences with α <0.05 for RR and WR ear phenotypes across the generations.

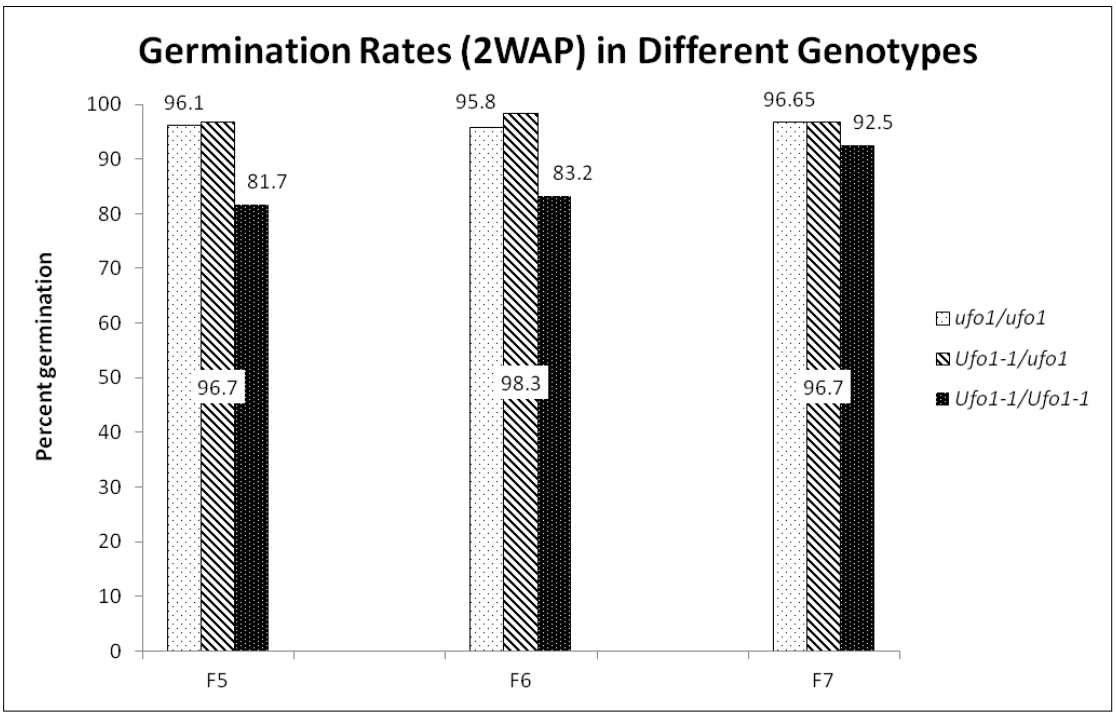


Figure 2-9: Percent germination in homozygous (*Ufo1-1/Ufo1-1*), heterozygous (*Ufo1-1/ufo1*) and wild type (*ufo1/ufo1*) in three filial generations, F_5, F_6 and F_7. Germination rate was calculated as percentage of emerged/planted seedlings 2 weeks after planting (WAP).

Table 2-1: Inheritance of *Ufo1-1* in independent families of the backcross (BC_1F_1 - BC_3F_1) Population I and the self- pollinated (F_1 -F_6) Population III from 2011 study.

Population	Generation	Family	Total Plants	Expected Plant Genotypes Ratio		Expected Plant Genotypes Frequency		Observed Plant Genotypes		X^2 (P)[a]
				Ufo1-1 /ufo1	*ufo1 /ufo1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	
Population I	BC_1 F_1	1	33	1	1	16.5	16.5	19	14	0.485 (0.486)
		2	14	1	1	7	7	7	7	0.000 (1.000)
	BC_2 F_1	1	40	1	1	20	20	22	18	0.225 (0.635)
		2	44	1	1	22	22	27	17	1.841 (0.175)
	BC_3 F_1	1	43	1	1	21.5	21.5	26	17	1.488 (0.223)
		2	38	1	1	19	19	17	21	0.237 (0.626)

[a] The null hypothesis: *Ufo1-1/ufo1* and *ufo1/ufo1* genotypes segregate at 1:1 ratio in the backcross (BC_1F_1 - BC_3F_1)

Population	Generation	Family	Total Plants	Expected Plant Genotypes Ratio			Expected Plant Genotypes Frequency			Observed Plant Genotypes			X^2 (P) [b/c]
				Ufo1-1 /Ufo1-1	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /Ufo1-1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /Ufo1-1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	
Population III	F_1	1	14		1			14			14		0.000 (1.000) [b]
		2	12		1			12			12		0.000 (1.000) [b]
	F_2	1	43	1	2	1	10.75	21.5	10.75	9	27	7	3.000 (0.223) [c]
		2	44	1	2	1	11	22	11	11	26	7	2.182 (0.336) [c]
	F_3	1	37	1	2	1	9.25	18.5	9.25	5	26	6	6.135 (0.047) [c]*
		2	46	1	2	1	11.5	23	11.5	5	33	8	9.087 (0.011) [c]*
	F_4	1	37	1	2	1	9.25	18.5	9.25	7	24	6	3.324 (0.190) [c]
		2	23	1			23			23			0.000 (1.000) [b]
	F_5	1	14	1	2	1	3.5	7	3.5	2	10	2	1.464 (0.481) [c]
		2	25	1	2	1	6.25	12.5	6.25	4	18	3	3.700 (0.157) [c]
	F_6	1	34	1	2	1	8.5	17	8.5	4	25	5	6.250 (0.044) [c]*
		2	31	1			31			31			0.000 (1.000) [b]

[b] The null hypothesis: all progenies fall into one category; either heterozygous *Ufo1-1/ufo1* (F_1 generation) or homozygous *Ufo1-1/Ufo1-1* (F_4 and F_6 generation)

[c] The null hypothesis: *Ufo1-1/Ufo1-1*, *Ufo1-1/ufo1,* and *ufo1/ufo1* genotypes segregate at 1:2:1 ratio

*P<0.05 and **P<0.001

Table 2-2: Inheritance of *Ufo1-1* in the pooled backcross (BC_1F_1 - BC_3F_1) Population I and in the pooled self- pollinated (F_1 -F_6) Population III from 2011 study.

Population	Generation	Total Plants	Expected Plant Genotypes Ratio		Expected Plant Genotypes Frequency		Observed Plant Genotypes		X^2 (P)[a]
			Ufo1-1 /ufo1	*ufo1 /ufo1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	
Population I	BC_1 F_1	47	1	1	23.5	23.5	26	21	0.340 (0.560)
	BC_2 F_1	84	1	1	42	42	49	35	2.012 (0.156)
	BC_3 F_1	81	1	1	40.5	40.5	43	38	0.198 (0.656)

[a] The null hypothesis: *Ufo1-1/ufo1* and *ufo1/ufo1* genotypes segregate at 1:1 ratio in the backcross (BC_1F_1 - BC_3F_1)

Population	Generation	Total Plants	Expected Plant Genotypes Ratio			Expected Plant Genotypes Frequency			Observed Plant Genotypes			X^2 (P) [b/c]
			Ufo1-1 /Ufo1-1	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /Ufo1-1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /Ufo1-1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	
Population III	F_1	26		1			26			26		0.000 (1.000) [b]
	F_2	87	1	2	1	21.75	43.5	21.75	20	53	14	4.977 (0.083) [c]
	F_3	83	1	2	1	20.75	41.5	20.75	10	59	14	15.145 (0.001) [c]**
	F_{4} ‡	37	1	2	1	9.25	18.5	9.25	7	24	6	3.324 (0.190) [c]
		23	1			23			23			0.000 (1.000) [b]
	F_5	39	1	2	1	9.75	19.5	9.75	6	28	5	7.462 (0.024) [c]*
	F_{6} ‡	34	1	2	1	8.5	17	8.5	4	25	5	6.250 (0.044) [c]*
		31	1			31			31			0.000 (1.000) [b]

[b] The null hypothesis: all progenies fall into one category; either heterozygous *Ufo1-1/ufo1* (F_1 generation) or homozygous *Ufo1-1/Ufo1-1* (F_4 and F_6 generation)

[c] The null hypothesis: *Ufo1-1/Ufo1-1*, *Ufo1-1/ufo1,* and *ufo1/ufo1* genotypes segregate at 1:2:1 ratio

*P<0.05 and **P<0.001

‡ The families were proven to be heterogeneous by Test for Homogeneity, hence the data were analyzed individually without being pooled

Table 2-3: Inheritance of *Ufo1-1*-induced gain of pericarp pigmentation in independent families of the backcross (BC_1F_1 - BC_3F_1) Population I and the self- pollinated (F_1 -F_6) Population III from 2011 study.

Population	Generation	Family	Total Ears	Expected Ear Phenotypes Ratio		Expected Ear Phenotypes Frequency		Observed Ear Phenotypes		X^2 (P)[a]
				RR	WR	RR	WR	RR	WR	
Population I	BC_1F_1	1	33	1	1	16.5	16.5	13	20	1.091 (0.296)
		2	14	1	1	7	7	5	9	0.643 (0.423)
	BC_2F_1	1	40	1	1	20	20	16	24	1.225 (0.268)
		2	44	1	1	22	22	18	26	1.1136 (0.291)
	BC_3F_1	1	43	1	1	21.5	21.5	22	21	0.0000 (1.000)
		2	38	1	1	19	19	8	30	11.605 (0.001)**

[a] The null hypothesis: RR and WR phenotypes segregate at 1:1 ratio in the backcross (BC_1F_1 - BC_3F_1)

Population	Generation	Family	Total Ears	Expected Ear Phenotypes Ratio		Expected Ear Phenotypes Frequency		Observed Ear Phenotypes		X^2 (P) [b/c]
				RR	WR	RR	WR	RR	WR	
Population III	F_1	1	14	1		14		14		0.000 (1.000) [b]
		2	12	1		12		12		0.000 (1.000) [b]
	F_2	1	42	3	1	31.5	10.5	31	11	0.000 (1.000) [c]
		2	42	3	1	31.5	10.5	22	20	10.285 (0.001) [c]**
	F_3	1	37	3	1	27.75	9.25	28	9	0.009 (0.924) [c]
		2	45	3	1	33.75	11.25	27	18	4.629 (0.031) [c]*
	F_4	1	37	3	1	27.75	9.25	21	16	5.631 (0.018) [c]*
		2	23	1		23		23		0.000 (1.000) [b]
	F_5	1	14	3	1	10.5	3.5	8	6	1.524 (0.217) [c]
		2	25	3	1	18.75	6.25	9	16	18.253 (0.001) [c]**
	F_6	1	33	3	1	24.75	8.25	11	22	28.373 (0.001) [c]**
		2	31	1		31		12	19	352.945 (0.001) [b]**

[b] The null hypothesis: all progenies fall into one category, RR phenotypes (F_1, F_4, and F_6 generation)

[c] The null hypothesis: RR and WR phenotypes segregate at 3:1 ratio

*P<0.05 and **P<0.001

Table 2-4: Inheritance of *Ufo1-1*-induced gain of pericarp pigmentation in the pooled backcross (BC_1F_1 - BC_3F_1) Population I and in the pooled self- pollinated (F_1 -F_6) Population III from 2011 study.

Population	Generation	Total Ears	Expected Ear Phenotypes Ratio		Expected Ear Phenotypes Frequency		Observed Ear Phenotypes		X^2 (P)[a]
			RR	WR	RR	WR	RR	WR	
Population I	BC_1 F_1	47	1	1	23.5	23.5	18	29	2.128 (0.145)
	BC_2 F_1	84	1	1	42	42	34	50	2.679 (0.102)
	BC_3 F_1 ‡	43	1	1	21.5	21.5	22	21	0.000 (1.000)
		38	1	1	19	19	8	30	11.605 (0.001)**

[a] The null hypothesis: RR and WR phenotypes segregate at 1:1 ratio in the backcross (BC_1F_1 - BC_3F_1)

Population	Generation	Total Ears	Expected Ear Phenotypes Ratio		Expected Ear Phenotypes Frequency		Observed Ear Phenotypes		X^2 (P) [b/c]
			RR	WR	RR	WR	RR	WR	
Population III	F_1	26	1		26		26		0.000 (1.000) [b]
	F_2 ‡	42	3	1	31.5	10.5	31	11	0.000 (1.000) [c]
		42	3	1	31.5	10.5	22	20	10.285 (0.001) [c]**
	F_3	82	3	1	61.5	20.5	55	27	2.341 (0.126) [c]
	F_4 ‡	37	3	1	27.75	9.25	21	16	5.631 (0.018) [c]*
		23	1		23		23		0.000 (1.000) [b]
	F_5	39	3	1	29.25	9.75	17	22	18.880 (0.001) [c]**
	F_6 ‡	33	3	1	24.75	8.25	11	22	28.373 (0.001) [c]**
		31	1		31		12	19	352.945 (0.001) [b]**

[b] The null hypothesis: all progenies fall into one category, RR phenotypes (F_1, F_4, and F_6 generation)

[c] The null hypothesis: RR and WR phenotypes segregate at 3:1 ratio

*P<0.05 and **P<0.001

‡ The families were proven to be heterogeneous by Test for Homogeneity, hence the data were analyzed individually without being pooled

Table 2-5: Genotypic and phenotypic inheritance of *Ufo1-1* in the backcross and the self-pollinated populations from 2011 study.

	Generation	Total Plants	Expected Plant Genotypes			Observed Plant Genotypes			X^2	Total Ears	Expected Ear Phenotypes		Observed Ear Phenotypes		X^2
Population I			*Ufo1-1 /Ufo1-1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /Ufo1-1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*			RR	WR	RR	WR	
	$BC_1 F_1$	47		23.5	23.5		26	21	0.34	47	23.5	23.5	18	29	2.13
	$BC_2 F_1$	84		42	42		49	35	2.01	84	42	42	34	50	2.68
	$BC_3 F_1$ ‡	43		21.5	21.5		26	17	1.49	43	21.5	21.5	22	21	0.00
		38		19	19		17	21	0.24	38	19	19	8	30	11.61 **
Population III															
	F_1	26		26			26		0.00	26	26		26		0.00
	F_2 ‡	43	10.75	21.5	10.75	9	27	7	3.00	42	31.5	10.5	31	11	0.00
		44	11	22	11	11	26	7	2.182	42	31.5	10.5	22	20	10.29**
	F_3	83	20.75	41.5	20.75	10	59	14	15.15**	82	61.5	20.5	55	27	2.34
	F_4 ‡	37	9.25	18.5	9.25	7	24	6	3.32	37	27.75	9.25	21	16	5.63*
		23	23			23			0.00	23	23		23		0.000
	F_5	39	9.75	19.5	9.75	6	28	5	7.46*	39	29.25	9.75	17	22	18.88**
	F_6 ‡	34	8.5	17	8.5	4	25	5	6.25*	33	24.75	8.25	11	22	28.37**
		31	31			31			0.00	31	31		12	19	352.95**

*P<0.05 and **P<0.001

‡ The families were proven to be heterogeneous by Test for Homogeneity, hence the data were analyzed individually without being pooled

Table 2-6: Inheritance of *Ufo1-1* in independent families of backcross populations (Population I and Population II) from 2012 study.

	Generation (Parent's Genotype)	Family	Total Plants	Expected Plant Genotypes Ratio		Expected Plant Genotypes Frequency		Observed Plant Genotypes		X^2 (P)
				Ufo1-1 /ufo1	*ufo1 /ufo1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	
Population I [B73]	BC_2F_1 (*Ufo1-1/ufo1*)	1	116	1	1	58	58	58	58	0.000 (1.000) [a]
	BC_2 F_1 *(ufo1/ufo1)*	1	95		1		95		95	0.000 (1.000) [b]
	BC_4 F_1 (*Ufo1-1/ufo1*)	1	113	1	1	56.5	56.5	48	65	2.266 (0.132) [a]
	BC_4 F_1 *(ufo1/ufo1)*	1	83		1		83		83	0.000 (1.000) [b]
Population II [W23]	BC_6 F_1 (*Ufo1-1/ufo1*)	1	110	1	1	55	55	56	54	0.009 (0.924) [a]
	BC_{10} F_1 (*Ufo1-1/ufo1*)	1	113	1	1	56.5	56.5	50	63	1.274 (0.259) [a]

[a] The null hypothesis: *Ufo1-1/ufo1* and *ufo1/ufo1* genotypes segregate at 1:1 ratio in the backcross population

[b] The null hypothesis: all progenies fall into one category; homozygous *ufo1/ufo1*

*P<0.05 and **P<0.001

Table 2-7: Inheritance of *Ufo1-1* in independent families of the self-pollinated population (Population III) from 2012 study.

Generation (Parent's Genotype)	**Family**	**Total Plants**	**Expected Plant Genotypes Ratio**			**Expected Plant Genotypes Frequency**			**Observed Plant Genotypes**			**X^2 (P) [b/c]**
			Ufo1-1 /Ufo1-1	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /Ufo1-1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /Ufo1-1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	
F_5 (*Ufo1-1/ufo1*)	1	56	1	2	1	14	28	14	14	27	15	0.107 (0.948) [c]
F_5 (*Ufo1-1/Ufo1-1*)	1	43	1			43			43			0.000 (1.000) [b]
F_5 (*Ufo1-1/Ufo1-1*)	2	48	1			48			48			0.000 (1.000) [b]
F_5 *(ufo1/ufo1)*	1	59			1			59			59	0.000 (1.000) [b]
F_5 *(ufo1/ufo1)*	2	55			1			55			55	0.000 (1.000) [b]
F_5 *(ufo1/ufo1)*	3	56			1			56			56	0.000 (1.000) [b]
F_6 (*Ufo1-1/ufo1)*	1	58	1	2	1	14.5	29	14.5	15	25	18	1.414 (0.493) [c]
F_6 *(Ufo1-1/Ufo1-1)*	1	59	1			59			59			0.000 (1.000) [b]
F_6 *(Ufo1-1/Ufo1-1)*	2	62	1			62			62			0.000 (1.000) [b]
F_6 *(Ufo1-1/Ufo1-1)*	3	47	1			47			47			0.000 (1.000) [b]
F_6 *(ufo1/ufo1)*	1	114			1			114			114	0.000 (1.000) [b]
F_7 *(Ufo1-1/ufo1)*	1	58	1	2	1	14.5	29	14.5	12	26	20	2.828 (0.2432) [c]
F_7 *(Ufo1-1/ufo1)*	2	27	1	2	1	6.75	13.5	6.75	5	15	7	0.630 (0.730) [c]
F_7 (*Ufo1-1/Ufo1-1*)	1	36	1			36			36			0.000 (1.000) [b]
F_7 (*Ufo1-1/Ufo1-1*)	2	57	1			57			57			0.000 (1.000) [b]
F_7 *(ufo1/ufo1)*	1	60			1			60			60	0.000 (1.000) [b]
F_7 *(ufo1/ufo1)*	2	55			1			55			55	0.000 (1.000) [b]

[b] The null hypothesis: all progenies fall into one category; homozygous *Ufo1-1/Ufo1-1* or homozygous *ufo1/ufo1*

[c] The null hypothesis: *Ufo1-1/Ufo1-1*, *Ufo1-1/ufo1,* and *ufo1/ufo1* genotypes segregate at 1:2:1 ratio

*P<0.05 and **P<0.001

Table 2-8: Inheritance of *Ufo1-1* in the pooled self- pollinated (F_5 –F_7) Population III from 2012 study.

Generation (Parent's Genotype)	Total Plants	Expected Plant Genotypes Ratio			Expected Plant Genotypes Frequency			Observed Plant Genotypes			X^2 (P) [b/c]
		Ufo1-1 /Ufo1-1	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /Ufo1-1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	*Ufo1-1 /Ufo1-1*	*Ufo1-1 /ufo1*	*ufo1 /ufo1*	
F_5 (*Ufo1-1/ufo1*)	56	1	2	1	14	28	14	14	27	15	0.107(0.948) [c]
F_5 (*Ufo1-1/Ufo1-1*)	91	1			91			91			0.000(1.000) [b]
F_5 *(ufo1/ufo1)*	170			1			170			170	0.0000(1.000) [b]
F_6 (*Ufo1-1/ufo1)*	58	1	2	1	14.5	29	14.5	15	25	18	1.414(0.493) [c]
F_6 *(Ufo1-1/Ufo1-1)*	168	1			168			168			0.000(1.000) [b]
F_6 *(ufo1/ufo1)*	114			1			114			114	0.000(1.000) [b]
F_7 *(Ufo1-1/ufo1)*	85	1	2	1	21.25	42.5	21.25	17	41	27	2.459(0.293) [c]
F_7 (*Ufo1-1/Ufo1-1*)	93	1			93			93			0.000(1.000) [b]
F_7 *(ufo1/ufo1)*	115			1			115			115	0.000(1.000) [b]

[b] The null hypothesis: all progenies fall into one category; homozygous *Ufo1-1/Ufo1-1* or homozygous *ufo1/ufo1*

[c] The null hypothesis: *Ufo1-1/Ufo1-1*, *Ufo1-1/ufo1,* and *ufo1/ufo1* genotypes segregate at 1:2:1 ratio

*P<0.05 and **P<0.001

Table 2-9: Inheritance of *Ufo1-1*-induced gain of pericarp pigmentation in independent families of backcross (Population I and Population II) from 2012 study.

	Generation (Parent's Genotype)	Total Ears	Expected Ear Phenotypes Ratio		Expected Ear Phenotypes Frequency		Observed Ear Phenotypes		X^2 (P)[a/b]
			RR	WR	RR	WR	RR	WR	
Population I [B73]	BC_2F_1 (*Ufo1-1*/ufo1)	115	1	1	57.5	57.5	49	66	2.226 (0.136) [a]
	BC_2 F_1 *(ufo1/ufo1)*	95		1		95		95	0.000(1.000) [b]
	BC_4 F_1 (*Ufo1-1*/ufo1)	113	1	1	56.5	56.5	8	105	81.558 (<0.0001) [a] **
	BC_4 F_1 *(ufo1/ufo1)*	83		1		83		83	0.0000(1.0000) [b]
Population II [W23]	BC_6 F_1 (*Ufo1-1*/ufo1)	109	1	1	54.5	54.5	27	82	26.752 (<0.0001) [a] **
	BC_{10} F_1 (*Ufo1-1*/ufo1)	111	1	1	55.5	55.5	38	73	10.414 (<0.0001) [a] **

[a] The null hypothesis: RR and WR phenotypes segregate at 1:1 ratio

[b] The null hypothesis: all progenies fall into one category, WR phenotypes

*P<0.05 and **P<0.001

Table 2-10: Inheritance of *Ufo1-1*-induced gain of pericarp pigmentation in independent families of self-pollinated population (Population III) from 2012 study.

Generation (Parent's Genotype)	**Family**	**Total Ears**	**Expected Ear Phenotypes Ratio**		**Expected Ear Phenotypes Frequency**		**Observed Ear Phenotypes**		**X^2 (P) [b/c]**
			RR	WR	RR	WR	RR	WR	
F_5 (*Ufo1-1*/ufo1)	1	56	3	1	42	14	21	35	40.024 (<0.0001) [c]**
F_5 (*Ufo1-1/Ufo1-1*)	1	43	1		43		43		0.000 (1.000) [b]
F_5 (*Ufo1-1/Ufo1-1*)	2	48	1		48		48		0.000 (1.000) [b]
F_5 *(ufo1/ufo1)*	1	59		1		59		59	0.000 (1.000) [b]
F_5 *(ufo1/ufo1)*	2	55		1		55		55	0.000 (1.000) [b]
F_5 *(ufo1/ufo1)*	3	56		1		56		56	0.000 (1.000) [b]
F_6 (*Ufo1-1/ufo1)*	1	58	3	1	43.5	14.5	27	31	23.540 (<0.0001) [c]**
F_6 *(Ufo1-1/Ufo1-1)*	1	59	1		59		32	27	713.95 (<0.0001) [b]**
F_6 *(Ufo1-1/Ufo1-1)*	2	56	1		56		19	37	1355.62 (<0.0001) [b]**
F_6 *(Ufo1-1/Ufo1-1)*	3	46	1		46		29	17	278.04 (<0.0001) [b]**
F_6 *(ufo1/ufo1)*	1	114		1		114		114	0.000 (1.000) [b]
F_7 *(Ufo1-1/ufo1)*	1	58	3	1	43.5	14.5	10	48	100.14 (<0.0001) [c]**
F_7 *(Ufo1-1/ufo1)*	2	26	3	1	19.5	6.5	4	22	46.154 (<0.0001) [c]**
F_7 (*Ufo1-1/Ufo1-1*)	1	36	1		36		21	15	215.93 (<0.0001) [b]**
F_7 (*Ufo1-1/Ufo1-1*)	2	57	1		57			57	3247.29 (<0.0001) [b]**
F_7 *(ufo1/ufo1)*	1	60		1		60		60	0.000 (1.000) [b]
F_7 *(ufo1/ufo1)*	2	55		1		55		55	0.000 (1.000) [b]

[b] The null hypothesis: all progenies fall into one category; either RR or WR

[c] The null hypothesis: RR and WR phenotypes segregate at 3:1 ratio

*P<0.05 and **P<0.001

Table 2-11: Inheritance of *Ufo1-1*-induced gain of pericarp pigmentation in the pooled self- pollinated (F_5 –F_7) Population III from 2012 study.

Generation (Parent's Genotype)	Total Ears	Expected Ear Phenotypes Ratio		Expected Ear Phenotypes Frequency		Observed Ear Phenotypes		X^2 (P) [b/c]
		RR	WR	RR	WR	RR	WR	
F_5 (*Ufo1-1*/ufo1)	56	3	1	42	14	21	35	40.024 (<0.0001)[c]**
F_5 (*Ufo1-1/Ufo1-1*)	91	1		91		91		0.000 (1.0000)[b]
F_5 *(ufo1/ufo1)*	170		1		170		170	0.000 (1.0000)[b]
F_6 (*Ufo1-1/ufo1)*	58	3	1	43.5	14.5	27	31	23.540 (<0.0001)[c] **
F_6 *(Ufo1-1/Ufo1-1)*	161	1		161		80	81	6847.25 (<0.0001)[b] **
F_6 *(ufo1/ufo1)*	114		1		114		114	0.000 (1.0000)[b]
F_7 *(Ufo1-1/ufo1)*	84	3	1	63	21	14	70	149.349 (<0.0001)[c] **
F_7 (*Ufo1-1/Ufo1-1*)	93	1		93		21	72	5311.00 (<0.0001)[b] **
F_7 *(ufo1/ufo1)*	115		1		115		115	0.000 (1.0000)[b]

[b] The null hypothesis: all progenies fall into one category; either RR or WR

[c] The null hypothesis: RR and WR phenotypes segregate at 3:1 ratio

*P<0.05 and **P<0.001

Table 2-12: Genotypic and phenotypic inheritance of *Ufo1-1* in the backcross and the self-pollinated populations from 2012 study

	Generation (Parent's Genotype)	Total Plants	Expected Plant Genotypes			Observed Plant Genotypes			X^2	Total Ears	Expected Ear Phenotypes		Observed Ear Phenotypes		X^2
			Ufo1-1 / *Ufo1-1*	*Ufo1-1* /ufo1	ufo1 /ufo1	*Ufo1-1* /*Ufo1-1*	*Ufo1-1* /ufo1	ufo1 /ufo1			RR	WR	RR	WR	
Population I	BC_2F_1 (*Ufo1-1/ufo1*)	116		58	58		58	58	0.00	115	57.5	57.5	49	66	2.23
	BC_2 F_1 (*ufo1/ufo1*)	95			95			95	0.00	95		95		95	0.00
	BC_4 F_1 (*Ufo1-1/ufo1*)	113		56.5	56.5		48	65	2.27	113	56.5	56.5	8	105	81.56**
	BC_4 F_1 (*ufo1/ufo1*)	83			83			83	0.00	83		83		83	0.00
Population II	BC_6F_1 (W23) (*Ufo1-1/ufo1*)	110		55	55		56	54	0.01	109	54.5	54.5	27	82	26.75**
	BC_{10} F_1 (W23) (*Ufo1-1/ufo1*)	113		56.5	56.5		50	63	1.27	111	55.5	55.5	38	73	10.41*
Population III	F_5 (*Ufo1-1/ufo1*)	56	14	28	14	14	27	15	0.11	56	42	14	21	35	40.02**
	F_5 (*Ufo1-1/Ufo1-1*)	91	91			91			0.00	91	91		91		0.00
	F_5 (*ufo1/ufo1*)	170			170			170	0.00	170		170		170	0.00
	F_6 (*Ufo1-1/ufo1*)	58	14.5	29	14.5	15	25	18	1.41	58	43.5	14.5	27	31	23.54**
	F_6 (*Ufo1-1/Ufo1-1*)	168	168			168			0.00	161	161		80	81	6847**
	F_6 (*ufo1/ufo1*)	114			114			114	0.00	114		114		114	0.00
	F_7 (*Ufo1-1/ufo1*)	85	21.25	42.5	21.25	17	41	27	2.46	84	63	21	14	70	149.4**
	F_7 (*Ufo1-1/Ufo1-1*)	93	93			93			0.00	93	93		21	72	5311 **
	F_7 (*ufo1/ufo1*)	115			115			115	0.00	115		115		115	0.00

*P<0.05 and **P<0.001

	Generation	Total Ears Examined	Number of expressers		Penetrance (%)
			Expected	Observed	
2011 Field	$BC_1 F_1$ [B73]	97	48	41	85.4
	$BC_2 F_1$ [B73]	84	42	34	80.9
	$BC_3 F_1$ [B73]	81	40	30	75.0
2012 Field	BC_2F_1 [B73]	115	57	49	85.9
	$BC_4 F_1$ [B73]	113	56	8	14.3
	$BC_6 F_1$ [W_{23}]	109	54	27	50.0
	$BC_{10} F_1$ [W_{23}]	111	55	38	69.1
2011 Field	F_1	26	26	26	100
	F_2	84	63	53	84.1
	F_3	45	33	27	81.8
	F_4	37	27	21	77.8
	F_5	39	29	17	58.6
	F_6	33	24	11	45.8
2012 Field	F_5	56	42	21	50.0
	F_6	58	43	27	62.8
	F_7	84	63	14	22.2

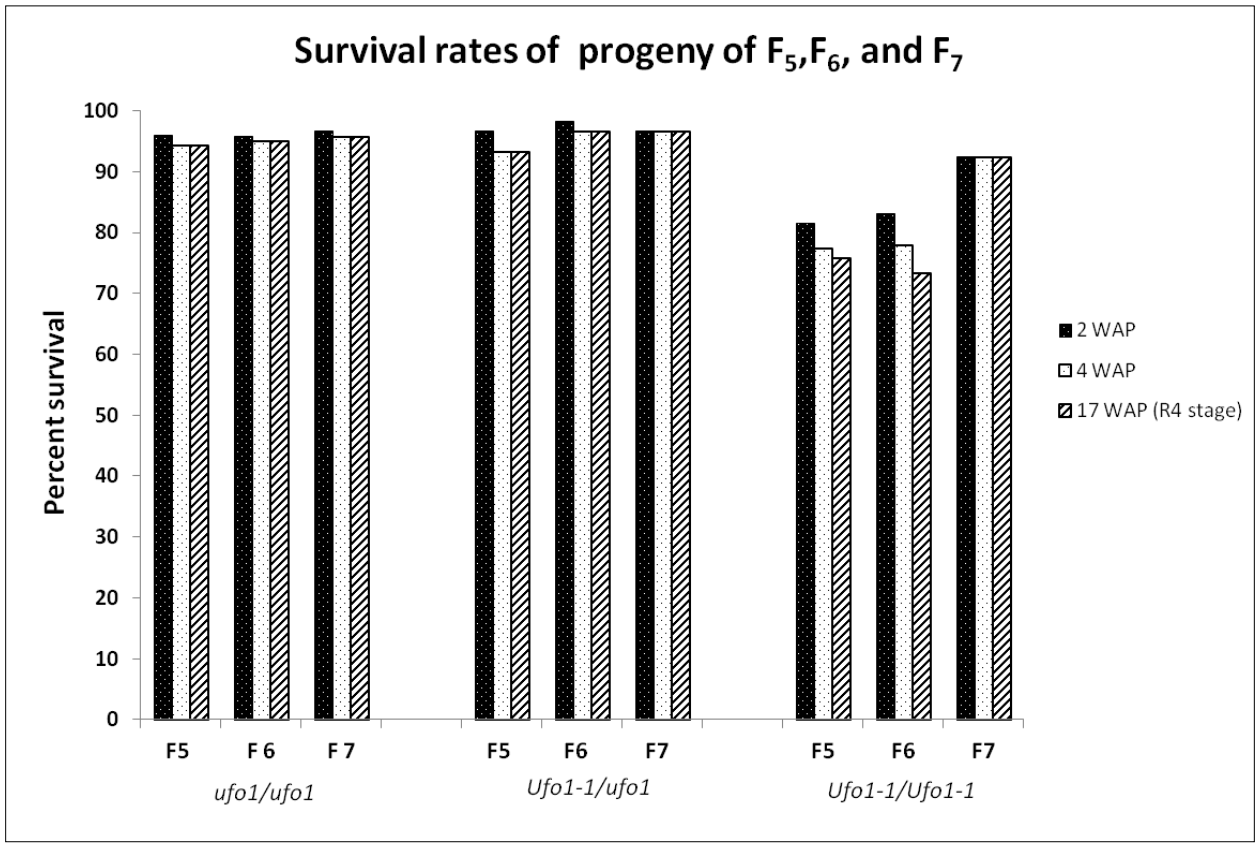
Table 2-13: Penetrance of *Ufo1-1* in different genetic backgrounds. Expected phenotypes are based on assumption of complete penetrance of *Ufo1-1*; half of the progeny of the backcross populations is expected to exhibit *Ufo1-1*-induced phenotypes. In filial populations, all F_1 progenies are expected to show gain of pericarp pigmentation. In subsequent filial populations, 75% of the F_n individuals are expected to be *Ufo1-1* expresser plants (only segregating families were included in penetrance analysis of self-pollinated populations). Penetrance was calculated as percentage of observed/expected *Ufo1-1*-expressers.

Supplemental Table/ Figure

Table S2-1: Primer sequences used for genotyping of *Ufo1-1* locus

Name	Forward Primer	Reverse Primer
SSR31	TCACAATCAAACAGGGCTGA	CTGTCTCAACCTCTGCACCA
CAPS5	GCTAATGGGTACGTGGTCGT	ACAGCGGAACAACCGTAATC

Figure S2-1: Survival rates of progeny from families of wild type *ufo1*, heterozygous (*Ufo1-1*/*ufo1)*, and homozygous (*Ufo1-1/Ufo1-1*) in three filial generations, F_5 ,F_6 and F_7



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Chapter 3

Relative quantification of DNA methylation level of *P1-wr* allele

Abstract

DNA methylation is the most widely studied epigenetic mark and plays a major role in transcriptional gene silencing. Phenotypic differences exhibited by distinct *p1* alleles, *P1-wr* and *P1-rr*, have been attributed to differences in methylation states of their distal enhancer (DE) regions. *P1-wr* is hypermethylated and thus is silenced in the pericarp, whereas *P1-rr* is hypomethylated and expressed in both the pericarp and cob glumes. *Unstable factor for orange1* (*Ufo1*) activates *P1-wr* expression resulting in abnormal phlobaphenes accumulation in pericarp, cob glumes, leaf sheath, husk, and tassel glumes. Although the *Ufo1-1* allele is stably inherited, the *Ufo1-1* effects fade away in later generations of backcross and self– pollinated populations. Herein, we report methylation-specific qRT-PCR based assay to investigate the DNA methylation level in *P1-wr; Ufo1-1* plants in advanced generations. Our result indicates that the non-expresser plants were significantly hypomethylated as compared to the naïve *P1-wr*. This is a very interesting finding as loss of methylation at the DE regions has been previously associated with gain of pericarp pigmentation. Our study provides a new insight on possible role of *ufo1* in epigenetic regulation as the DNA methylation may not be directly involved in the *ufo1*-mediated pathway. However, further validations on this new qRT-PCR approach need to be performed before we are able to make such conclusion. Additional studies exploring other epigenetic mechanisms such as histone modifications and nucleosome occupancy would be informative to clarify the role of *ufo1* in maintaining tissue-specific gene silencing.

Introduction

DNA methylation has long been identified as a bona fide epigenetic mark and has been observed in both prokaryotes and eukaryotes. In prokaryotes, methylation can occur on both cytosines and adenines and this methylation is responsible for various biological processes such as DNA replication, DNA repair, and bacterial pathogenicity (REISENAUER *et al.* 1999). In contrast, in eukaryotes methylation occurs almost exclusively at cytosines and it is found in mammals, plants, and fungi. Cytosine methylation, the addition of a methyl group at the 5' position of the pyrimidine ring, is one of the molecular mechanisms for the inheritance of epigenetic information. In mammals, cytosine methylation is found in the symmetrical CG context (OKANO *et al.* 1999) and this methylation is involved in transposon immobilization, X-chromosome inactivation, genomic imprinting, and normal mammalian development [see review (JONES and TAKAI 2001; SENNER 2011)]. Interestingly, cytosine methylation occurs on both the symmetrical, CG, CHG, and the asymmetrical CHH sequence context (where H= A, T or C) in plants [see review (HENDERSON and JACOBSEN 2007)] and fungi (KOUZMINOVA and SELKER 2001). In plants, this epigenetic modification is commonly associated with suppression of transposon mobility (KATO *et al.* 2003), repression of gene expression (ZILBERMAN *et al.* 2007), and maintenance of genome integrity (FINNEGAN *et al.* 1996).

Cytosine methylation can be divided into two categories; *de novo* methylation which refers to establishment of new methylation marks on previously non-methylated cytosine bases, while maintenance of DNA methylation involves copying preexisting methylation marks on the parental strand of DNA into the newly synthesized strand during DNA replication (restoring the hemimetylated DNA back to the fully methylated state) [see review (LAW and JACOBSEN 2010)]. Owing to the relatively small and less complex genome of *Arabidopsis*, the majority of the genome-wide methylation mapping studies has been performed in this model organism. There are three major DNA methyltransferase enzymes in

Arabidopsis, which actively transfer and covalently attach methyl groups onto the cytosine residues. DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE) is the only DNA methyltransferase responsible for *de novo* cytosine methylation in all sequence contexts (CAO and JACOBSEN 2002). However, maintenance of DNA methylation is regulated by different pathways; MET1 (METHYLTRANSFERASE1) maintains the symmetrical CG methylation (AUFSATZ *et al.* 2004),whereas CMT3 (CHROMO METHYLATRANSFERASE3) preserves the symmetrical CHG methylation (SIMON *et al.* 2005), and the asymmetric CHH methylation is sustained through constant *de novo* methylation by DRM2 [see review (LAW and JACOBSEN 2010)].

The 5-methylcytosines are widely distributed in plant genomes, occurring in transposable elements (TEs), repetitive sequences, protein-coding genes, and non-repetitive intergenic regions (ZHANG *et al.* 2006). Newly developed whole-genome tilling microarrays and high-throughput sequencing technologies have allowed investigation of DNA methylation at a genome-wide scale. Recent studies show that approximately 20% of the *Arabidopsis* genome is methylated, where DNA methylation is largely distributed on TEs and repetitive sequences, while a significant amount of methylation is also observed on genic and intergenic regions (ZHANG *et al.* 2006). Generally, in plants, DNA methylation often acts to suppress the transposon mobility and to control the expression of repetitive sequences, pseudogenes, and in some instances active genes [see review (SIMON *et al.* 2005; SLOTKIN and MARTIENSSEN 2007)]. Previous evidences show that methylated transposons are immobile and loss of methylation results in a severe transcriptional reactivation of transposon and transposition (MIURA *et al.* 2001; SINGER *et al.* 2001; ZILBERMAN *et al.* 2007). In addition*, Arabidopsis* genome-wide DNA methylation mappings also show that cytosine methylation is widespread in repetitive sequences, where more than 50% of interspersed, tandem, and inverted repeats are heavily methylated (ZHANG *et al.* 2006). The roles of DNA methylation in gene silencing of repetitive elements have been demonstrated in numerous studies. For instances, the ribosomal RNA (rRNA) genes which consist of hundreds of copies that are

arranged in tandem arrays are stochastically silenced by DNA methylation (LAWRENCE *et al.* 2004). Another example is *FWA* (*Flowering Wageningen*) silencing by cytosine methylation in *Arabidopsis.* The *FWA* gene encodes a transcription factor that is responsible for regulating flowering time (KINOSHITA *et al.* 2004; SOPPE *et al.* 2000). This imprinted *FWA* gene is specifically expressed in the female gametophyte and endosperm tissues, in which transcription of *FWA* is dependent on demethylation of tandem repeats present at the transcription start site (FUJIMOTO *et al.* 2008; KINOSHITA *et al.* 2007). This loss of cytosine methylation can be induced by mutation of *Methyltransferase1* (*MET1*) (KINOSHITA *et al.* 2007) or by DNA glycosylase, DEMETER (DME) activity (CHOI *et al.* 2002).

Regulation of endogenous gene expression is strongly dependent on the location of methylation relative to a given gene. Genic methylation is observed at a lower frequency compared to DNA methylation in TEs and repetitive sequences (VAUGHN *et al.* 2007). Moreover, gene body methylation is strongly biased away from the 5' end (promoter regions) and 3' flanking sequences, indicating that there is an association between methylation and inhibition of transcriptional elongation, for example, methylation may impede RNA polymerase transit (ZILBERMAN *et al.* 2007). Commonly, methylation in the promoter region shows a negative correlation to gene expression through inhibition of transcriptional initiation. Genes methylated in the in the promoter region are often expressed in tissue-specific patterns. Methylation of transcribed regions (body-methylated) is observed at a higher frequency than promoter methylation. Interestingly, body-methylated genes are constitutively expressed and highly transcribed (ZHANG *et al.* 2006). However, loss of methylation in this open-reading frame results in slightly up-regulated transcription and has fewer effects than demethylation of transposons that causes their drastic remobilization (ZILBERMAN *et al.* 2007).

Recent work demonstrates that silent TEs are heavily methylated and these epigenetic marks are stably inherited from one generation to the next. In contrast, although genic methylation is

heritable, the DNA methylation pattern is frequently lost in segregating families and thus leads to epigenetic variation in the population (VAUGHN *et al.* 2007). Genic methylation is solely maintained by DNA methyltransferases (METI and CMT3) and thus sporadic failure of these DNA methyltransferase may account for the observed instability. In contrast, methylation of TEs is actively maintained and restored by siRNA, *de novo* methyltransferases and histone modification. Therefore, DNA methylation patterns on TEs are much more stable than genic methylation (VAUGHN *et al.* 2007).

To understand the inheritance of DNA methylation and its epigenetic roles in gene silencing, we have used the well-characterized maize flavonoid biosynthetic pathway. In maize, *unstable factor for orange1* (*ufo1*) plays a significant role in maintaining transcriptional silencing of the *pericarp color1* (*p1*) gene (CHOPRA *et al.* 2003). The *p1* gene encodes an R2R3 *Myb*-like transcription factor that activates transcription of the structural genes of phlobaphenes biosynthesis, leading to the accumulation of brick red phlobaphene pigment (GROTEWOLD *et al.* 1994). Remarkably, there are more than 100 natural *p1* alleles and epialleles, which are easily distinguished by their distinct pigmentation in pericarp and cob glumes (BRINK and STYLES 1966). For example, *P1-wr* specifies *w*hite pericarp and *r*ed cob glumes, whereas *P1-rr* conditions *r*ed pericarp and *r*ed cob glumes. Interestingly, *P1-rr* and *P1-wr* have 99.9% DNA sequence similarity in their coding and promoter regions. However, these two alleles differ in gene structure; *P1-rr* contains only one copy, whereas *P1-wr* carries more than six copies of a gene unit that are arranged in a head-to-tail tandem repeat complex (CHOPRA *et al.* 1998). Studies on *p1* expression in pericarp tissues show that *P1-wr* transcript is reduced to 30% of that in *P1-rr* pericarp (CHOPRA *et al.* 1996). Additional studies have revealed that the multicopy *P1-wr* is hypermethylated as compared to *P1-rr* (CHOPRA *et al.* 1998). Moreover, further transgenic experiments confirmed that the differential gene expression of *P1-wr* and *P1-rr* in pericarp tissue was not attributed to the differences in promoter or coding region that are present in these two alleles (COCCIOLONE *et al.* 2001). These evidences suggested that the phenotypic difference between *P1-rr* and *P1-wr* is attributed to epigenetic

mechanisms. Other *p1* epialleles including *P1-pr*, *P1-rr'* and *P1-wr** have also been correlated with epigenetic regulation through DNA methylation and chromatin condensation (RHEE *et al.* 2010; SEKHON *et al.* 2012).

A mutant allele of *ufo1, Ufo1-1* releases silencing of the *P1-wr* multicopy complex by hypomethylation at the *P1-wr* distal enhancer region and thus induces pericarp pigmentation of *P1-wr; Ufo1-1* plants. However, this *Ufo1-1*-induced gain of pericarp pigmentation has poor expressivity as the pericarp pigmentation levels vary among *P1-wr; Ufo1-1* plants. In addition, the F_1 progeny (*P1-wr;Ufo1-1/ufo1)* plants also show incomplete penetrance (CHOPRA *et al.* 2003). Recent work demonstrates that extended exposure to *Ufo1-1* induces progressive loss of DNA methylation in *P1-wr* sequences over several generations of backcrossing with the recurrent parent, *P1-wr* (SEKHON and CHOPRA 2009). This progressive hypomethylation is correlated with an increase in penetrance and improved expressivity of *Ufo1-1*-induced phenotypes. Surprisingly, results from Chapter 2 in this study show that *Ufo1-1*-induced phenotypic effects are lost in advanced generations of both self-pollinated and backcross populations. Therefore, DNA methylation level at *P1-wr* in different genotypes of *P1-wr; Ufo1-1* plants exhibiting *Ufo1-1*-induced phenotype (RR- hereafter called as expressers) and silenced expression (WR- hereafter called as non-expressers) warrants further investigation. Herein, we report quantitative real time PCR-based assay (qRT-PCR) to investigate the relative methylation levels of *P1-wr; Ufo1-1* plants. Briefly, methylation dependent restriction enzyme, *Msp*I, was used to treat the genomic DNA followed by qRT-PCR quantification using primers flanking the methylation sensitive sites on the distal enhancer of *p1* (see Supplemental Figure S3-1).

Materials and Methods

Genetic stocks

The original *Ufo1-1* stock in the *P1-wr* background was obtained from Dr. Derek Styles, University of Victoria (Victoria, BC, Canada). The *Ufo1-1* mutation was introgressed into an inbred line 4Co63 (genotype *p1-ww c1 r-r*, National Seed Storage Laboratory, Fort Collins, CO) as previously described (CHOPRA *et al.* 2003). Both the original *Ufo1-1* (*P1-wr/P1-wr; Ufo1-1/Ufo1-1*) stock and the introgressed *Ufo1-1* mutation (*p1-ww/p1-ww; Ufo1-1/Ufo1-1*) were used in this study. The B73 and W23 inbred lines carrying a *P1-wr* allele were obtained from Maize Genetics Cooperation Stock Center (Urbana, IL). The *P1-rr* allele used in this study was derived from the *P1-rr-4B2* genetic stock (GROTEWOLD *et al.* 1991). The paramutagenic *P1-rr'* allele was derived from a transgene-induced silent event that has been previously reported (SIDORENKO and PETERSON 2001). The *P1-rr'* (*P1-rr'/ P1-rr'*) stock used in this study was progeny of homozygous *P1-rr'* plant that exhibits extreme silencing in which the pericarp is colorless and cob glumes is light pink. *P1-pr*^{Tp}, a spontaneous epiallele of *P1-rr,* has been previously described (SIDORENKO and PETERSON 2001). All of the populations discussed here were planted at the Russell E. Larson Agricultural Research farm in Rock Springs, PA from May 2011 to October 2012 except for leaf materials of *P1-rr', P1-pr*^{Tp}, *P1-rr* and *P1-wr* [B73] used for validation of qRT-PCR assay. These materials were grown in greenhouse in ASI building, University Park, PA.

Genetic crosses

The self-pollinated population are derived from the crosses between the B73 (*P1-wr/P1-wr; ufo1/ufo1*) and the original *Ufo1-1*. For developing the self-pollinated population, F_1 plants (*P1-wr/P1-*

wr; Ufo1-1/ufo1) with strong *Ufo1-1*-induced pigmentation were selected for self-pollination to obtain the F_2 generation. The F_2 plants grown from these selected ears were subjected to subsequent cycles of self-pollination and selection to develop later generations (see Figure 2-3 in Chapter 2 for crossing scheme). Two set of experiments were done on these self-pollinated populations. The first set was carried out during summer 2011 involving six self-pollinated generations. F_2, F_4 and F_5 progeny plants from this first set were used for Southern blot and qRT-PCR based assay for DNA methylation studies. The second set was planted in summer 2012; progeny plants from advanced generations (F_4 and F_6) were analyzed by qRT-PCR-based assay only for DNA methylation.

The B73 backcross population from the crosses between B73 (*P1-wr/P1-wr; ufo1/ufo1*) and original *Ufo1-1* using B73 as a recurrent parent. For developing this B73 backcross population, F_1 plants (*P1-wr/P1-wr; Ufo1-1/ufo1*) with strong *Ufo1-1*-induced phenotypes were backcrossed to B73 to generate BC_1F_1 ears. Strong *Ufo1-1* expressers (uniform dark orange pericarp pigmentation) in BC_1F_1 ears were grown and were subjected to subsequent cycles of backcrossing and selection to develop later generations (see Figure 2-2A in Chapter 2 for crossing scheme). Progeny plants from BC_4F_1 were used in the DNA methylation study using qRT-PCR.

The W23 backcross population from the crosses between W23 inbred lines (*P1-wr/P1-wr; ufo1/ufo1*) and the introgressed *Ufo1-1* mutation. The W23 backcross population was developed by backcrossing the F_1 plants (*P1-wr/P1-ww; Ufo1-1/ufo1*) with W23 as a recurrent parent and the BC_1F_1 ears demonstrating intense *Ufo1-1*-induced pericarp pigmentation were chosen for next backcross cycles (see Figure 2-2B in Chapter 2 for crossing scheme). Progeny plants from BC_8F_1 generation were analyzed in this study.

DNA extraction and quantitative Real -Time PCR (qRT-PCR)

Leaf samples were harvested at v6 (vegetative growth with 6th leaf fully expanded) stage, while pericarp samples were collected 18 days after pollination (DAP). Both tissue samples were flash frozen in liquid nitrogen and kept in a freezer at – 80°C until ready to use. The samples were manually ground into a fine powder using a mortar and pestle in liquid nitrogen. Genomic DNA was isolated using a modified CTAB method (SAGHAI-MAROOF *et al.* 1984). DNA was precipitated with 7.5M ammonium acetate and the pellet was re-suspended in 50 µl of ddH_2O. Genomic DNA was quantified using Qubit 2.0 Fluorometer. 300 ng of genomic DNA was digested with MspJI at 37°C for 16 hours (New England Biolabs, Ipswich, *MA*). Mock digestion was performed by substituting the restriction enzyme with 60% glycerol. Quantitative real-time PCR (qRT-PCR) was carried out using an Applied Biosystems 7500 (ABI7500) Fast Real-Time PCR System (Foster City, CA). qRT-PCR reactions were performed using 20 ng of DNA and the FastStart Universal SYBR Green RT-PCR Master Mix kit as the detector (Roche Applied Science, Indianapolis, IN). The qRT-PCR reaction mix was 2 µl of DNA, 3 µl of 333 nM forward and reverse primer mix, and 5 µl of SYBR Green in a total volume of 10 µl. The default program for the qRT-PCR were as follows; pre-incubation at 95°C for 10 minutes followed by 40 cycles of 95°C (15 sec) and 60°C (1 min). After each cycle, a dissociation curve was run consisting of 95°C (15 sec), 60°C (1 min), and 95°C (15 sec). The primers used are specific to *p1* distal enhancer (PW_RTF15-1_Fw, PW_RTF15-1_Rev, PW_RTF15-2_Fw, PW_RTF15-2_Rev) and are listed in Supplemental Table S3-1. Positions of these primers on the *P1-wr* gene structure are illustrated in Figure 3-5A. Validation of qRT-PCR assay using different alleles of *p1* was performed using PW_RTF15-2 Forward and Reverse primer pairs. All other qRT-PCR assays were performed using the other set of primer, PW_RTF15-1. The difference between digested C(t) and mock C(t) was calculated for each sample. The relative fold change of DNA demethylation level was calculated using $2^{-\Delta\Delta Ct}$ method (LIVAK and SCHMITTGEN 2001).

***Ufo1-1* genotyping using SSR and CAPS marker**

PCR reactions were carried out using GoTaq Green Master Mix (Promega, Madison, WI) in a total reaction volume of 20 µl. Annealing temperature is dependent on the melting temperature (T_m) of specific SSR or CAPS primer used. The primers used for genotyping are listed in Supplemental Table S3-2. The PCR conditions for SSR31 were as follows: 94°C for 4 minutes, 35 cycles of 94°C (45 seconds), annealing temperature of 59°C (45 seconds), 72°C (45 seconds) and final extension at 72°C for 7 minutes. The PCR condition for CAPS 5 are: 94°C for 4 minutes, 35 cycles of PCR amplification and each cycle consisted of 94°C (30 seconds), annealing temperature of 54°C (30 seconds), 72°C (30 seconds), and final extension at 72°C for 7 minutes. The PCR products of CAPS5 were first digested with *Eco*RI for 2 hours at 37°C before separating on a 2% agarose gel at a constant voltage of 70 Volts. The PCR products of SSR31 were directly loaded on to a 4% SFR agarose gel and run with constant voltage of 80 Volts. After electrophoresis, genotype of each sample was determined by distinguishing homozygous (*Ufo1-1/Ufo1-1*), heterozygous (*Ufo1-1/ufo1*) and wild type (*ufo1*/*ufo1*) bands. SSR31 shows polymorphism in B73 population, whereas CAPS5 is polymorphic in both B73 and W23 populations. The SSR31 PCR product is approximately 121 bp. The CAPS5 PCR product is 315-bp in size and contains one restriction enzyme cutting site yielding to 100-bp and 215-bp bands in *Ufo1-1* allele.

DNA gel blot analysis

Leaf genomic DNA was isolated using a modified CTAB method (SAGHAI-MAROOF *et al.* 1984). DNA was precipitated with 7.5M ammonium acetate and the pellet was re-suspended in 50 µl of ddH_2O. Genomic DNA was digested overnight with *Hpa*II (Promega, Madison, WI). The digested DNA was separated on 0.8% agarose gel, and then transferred onto nylon membrane. The membrane was then

hybridized with ^{32}P-α-dCTP labeled DNA probe as previously described (SEKHON *et al.* 2007). *p1* gene fragment 15 was used as a radioactively labeled probe in this study (LECHELT *et al.* 1989).

Bisulfite sequencing analysis

Bisulfite treatment was performed on genomic DNA of two independent samples of each genotype using EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). The bisulfite-treated genomic DNA was then amplified using gene-specific primers as listed in Supplemental Table S3-3. A nested PCR amplification which requires external and internal primer pairs was done to ensure high quantity of PCR products. The PCR products of external primers serve as templates for the internal primers. The final PCR products were then sub-cloned into pSC-A-amp/kan vector using StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA). Transformed *E-coli* cultures were grown overnight in liquid LB at 38°C. The plasmid DNA was then isolated using StrataPrep Plasmid Miniprep Kit (Agilent Technologies, Santa Clara, CA). The isolated plasmid DNA was concentrated before it was sent to Penn State Nucleic Acid Facility for sequencing. All DNA sequencings were performed using ABI Hitachi 3730XL DNA Analyzer. The DNA sequence was first aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) before the DNA methylation patterns were exploited using CyMATE program (HETZL *et al.* 2007).

Results

Methylation-specific qRT-PCR based assay offers a rapid, high-throughput DNA methylation analysis

DNA methylation is common and is the most well studied epigenetic signaling tool that plays a role in gene silencing. It was previously demonstrated that the degree of enhanced pericarp

pigmentation in *P1-wr;Ufo1-1* plants was strongly correlated with the extent of *P1-wr* demethylation (CHOPRA *et al.* 2003). Moreover, a previous study showed that *Ufo1-1*-induced gain of pericarp pigmentation is associated with the reduced methylation (CG and CNG) at the distal enhancer (DE) region of the *P1-wr* (SEKHON and CHOPRA 2009). Therefore, we expected that the silencing of *p1* in *Ufo-1-1* non-expressers (NE) plants of advanced generations is correlated with the hypermethylation at the DE region. To investigate the association between DNA methylation and pericarp pigmentation, we analyzed the relative DNA methylation levels in *Ufo1-1* expressers (E) and non-expressers (NE) plants using methylation-specific qRT-PCR based assay. To ensure the validity of this method, we first performed this assay on *p1* alleles with known methylation status; *P1-rr*, *P1-rr'*, *P1-pr*^{Tp} and *P1-wr* [B73]. *P1-wr* has previously been reported to be hypermethylated relative to *P1-rr* (CHOPRA *et al.* 1998). In addition, both silenced *p1* epialleles, *P1-rr'* and *P1-pr*^{Tp} which are derived from a common *P1-rr* ancestor have been shown to be methylated at the DE region (CHOPRA *et al.* 2003; SEKHON *et al.* 2012; SIDORENKO and PETERSON 2001). As expected, our result showed that the multicopy *P1-wr* allele has higher methylation level in comparison to *P1-rr*. This was indicated by smaller fold change of DNA demethylation in *P1-wr* as compared to *P1-rr* (Figure 3-1A). Heavily methylated genomic DNA would have low amount of qRT-PCR templates as the restriction enzyme, *Msp*I, would have cleaved the methylated sites. Hence, the hypermethylated DNA would have high C(t) value and thus would have large Δ C(t) (difference between the digested C(t) and mock C(t)). Since relative demethylation is calculated by $2^{-\Delta\Delta Ct}$ method, higher Δ C(t) would result in lower fold change of DNA demethylation. Based on our result, in pericarp, the DE region of *P1-rr'*, *P1-pr*^{Tp} and *P1-wr* are more methylated than *P1-rr*. This result is in agreement with the observed pericarp phenotypes as shown in Figure 3-1A. We further performed the qRT-PCR assay on the leaf genomic DNA of *P1-rr*, *P1-rr'*, *P1-pr*^{Tp} and *P1-wr* [B73] to test if DNA methylation would differ in the leaf as compared to the pericarp. Similarly to pericarp, *P1-rr* leaf DNA is hypomethylated as compared to *P1-rr'*, *P1-pr*^{Tp} and *P1-wr* (Figure 3-1B). However, the

methylation levels in leaf samples are more variable as indicated by large standard errors. Taken together, these results suggest that methylation-specific qRT-PCR assay is able to determine the relative methylation levels at *p1* alleles.

Ufo1-1-induced hypomethylation at _P1-wr_ does not correlate with pericarp pigmentation in advanced generations

Ufo1-1 induces pericarp pigmentation in *P1-wr* plants which results in an RR phenotype. To examine if *Ufo1-1*-induced pericarp pigmentation in *P1-wr* plants would show demethylation of the DE, qRT-PCR was performed on BC_8F_1 plants. Pericarp genomic DNA of two plants from each class, *P1-wr*; *Ufo1-1* expressers (E) and non-expressers (NE), were subjected to this assay. As predicted, *P1-wr*; *Ufo1-1* E plants are hypomethylated (about two-fold increase in DNA demethylation) as compared to the *P1-wr* plants (Figure 3-1C). Interestingly, NE plants also show slight reduction in DNA methylation level with approximately 1 fold increase in relative demethylation in comparison to *P1-wr.* We further tested the DNA methylation level in different genotypes of *P1-wr; Ufo1-1* plants regardless of their phenotypes. qRT-PCR assay was performed on leaf genomic DNA of BC_4F_1 *P1-wr; Ufo1-1* and *P1-wr; Ufo1-1/ufo1*. As anticipated, our result showed that *P1-wr* plants carrying heterozygous *Ufo1-1* are hypomethylated (approximately a five-fold change of DNA demethylation) as compared to *P1*-w*r* (Figure 3-1D). In addition, *P1-wr* plants carrying wild type *ufo1* also exhibited slight reduction of methylation; around 1 fold increase in DNA demethylation. The relative demethylation in *P1-wr; ufo1* leaf tissues (0.83) is close to relative demethylation in pericarp tissues of NE plants (0.95). Based on these results, we concluded that the use of leaf genomic DNA would be sufficient in the qRT-PCR analysis to represent the DNA methylation level associated with the gain of pericarp pigmentation. This is beneficial for further analysis of DNA methylation using high-throughput techniques due to the impracticality of collecting a large amount of pericarp samples from numerous plants.

Since silencing of *p1* was observed in majority of the progeny in advanced generations of the self-pollinated population, qRT-PCR analysis was used to analyze the DNA methylation status of F_6 plants. Five categories of plants were studied; *P1-wr; Ufo1-1* (E), *P1-wr; Ufo1-1* (NE), *P1-wr; Ufo1-1/ufo1* (E), *P1-wr;Ufo1-1/ufo1* (NE) and *P1-wr;ufo1* (NE). The methylation levels of these categories were compared with *P1-wr* [B73]. Surprisingly, all five categories showed a high-fold increase in DNA demethylation regardless of the pericarp phenotypes (Figure 3-2). E plants carrying homozygous *Ufo1-1* exhibited the highest fold increase (eight folds) of DNA demethylation relative to *P1-wr*. *P1-wr; Ufo1-1* (NE) plants showed three-fold increase in demethylation at DE region as compared to *P1-wr*. *P1-wr; Ufo1-1/ufo1* (E) plants showed approximately a six-fold increase in demethylation in comparison to *P1-wr*. NE plants carrying heterozygous *Ufo1-1* exhibited about seven-fold more demethylation than *P1-wr.* Negative segregants, *P1-wr; ufo1* plants with no pericarp pigmentation, also exhibited increase in DNA demethylation (about four folds) relative to *P1-wr*. Although all of the classes show large standard errors indicating vast variation among samples, the E plants have the highest variability in DNA demethylation. This observation is consistent with the result of qRT-PCR analysis done on the backcross plants; higher variability of demethylation was observed in the leaf tissues of the E plants as compared to the pericarp tissues of the E plants (see Figure 3-1C & D). Importantly, the reduction of methylation in NE plants may imply that the gain of pericarp pigmentation in the advanced generations is not directly associated with loss of DNA methylation at the DE region.

An observation similar to the previously published work (SEKHON and CHOPRA 2009) was made when we compared DNA methylation levels between F_4 and F_6 generations. Prolonged exposure to the *Ufo1-1* mutation induces progressive hypomethylation at the DE region of *P1-wr* over generations. Our result showed that the demethylation levels in F_6 plants are higher than that in the F_4 plants (Figure 3-3). Overall, in F_4, both E and NE plants are hypomethylated as compared to the naïve *P1-wr* (which has never been exposed to the *Ufo1-1* mutation). The demethylation level in F_4 E plants (carrying either

Ufo1-1/Ufo1-1 or *Ufo1-1/ufo1* mutation) are about four-fold higher than that observed in the wild type (*P1-wr; ufo1*) plants. Segregating *P1-wr; ufo1/ufo1* NE plants showed 2.5-fold increase of demethylation relative to the wild type *P1-wr*; *ufo1.* In summary, our qRT-PCR analysis showed that the relative demethylation of F_4 is always lower than that of F_6 in any of the segregating category. Our DNA gel blot analysis for E and NE plants derived from F_2, F_4 and F_5 generations validates this observation of progressive loss of methylation over several generations (Figure 3-4A). Additional small molecular weight bands (~3.4 kb, ~2.8 kb, ~2.2 kb, ~0.6 kb and ~0.5 kb) were observed in E plants (Figure 3-4A; lanes labeled as 179, 315 & 350), but were not seen in NE (see lanes labeled as 200, 304 & 347) and *P1-wr* [B73] plants. These results indicate possible hypomethylation of additional *Hpa*II sites in the E as compared to the NE plants. Moreover, the intensity of *Hpa*II bands in the E plants increases from F_2 through F_5 suggesting that advanced generations are more hypomethylated. However, further analysis on the DNA methylation level of these samples using qRT-PCR assay yielded inconsistent results (Figure 3-4B). Although all the NE plants are hypermethylated and consistent with the gel blot results, only the F_2 E plant (lane 179) showed loss of methylation (about five-fold change of demethylation) as compared to the *P1-wr* plants. Two of the expressers (lane 315 and 350) are hypermethylated, which is in contrast to the observation made from the DNA gel blot. This discrepancy between the gel blot and qRT-PCR results can be attributed to differential hypomethylation among distinct *P1-wr* gene copies or in individual cells. This scenario has been observed in DNA methylation analysis obtained by bisulfite sequencing where differential methylation patterns were seen among different clones (SEKHON and CHOPRA 2009). In addition, the qRT-PCR assay analyzes a small portion of the DE region; ~ 60-bp of 499-bp of the distal enhancer. This sixty base region covers only three cytosines in CG and CHG context (See Supplemental Figure S3-2). Therefore, our qRT-PCR assay might not be able to identify methylation status of cytosines outside the PCR amplified region. To overcome this issue, additional qRT-PCR primers need to be designed around the entire DE region for complete DNA methylation analysis.

Another possible way to verify the qRT-PCR result is to perform bisulfite sequencing spanning the entire distal enhancer region. Previous work in our laboratory has shown that there was significant reduction of methylation (CG and CNG contexts) in BC_4F_1 leaf tissues (SEKHON and CHOPRA 2009). However, only two E BC_4F_1 plants were included in this previous study. Thus, it will be interesting to see whether NE plants would show similar reductions of cytosine methylation within the entire DE. We have performed bisulfite sequencing on cob tissues of *P1-wr* [B73] and BC_8F_1 *P1-wr; Ufo1-1* E and NE plants (Figure 3-5). Our findings show that there are no significant DNA methylation changes at the DE region between E and NE plants in cob tissue. Moreover, no specific site was differentially affected between E and NE plants as shown in supplemental Figure S3-3. The absence of a significant change in DNA methylation could be because the cob glumes of E and NE plants have substantial *p1* expression. However, bisulfite sequencing investigating methylation level of the DE using pericarp tissues is in progress and will be compared with DNA methylation levels in the cob glumes. In addition, we will perform qRT-PCR methylation assays on pericarp and cob glumes DNA to compare and validate the results. If the results obtained from bisulfite sequencing can be validated with simple and quick qRT-PCR analysis, then in the future it will be efficient to use this method for studying DNA methylation.

Collectively, our results suggested that extended exposure to *Ufo1-1* mutation increases the relative demethylation in *P1-wr* plants and suggests that DNA methylation pattern can accumulate and be passed on to the next generations. Furthermore, our findings show that the loss of methylation at the DE region does not exactly correlate with the *Ufo1-1*-induced pericarp pigmentation. Further analysis of *p1* transcript level in both E and NE plants may provide valuable information to explain this finding. Additional studies on other epigenetic silencing mechanisms such as chromatin remodeling and histone modifications need to be explored to clarify the role of *ufo1* in the maintenance of gene silencing.

Discussion

Ever since cytosine methylation was discovered to play major role in gene silencing, various techniques have been designed to analyze genomic DNA methylation. There are two main reasons for developing methods for DNA methylation studies; one is to assess global or overall levels of methylcytosine, and second is to detect the methylation state of specific DNA sequences. Global DNA methylation can be quantified by high performance separation techniques such as HPLC (EICK *et al.* 1983) and HPCE (FRAGA *et al.* 2000) or by enzymatic means including a methyl-acceptor assay (WU *et al.* 1993). Moreover, overall methylcytosine levels in genomes can also be detected using a fluorescent assay that requires chloroacetaldehyde modification [see review (OAKELEY 1999)]. Recent developments in microarray and high-throughput sequencing technologies have led to the discovery of advanced methods to study genome-wide DNA methylation at very high-resolution. Such sophisticated methods involving whole-genome tilling microarrays or methylC-seq were reported in numerous studies of DNA methylation profiling in *Arabidopsis* (SHEN *et al.* 2012; VAUGHN *et al.* 2007; ZHANG *et al.* 2006).

Experimental approaches to study site-specific DNA methylation can be divided into two types; non-bisulfite and bisulfite treatment methods. Non-bisulfite methods are based on enzymatic activity of methylation-sensitive restriction endonucleases (MS-REs) and qualitatively measured using Southern blotting or PCR. Bisulfite treatment methods involve deamination of unmethylated cytosine to uracil which changes the DNA sequence. Hence, this conversion enables distinguishing cytosine from methylcytosine at a single nucleotide level. This bisulfite-modified DNA requires PCR amplification before the methylation status can be detected by either direct sequencing, methylation-specific PCR (MSP), MS-REs (COBRA), nucleotide extension assays (MS-SnuPE) or MethyLight [see review (FRAGA and ESTELLER 2002)].

Previously, Southern blotting and clone-based bisulfite genomic sequencing analyses were successfully performed to investigate the cytosine methylation level of *P1-wr; Ufo1-1* plants (SEKHON and CHOPRA 2009). These studies using early generations showed a positive correlation between loss of DNA methylation and gain of pigmentation. However, Southern blot analysis requires large amounts of high quality DNA and is very labor intensive. Therefore, using this method to analyze DNA methylation of large number of samples is highly impractical. Although bisulfite sequencing yields valuable single-base resolution of methylation status, performing this technique in a high-throughput manner remains technically challenging. Hence, we established methylation- specific quantitative real-time PCR (qRT-PCR) based assay to study DNA methylation level at *p1* distal enhancer region. Our findings show that loss of DNA methylation is not directly correlated with the gain of pericarp pigmentation in the advanced generations. Both E and NE plants show high level of demethylation as compared to the naïve *P1-wr*. Besides, significant reduction of methylation was also observed in the NE negative segregants carrying wild type *ufo1*/*ufo1*. Thus, we propose that *Ufo1-1* may transiently release transcriptional silencing of *P1-wr* and the associated hypomethylation may be a secondary effect of the *Ufo1-1* interaction with the *P1-wr* allele. Silencing of the hypomethylated NE in later generations may imply loss-of-function of *Ufo1-1* mutation and the imposed DNA demethylation is merely a reminiscent of its previous interaction with the *P1-wr*. Further study on DNA methylation levels in the progeny of these NE segregants would be essential to prove this assumption. Taken together, our results may suggest that although *Ufo1-1*-induced hypomethylation of *P1-wr* is stably transmitted to the next generation, DNA methylation may not play the key roles in transcriptional silencing of *P1-wr* gene.

Lack of correlation between DNA methylation and pericarp pigmentation in advanced generations suggests that *ufo1*-mediated pathway may function in epigenetic mechanisms that do not involve DNA methylation as the cause. One possibility is that *ufo1* may be involved in chromatin remodeling. *ufo1* may encode a novel protein and/or regulate factor (s) in chromatin remodeling

complexes. Generally, chromatin remodeling complexes act as transcriptional co-activators by guiding transcription factors to the nucleosome. In plants, there are four families of chromatin remodeling complexes; (1) The SWI/SNF (Switching/Sucrose Non-Fermenting) family, (2) The ISWI (Imitation Switching) family, (3) The CHD (Chromodomain-Helicase DNA binding protein) family and (4) The INO80 (Inositol requiring 80) family [see review (CLAPIER and CAIRNS 2009)]. In *Arabidopsi*s, *MOM1* (Morpheus' molecule1) encodes a SWI2/SNF2-like protein that is essential for the maintenance of transcriptional silencing. The *mom1* mutation reactivates silenced loci that are hypermethylated and thus suggests that the chromatin remodeling and DNA methylation may act independently (AMEDEO *et al.* 2000). Furthermore, the enzymatic activity of *Arabidopsis* Deficient in DNA Methylation 1 (DDM1) protein (which acts as a chromatin remodeling factor) has been shown to be not affected by DNA methylation (BRZESKI and JERZMANOWSKI 2003). Additionally, a number of genes have been identified to encode essential proteins in chromatin remodeling complexes. For example, *SPLAYED* (*SYD*) gene encodes a SNF2/BRM chromatin remodeling subfamily member while *PICKLE* (*PKL*) encodes a protein of CHD subfamily. Mutation of these genes cause severe distortion in developmental phenotypes in *Arabidopsis* [see review (VERBSKY and RICHARDS 2001)]. In another study, *Arabidopsis FASCIATA1* (*FAS1*) and *FAS2* genes have been identified to encode protein subunits of chromatin assembly factor-1 (CAF1) which is involved in chromatin assembly following DNA replication. The *fas* mutants showed pleiotropic developmental abnormalities that are associated with loss of epigenetic regulation on *WUSCHEL* (*WUS*) and *SCARECROW* (*SCR*) expression in apical meristems (KAYA *et al.* 2001). Expresser *P1-wr* plants carrying *Ufo1-1* mutation also exhibited pleiotropic defects (in this study and (CHOPRA *et al.* 2003)) and thus may strengthen the hypothesis that *ufo1* may be involved in chromatin remodeling complexes.

Numerous cases indicating that DNA methylation alone is not capable of regulating gene silencing have been reported not only in maize (HOEKENGA *et al.* 2000), but also in *Arabidopsis* (AMEDEO *et al.* 2000; SCHEID *et al.* 1998) and rice (DING *et al.* 2007; OKANO *et al.* 2008). For example, maize *mop1*

(*mediator of paramutation1*) mutant causes gradual loss of methylation at *mudrA* (the transposase), but *mudrA* expression remains silenced until several generations of exposure to *mop1-1* (WOODHOUSE *et al.* 2006). This may imply that there is another epigenetic mechanism regulating the transcriptional silencing besides DNA methylation. Additionally, phenotypic differences between the maize *Pl-Blotched* (variegated pigmentation pattern) and its presumptive progenitor, *Pl-Rhoades* (uniformly dark purple) has been primarily attributed to their chromatin structure rather than cytosine methylation (HOEKENGA *et al.* 2000). In rice, hypermethylation of the promoter region of transgene and endogenous genes does not induce transcriptional suppression, but enrichment of Histone 3 lysine 9 di-methylation (H3K9me2) in the promoter was observed (OKANO *et al.* 2008). Similar observation was made in *Arabidopsi*s where loss-of-function effect of *SUVH2* (which encodes histone methyltransferase) reduces all heterochromatin-specific histone marks and thus leads to reactivation of hypermethylated Athila transposons (NAUMANN *et al.* 2005). Interplay between DNA methylation and chromatin modification has been disclosed in numerous studies. For instance, dependency of DNA methylation on histone modification has been reported in *SDG714* (*SET Domain Group Protein 714*) mutant in which loss of H3K9me2 mark at the *Tos17* locus reduces CG and CHG cytosine methylation in rice (DING *et al.* 2007).

Recent work in our laboratory have shown that gain of pericarp pigmentation of silenced *P1-wr**, an epiallele of *P1-wr*, is not associated with hypomethylation at the DE region (WANG 2012). Similarly, *Ufo1-1*-induced reactivation of other silenced *p1* alleles, *P1-rr'* and *P1-pr*^{TP}, has been shown to be not correlated to changes in DNA methylation alone (SEKHON *et al.* 2012). In these cases, the gain of pericarp pigmentation has been attributed to the loss of a suppressive histone mark, H3K9me2. Therefore, there is a possibility that the reactivation of *P1-wr*; *Ufo1-1* plants is also regulated by histone methylation, H3K9me2, rather than DNA methylation. Since the *Ufo1-1*-induced pericarp pigmentation is not stably inherited and reverted back to silenced state following segregation of *Ufo1-1*, *Ufo1-1* may temporarily disrupt the chromatin structure (relaxation of *P1-wr* gene structure from heterochromatin

to euchromatin) or transiently alter nucleosome occupancy of *P1-wr* multicopy gene. To test this hypothesis, the chromatin state of *P1-wr* in both E and NE plants need to be examined using chromatin immunoprecipitation assays and quantitative real-time PCR (ChIP-qPCR).

In conclusion, although previous studies showed that *ufo1*-mediated mechanism is often associated with DNA methylation, this study offers a new insight on other potential role of *ufo1* in maintenance of gene silencing. However, more analyses need to be done to investigate this possibility.

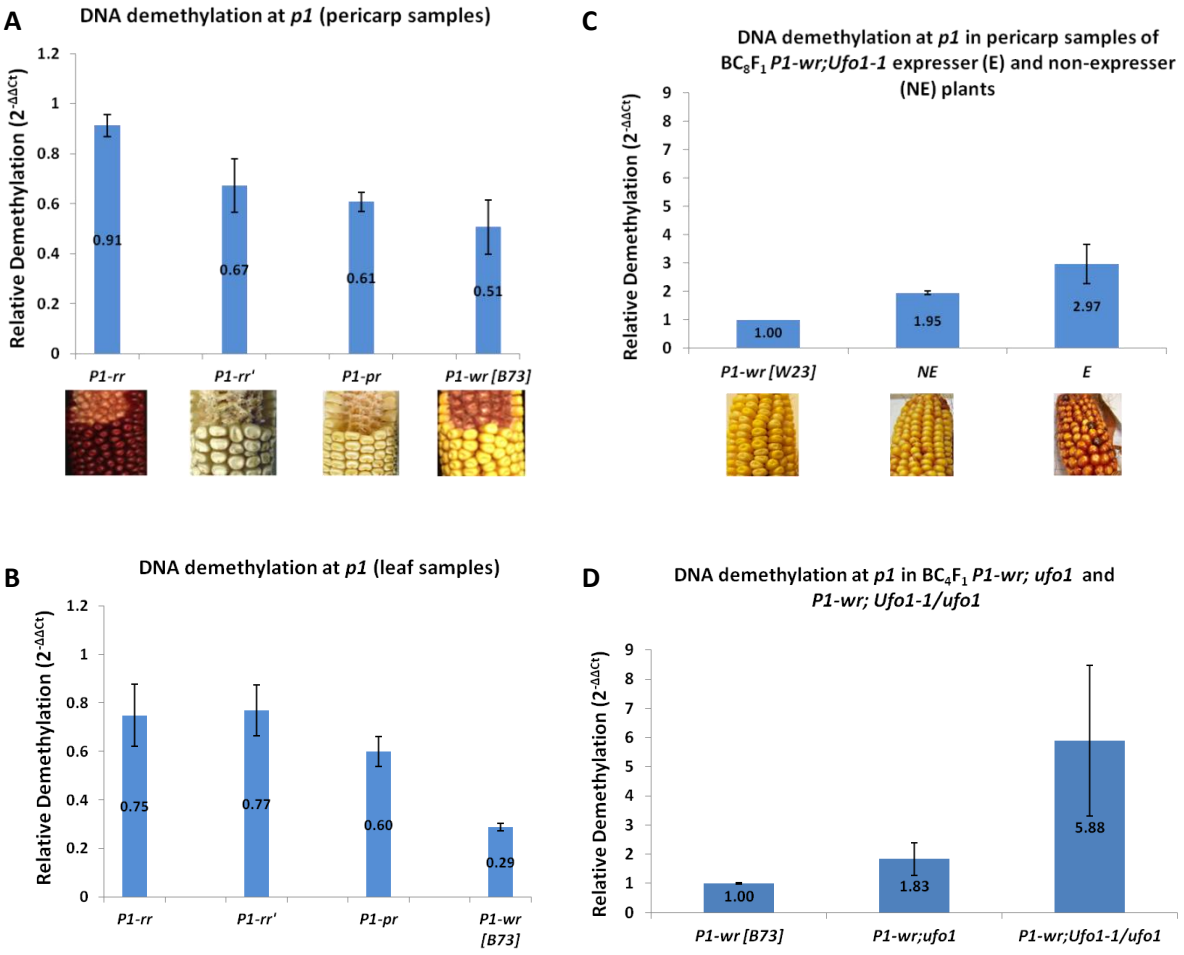

Figure 3-1: Comparison of the DNA demethylation level at *p1* distal enhancer using a qRT-PCR assay. (A) & (B) Validation of qRT-PCR assays were performed on *P1-rr*, *P1-rr', P1-pr* and *P1-wr* [B73] pericarp tissues (18 DAP) and leaf samples, respectively. Pericarp and leaf genomic DNA from at least three plants in each category was analyzed and average values along with standard errors are shown. PW_RTF15-2 Forward and Reverse primer pairs were used in these assays. DNA demethylation level was normalized to *P1-rr* DNA demethylation level. Relative demethylations were measured by calculation of $2^{-\Delta\Delta Ct}$. (C) Relative demethylation levels in pericarp DNA of BC_8F_1 *P1-wr; Ufo1-1* Expresser (E) and Non-Expresser (NE) were analyzed using qRT-PCR with a second set of primers (PW_RTF15-1 primer set was used). DNA demethylation level was normalized to *P1-wr* DNA demethylation level. Relative demethylations were measured by calculation of $2^{-\Delta\Delta Ct}$. (D) qRT-PCR assays were performed on leaf samples of BC_4F_1 plants carrying heterozygous (*Ufo1-1/ufo1*) and wild type (*ufo1/ufo1*). Plants were genotyped using CAPS5 marker followed by qRT-PCR assays (PW_RTF15-1 primer set was utilized). DNA demethylation level was normalized to *P1-wr* DNA demethylation level. Relative demethylations were measured by calculation of $2^{-\Delta\Delta Ct}$. Leaf genomic DNA from at least three plants in each category was analyzed and average values along with standard errors are shown. The ear pictures below the graph represent the corresponding pericarp phenotypes used in the qRT-PCR assays. See Figure 3-5A for position of primers used here.

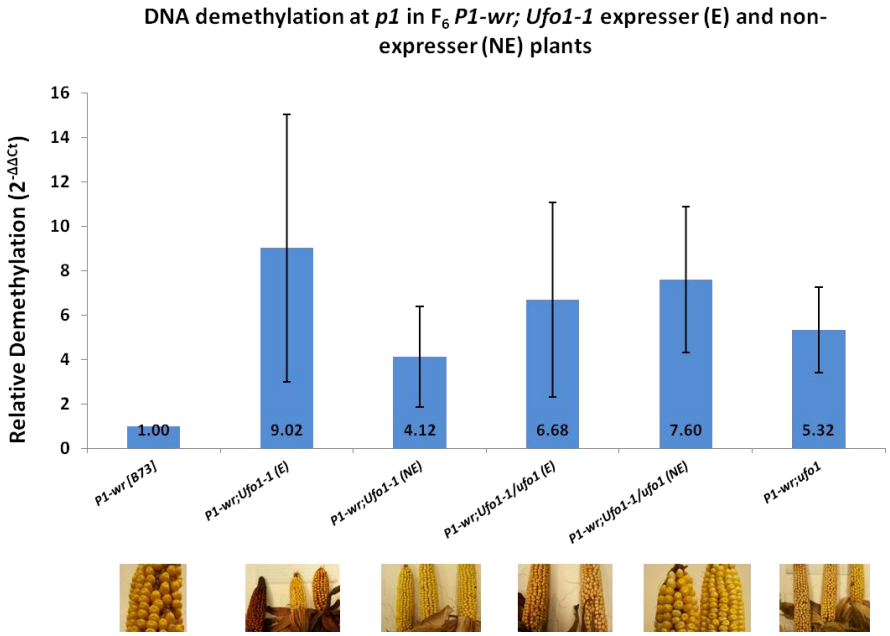

Figure 3-2: Relative DNA demethylation levels in F_6 Expresser (E) and Non-Expresser (NE) plants carrying homozygous (*Ufo1-1/Ufo1-1*), heterozygous (*Ufo1-1/ufo1*) and wild type (*ufo1/ufo1*). PW_RTF15-1 primer set was utilized in this assay. DNA demethylation level was normalized to *P1-wr* DNA demethylation level. Relative demethylations were measured by calculation of $2^{-\Delta\Delta Ct}$. Leaf genomic DNA from at least three plants in each category was analyzed and average values along with standard errors are shown.

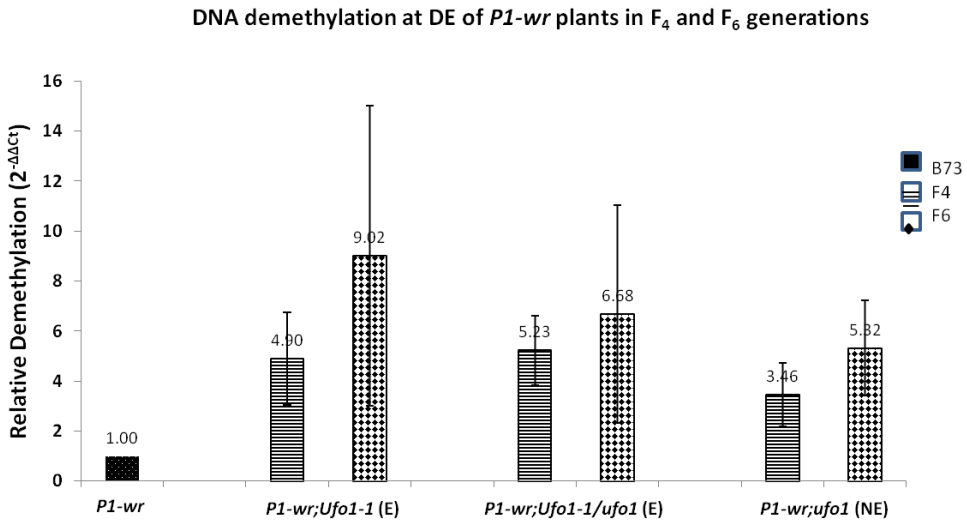

Figure 3-3: Relative demethylation levels in F_4 & F_6 *P1-wr; Ufo1-1* Expressers (E), *P1-wr; Ufo1-1/ufo1* Expressers (E) and *P1-wr; ufo1* Non-Expressers (NE). PW_RTF15-1 primer set was utilized in this assay. DNA demethylation level was normalized to *P1-wr* DNA demethylation level. Relative demethylations were measured by calculation of $2^{-\Delta\Delta Ct}$. Leaf genomic DNA from at least three plants in each category was analyzed and the averages are presented here.

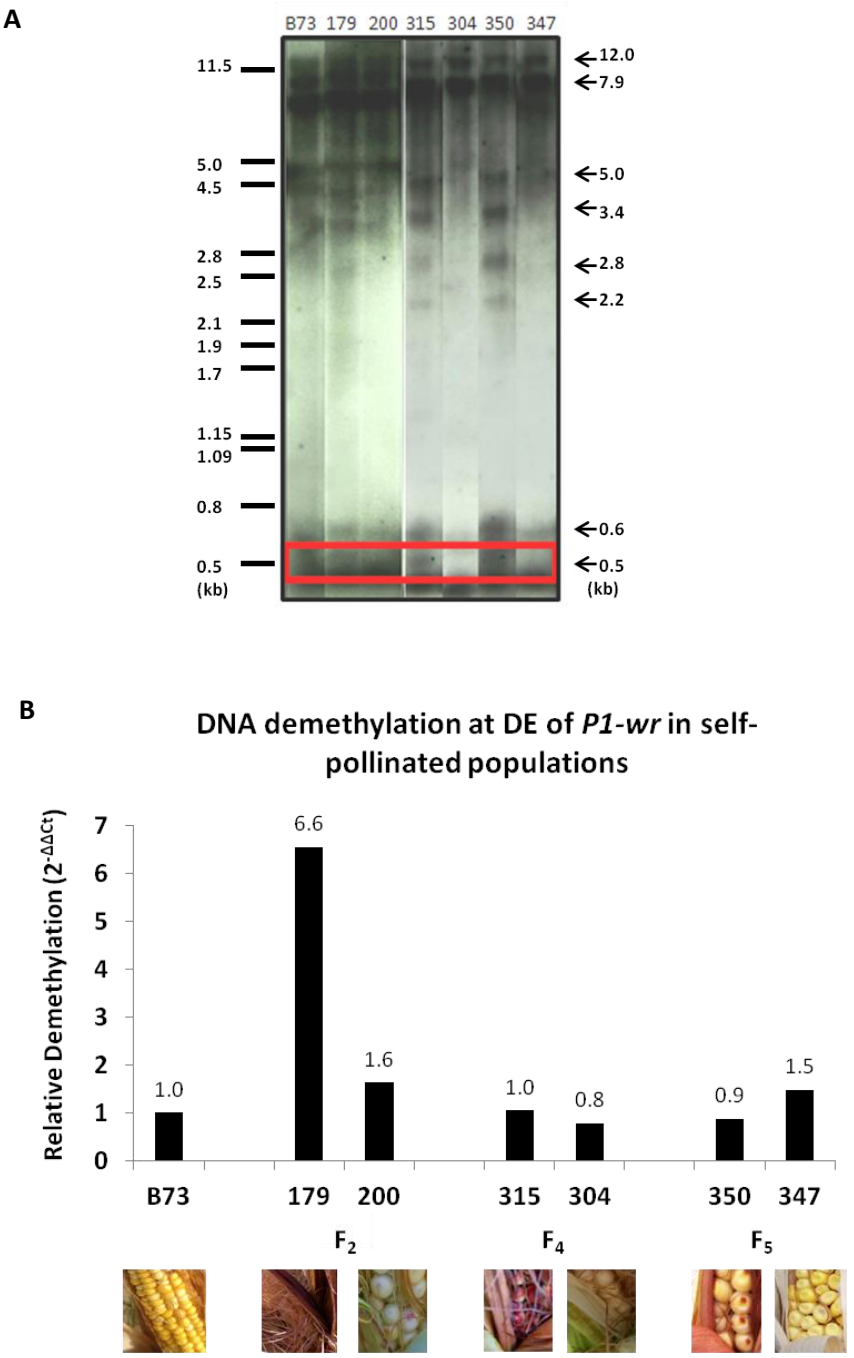

Figure 3-4: Validation of methylation-specific qRT-PCR assay using Southern blot approach. (A) Southern blot analysis was performed on F_2 (179 & 200), F_4 (315 & 304) and F_5 (350 & 347) *P1-wr/P1-wr; Ufo1-1/ufo1* plants. Leaf genomic DNA of six individuals (two from each generation) was digested with methylation-sensitive restriction enzyme, *Hpa*II. Gel blots were hybridized with *p1* distal enhancer F15 probe (see Figure 3-5A for the position of the probe on the *P1-wr* gene structure). (B) Leaf genomic DNA from the same 6 plants was digested with *MspJ*I and used in qRT-PCR assays. PW_RTF15-1 primer set was used in this assay. DNA demethylation level was normalized to *P1-wr* DNA demethylation level. Relative demethylations were measured by calculation of $2^{-\Delta\Delta Ct}$. The pictures below the bar graph represent the ear phenotypes of the plants.

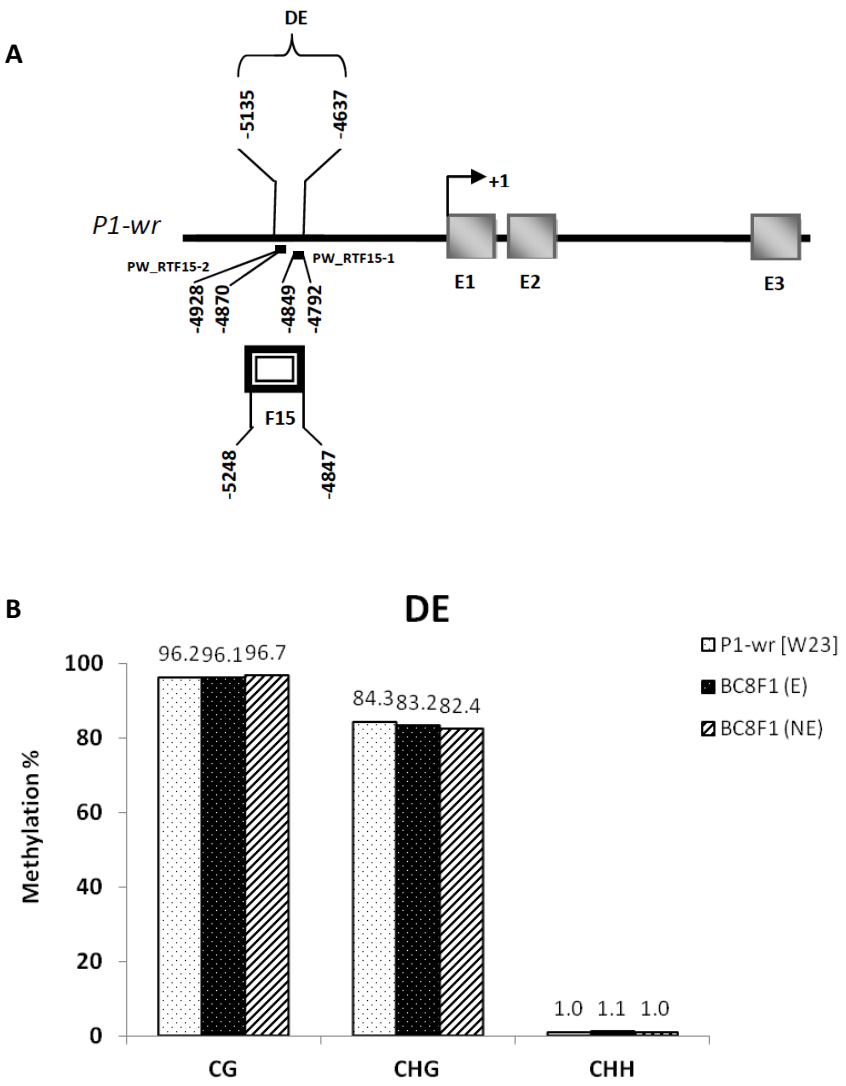

Figure 3-5: DNA methylation analysis using genomic bisulfite sequencing of distal enhancer (DE). Bisulfite sequencing assays were performed on cob tissues of BC_8F_1 *P1-wr; Ufo1-1* Expressers (E) & Non-Expressers (NE). (A) Line diagram of the *P1-wr* gene structure. The exons are shown as gray boxes. Bent arrow indicates the transcription start site. The 499-bp region marked as distal enhancer (DE) above the gene structure was analyzed by bisulfite sequencing. Empty box represents F15 probe for Southern blotting analysis. Coordinates for qRT-PCR (PW_RTF15-1 and PW_RTF15-2) primers are illustrated on the diagram. (B) Overall DNA methylation in CG, CHG and CHH (H is A, T, or G) contexts was analyzed and compared between *P1-wr* [W23], *P1-wr; Ufo1-1* Expressers (E) & Non-Expressers (NE). Overall methylation in each context was calculated by dividing the number of methylated cytosines with total cytosines in that context in all the clones. At least two plants were analyzed from each category and the averages are reported here.

Supplemental Table / Figure

Table S3-1: Primer sequences used for qRT-PCR methylation assays

Name	Sequence
PW_RTF15-1_Fw	TCGACGGTCATATGCATGGAT
PW_RTF15-1_Rev	ACCGAGAAGCCGCTGCTA
PW_RTF15-2_Fw	GACGTCTCACCGGCTCACA
PW_RTF15-2_Rev	ATGCAACGCAACGCTTTG

Table S3-2: Primer sequences used for *Ufo1-1* genotyping

Name	**Forward Primer**	**Reverse Primer**
SSR31	TCACAATCAAACAGGGCTGA	CTGTCTCAACCTCTGCACCA
CAPS5	GCTAATGGGTACGTGGTCGT	ACAGCGGAACAACCGTAATC

Table S3-3: Primer sequences used for bisulfite sequencing of the distal enhancer region

RBS8F	GGTTTGTTGTTTGTTTTTATTTTGTTT	External
RBS1R	CCAACCACAACAATATAAAACTCTATA	External
RBS9F	GATTTAAGAGATTTTAAGATATGTGTGT	Internal
RBS3R	ACAATAATACTATTAAACAATAAACATACA	Internal

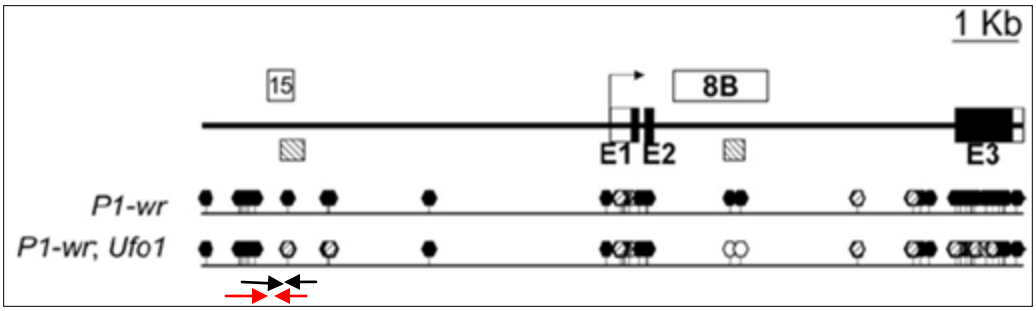

Figure S3-1: Line diagram of the *P1-wr* gene sequence and methylation map of *Hpa*II sites of *P1-wr* and *P1-wr; Ufo1*. Transcription start site is indicated by a bent arrow. Black boxes on the solid line represent three exons (E1, E2 and E3). Probe fragments F15 and F8B are indicated at the top of the gene structure. Stripped boxes represent region selected for bisulfite sequencing analysis. Open, striped, and solid hexagons represent unmethylated, partially methylated and completely methylated *Hpa*II sites, respectively. Figure is adapted from Sekhon and Chopra, 2009. Black arrows represent PW_RTF15-1 primer pairs, whereas red arrows represent PW_RTF15-2 primer pairs used in the qRT-PCR analysis.

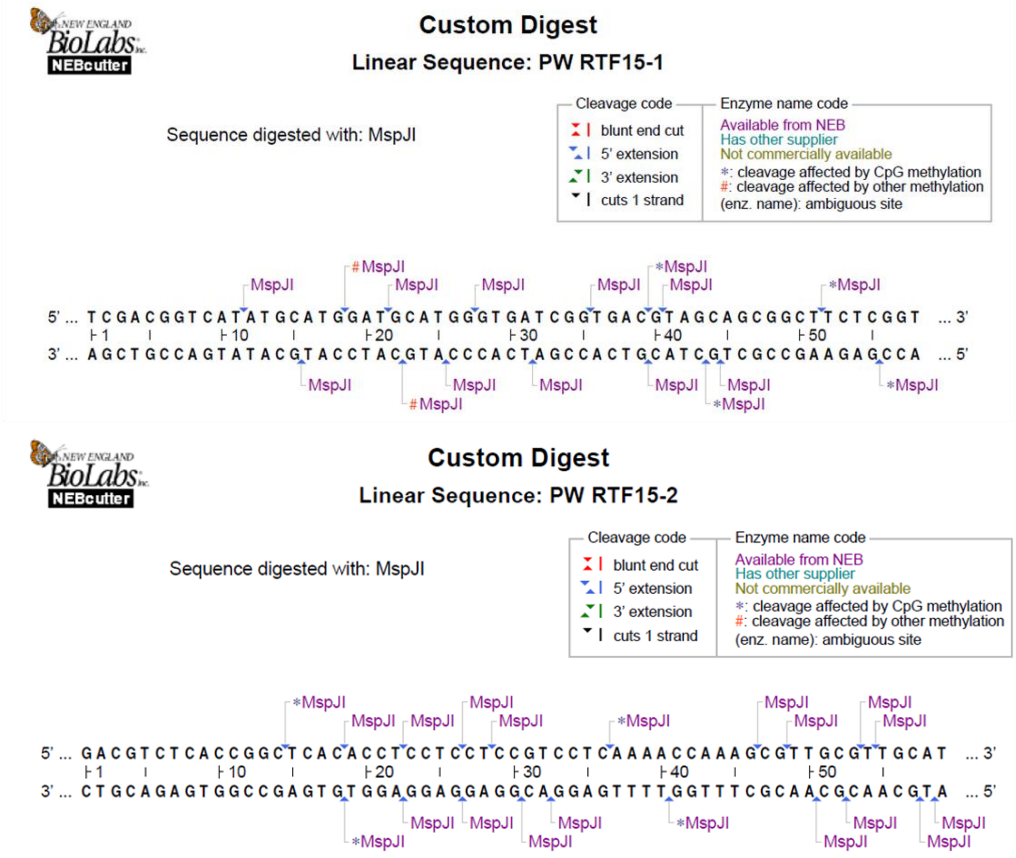

Figure S3-2: *MspJ*I sites on PW_RTF15-1 and PW_RTF15-2 PCR products as produced by using NEBcutter web interface

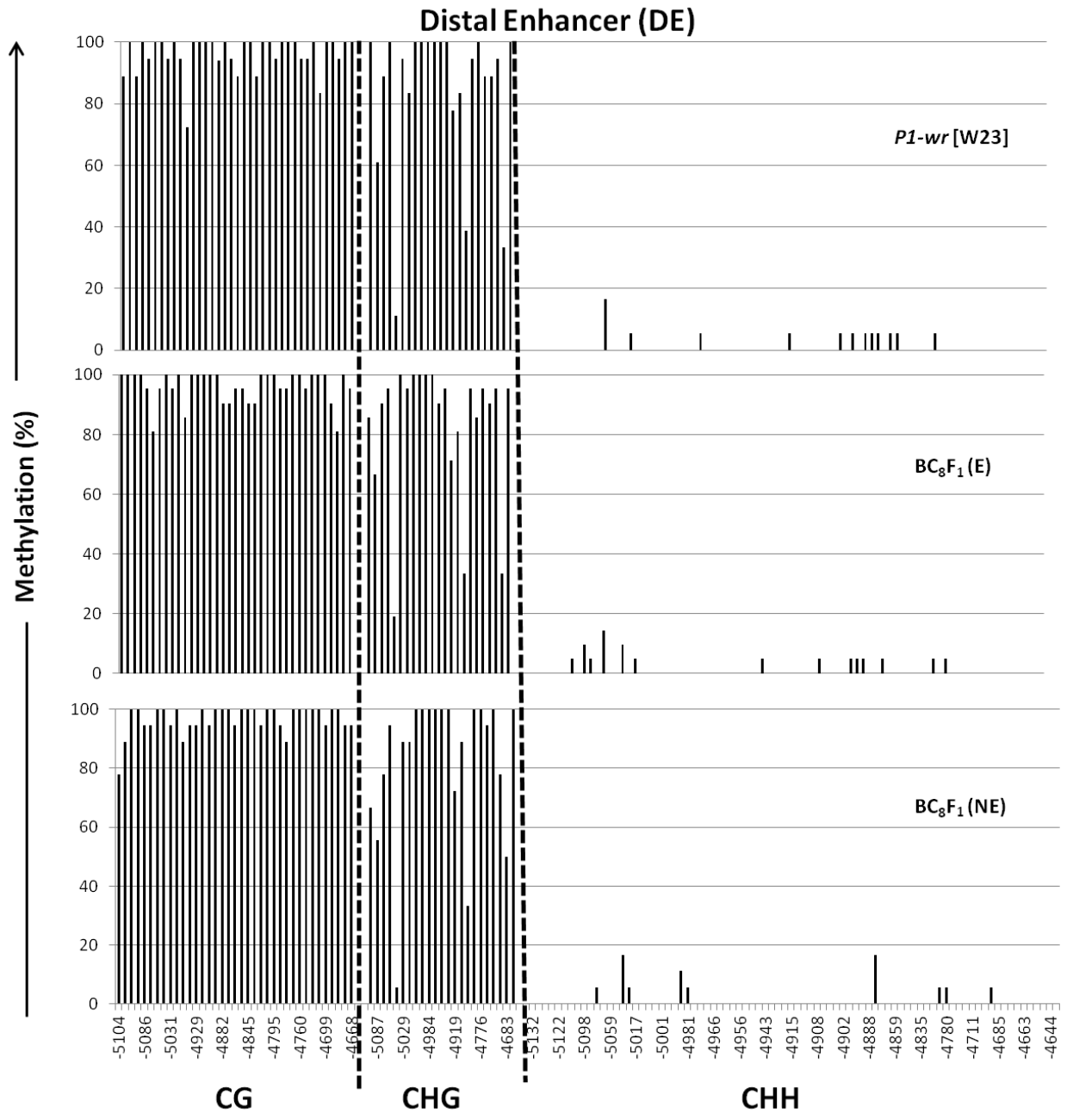

Figure S3-3: Site-specific methylation profile of 499-bp distal enhancer (DE) region obtained by bisulfite sequencing assay on cob tissues of *P1-wr* [B73] and BC_8F_1 *P1-wr; Ufo1-1* Expressers (E) & Non-Expressers (NE). DNA methylation at CG, CHG, and CHH contexts are shown on the x-axis. Percent methylation is shown on the y-axis.

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Chapter 4

Summary and perspectives

Phenotypic diversity is often linked to genetic differences or allelic variants where such variants are caused by changes in the underlying DNA sequences. These alterations could be small-scale changes at the base-pair level such as point mutations, insertions, and deletions or large-scale chromosomal rearrangements like transposon insertions, chromosomal deletions, inversions, and translocations. The cause and effect of allelic diversity has been the cornerstone of modern genetics research especially in the study of genetic diseases. However, in recent years, an increasing amount of phenotypic variation has been reported to be unrelated to genetic variation. Such phenotypic variabilities have been attributed to epigenetics changes where variations in observable traits are caused by mechanisms other than alteration in the primary nucleotide sequences. DNA methylation, histone modifications, histone variants, and nucleosome positioning are important epigenetic marks involved in transcriptional gene silencing. The dynamic of these epigenetic states is manifested in differential expression of genes that ultimately leads to the variation in phenotypes. Variability in pigmentation has been a focal point of epigenetic studies in maize owing to its easily visible phenotypes. For example, epigenetic phenomena such as imprinting and paramutation were studied at different loci that are involved in biosynthetic pathway of anthocyanins and phlobaphenes pigments. Such loci include *r1* (*red1*) (BRINK 1956), *b1* (*Booster1*) (COE 1959), *pl1* (*purple plant1*)(HOLLICK *et al.* 1995) and *p1* (*pericarp color1*)(SIDORENKO and PETERSON 2001).

The maize *p1* gene encodes an R2R3 Myb transcription factor that regulates structural genes in the flavonoid pathway of phlobaphenes biosynthesis (GROTEWOLD *et al.* 1991; GROTEWOLD *et al.* 1994). It

has more than 100 natural alleles and epialleles, many of which are easily distinguished based on their pigmentation patterns (BRINK and STYLES 1966; COCCIOLONE *et al.* 2001) . The *p1* alleles are commonly identified by a two letter suffix based on their expression in pericarp and cob glumes. For example, *P1-rr* confers *r*ed pericarp and *r*ed cob, whereas *P1-wr* conditions *w*hite pericarp and *r*ed cob. Distinct expression patterns expressed by *p1* gene have been attributed to stable inheritance of epigenetic marks on its diverse alleles (CHOPRA *et al.* 1998; CHOPRA *et al.* 2003; SEKHON and CHOPRA 2009; SEKHON *et al.* 2012). This study was aimed to investigate the inheritance of tissue-specific expression of *p1-wr* allele and also to understand the maintenance of epigenetic marks imposed on this allele. To understand the mechanism behind this tissue-specific expression of *P1-wr*, we utilized a spontaneous trans-acting dominant mutant, *Ufo1-1* (*Unstable factor for orange1*), that disrupts the *p1* expression pattern. The wild type *ufo1* is responsible for maintenance of transcriptional silencing of the *p1* gene and a mutant allele of *ufo1* perturbs the expression pattern of *p1* alleles and results in a change of phenotypes. *P1-wr* plants exhibit a white pericarp and red cob phenotype but in the presence of *Ufo1-1*, *p1* expression is hyperactivated and thus phlobaphenes are ectopically accumulated not only in both pericarp and cob glumes but also throughout the plant body. Previous work showed that this increased pigmentation was associated with loss of DNA methylation at the promoter regions of *p1* (CHOPRA et al. 2003). However, *Ufo1-1* has incomplete penetrance where only a subset of the F_1 progeny from the cross between *P1-wr/P1-wr; ufo1/ufo1 x P1-ww/P1-ww; Ufo1-1/Ufo1-1* shows gain of pericarp pigmentation. In addition to incomplete penetrance, *Ufo1-1* also exhibits poor expressivity where the extent of pigmentation is highly variable (SEKHON and CHOPRA 2009). Given the fact that *Ufo1-1* exhibits incomplete penetrance and low expressivity we investigated if *Ufo1-1* and its phenotypic effects were stably inherited over generations. We followed the inheritance of *Ufo1-1* allele by genotyping using *Ufo1-1* tightly linked markers and we also studied the *Ufo1-1* and *P1-wr* interaction for multiple generations in different genetic backgrounds. The genotypic and phenotypic inheritance studies on the

Ufo1-1 mutation and the wild type *ufo1* offer insight into how tissue-specific expression of *p1* is maintained across generations.

The results of the genotypic study show that the wild type *ufo1* and the *Ufo1-1* mutation are faithfully inherited following Mendelian genetics in both the backcross and self-pollinated populations and were not influenced by genetic backgrounds. As expected, the wild type *ufo1* maintained the *P1-wr* expression pattern across generations. However, *Ufo1-1*-induced phenotypes deviate from Mendelian expectations in the advanced generations of both the backcross and self-pollinated populations. In the later generations, the presence of *Ufo1-1* mutation is no longer capable of reactivating the expression of *p1* gene, and thus the phenotype of *P1-wr* plants reverted back to normal; white pericarp and red cob. The non-concordance between the results of genotypic and phenotypic inheritance in the advanced generations suggests that the *Ufo1-1* perturbation on *p1* tissue-specific expression is transient. The effects of *Ufo1-1* on *P1-wr* plants are strongly observed in the early generations and gradually disappeared in the later generations. It may have been the case of genetic redundancy, where multiple epigenetic mechanisms controlling silencing at *p1* locus could be present in the genome. In addition, the *Ufo1-1*-induced pericarp pigmentation is also influenced by genetic backgrounds. The *Ufo1-1* penetrance in B73 background decreases over generations, but increases in W23 background and thus suggests that the function of *Ufo1-1* may be affected by other modifiers in the backgrounds. We then took a step further to investigate the epigenetic mechanisms underlying the re-silencing of *p1* gene in the advanced generations. Using methylation specific qRT-PCR approach, the DNA methylation levels at the distal enhancer (DE) region of *p1* were studied in both expressers and non-expresser plants carrying the *Ufo1-1* mutation. Interestingly, non-expresser plants also displayed a significant fold increase in reduction of DNA methylation at a pericarp-specific regulatory element positioned in the DE region. On the basis of this result and recent work (WANG 2012), we concluded that gain of pericarp pigmentation is not associated with hypomethylation of the DE region of *p1* and may have been caused by other

epigenetic mechanisms such as histone modifications. *Ufo1-1* may have temporarily released transcriptional silencing of *P1-wr* by means other than loss of DNA methylation and the accompanied hypomethylation may be a secondary effect of its interaction with the *P1-wr* allele. This research emphasizes that *ufo1* has a role in transcriptional silencing preferably through epigenetic mechanisms upstream of DNA methylation.

As in with any scientific study, the inheritance analyses should be repeated to verify the results. Larger population sizes would also add statistical power to the study. In addition, the high-throughput phenotypic study could be improved by implementing a new color measurement method used by Goettel and Messing (GOETTEL and MESSING 2012). Furthermore, this study should be repeated in replicates to minimize the variance in data. It would also be informative to study the interaction between other pleiotropic defects caused by *Ufo1-1* and gain of pericarp pigmentation as this could elicit role of *ufo1* at multiple loci. Further validations on the methylation-specific qRT-PCR based assay are necessary to confirm its reliability in determining the DNA methylation level at promoter regions of *P1-wr*. Bisulfite sequencing analysis of the methylation states at the DE regions would be the best approach to verify the qRT-PCR results. Another interesting study would be to investigate the *p1* and *a1* transcript levels in the non-expresser *P1-wr; Ufo1-1* plants of the advanced generations. This could offer more information on the role of *ufo1* in regulation of gene expression as *ufo1* also may have been involved in post-transcriptional silencing. Finally, chromatin immunoprecipitation assays and quantitative real-time PCR (ChIP-qPCR) should be performed on these non-expresser *P1-wr; Ufo1-1* plants to learn about the chromatin state of *P1-wr; Ufo1-1*.

This study emphasizes the importance of *ufo1* in regulating tissue-specific expression patterns of *P1-wr* through the maintenance of gene silencing. However, the understanding of the underlying mechanism on which *ufo1* participates is still incomplete. My research rules out DNA methylation as the

primary mechanism in the *ufo1*-mediated pathway yet additional studies need to be explored to unravel the *ufo1*-mediated epigenetic regulation.

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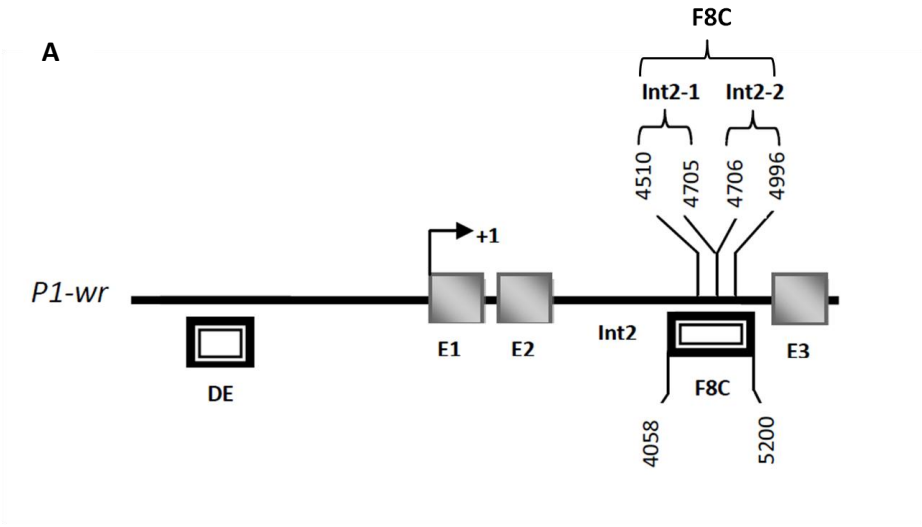
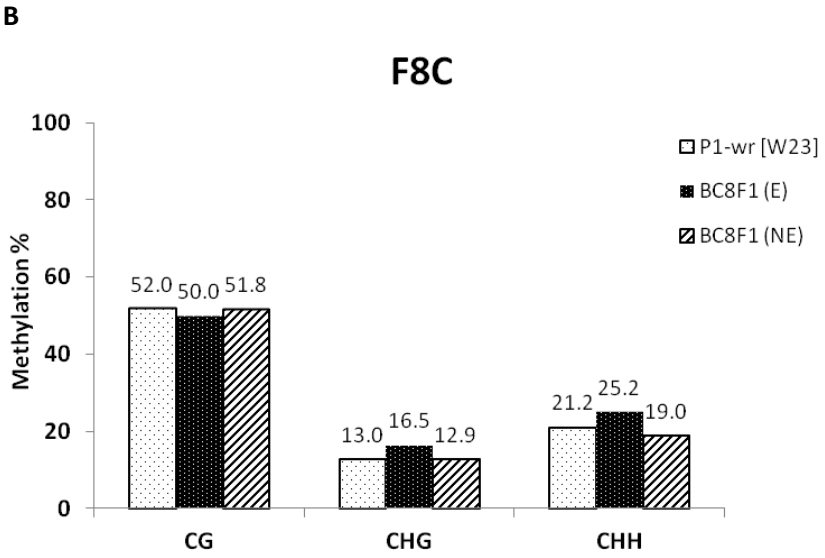
Appendix A

List of abbreviations

SSR	Simple Sequence Repeats
CAPS	Cleaved Amplified Polymorphic Sequences
SFR	Super Fine Resolution
HPLC	High-Performance Liquid Chromatography
HPCE	High-Performance Capillary Electrophoresis
LB	Lysogeny Broth

Appendix B

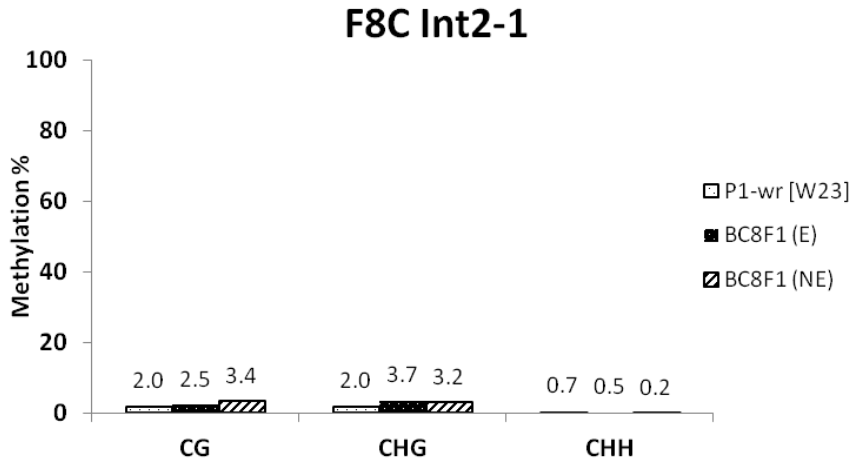




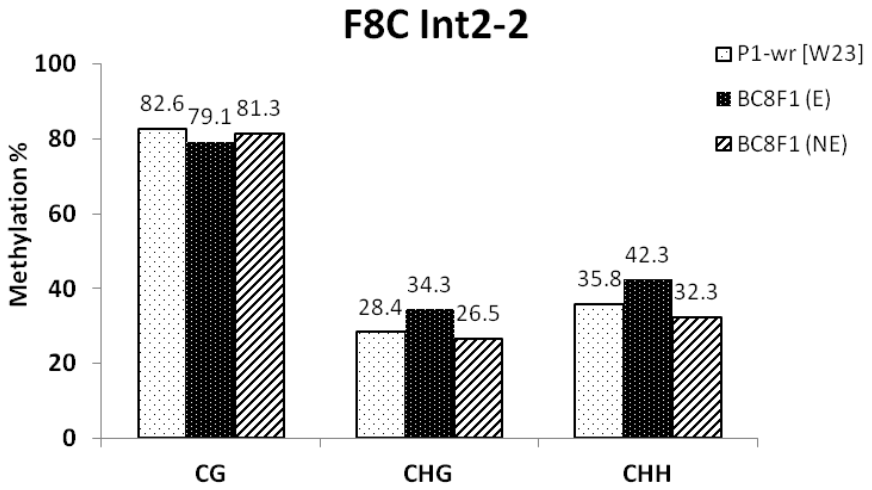
Appendix B: DNA methylation analysis using genomic bisulfite sequencing of intron 2 (F8C) region. Bisulfite sequencing assays were performed on cob tissues of BC_8F_1 *P1-wr; Ufo1-1* Expressers (E) & Non-Expressers (NE). (A) Line diagram of the *P1-wr* gene structure. The three exons (E1, E2 and E3) are shown as gray boxes. Bent arrow indicates the transcription start site. The 487-bp region marked as F8C above the gene structure was analyzed by bisulfite sequencing. Empty boxes represent F15 and F8C probes for Southern blotting analysis. (B) Overall DNA methylation in CG, CHG and CHH (H is A, T, or G) contexts was analyzed and compared between *P1-wr* [W23], *P1-wr; Ufo1-1* Expressers (E) & Non-Expressers (NE). Overall methylation in each context was calculated by dividing the number of methylated cytosines with total cytosines in that context in all the clones. At least two plants were analyzed from each category and the averages are reported here.

Appendix C

A

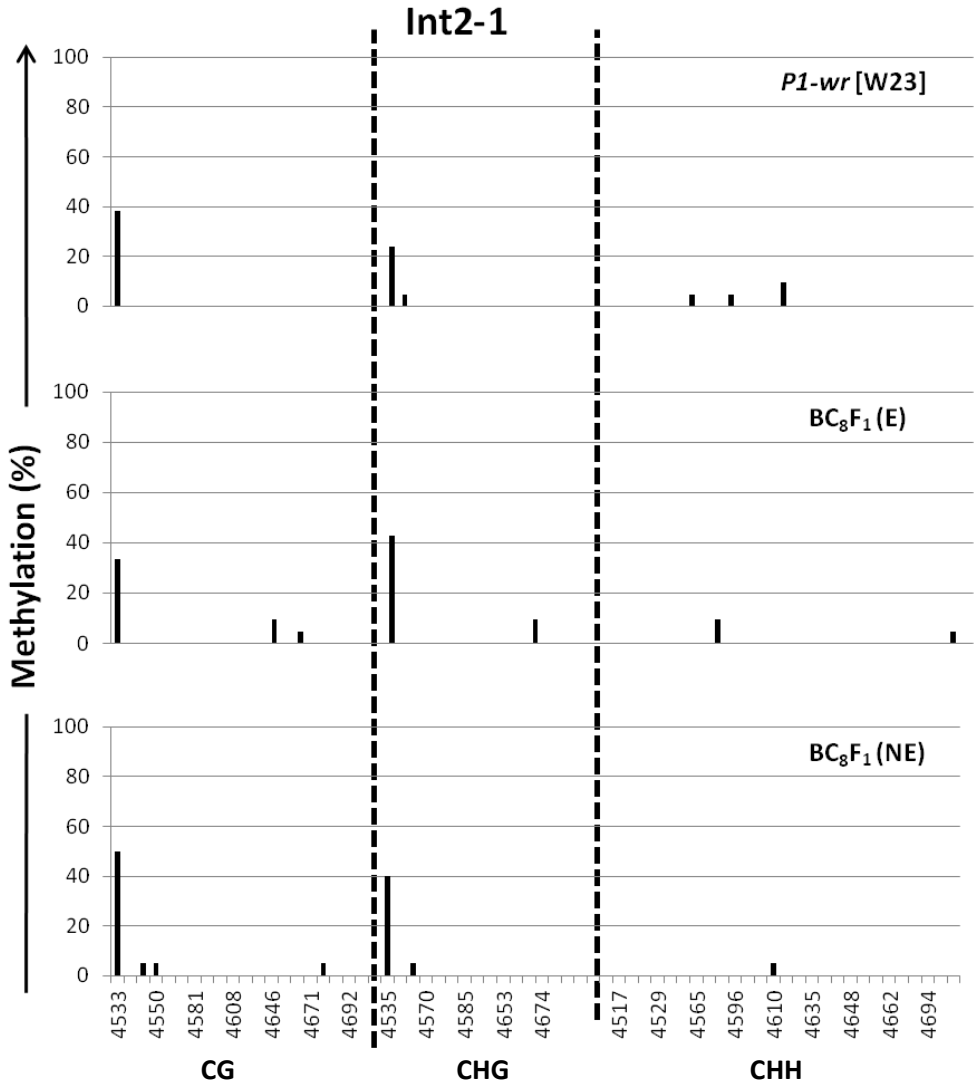


B



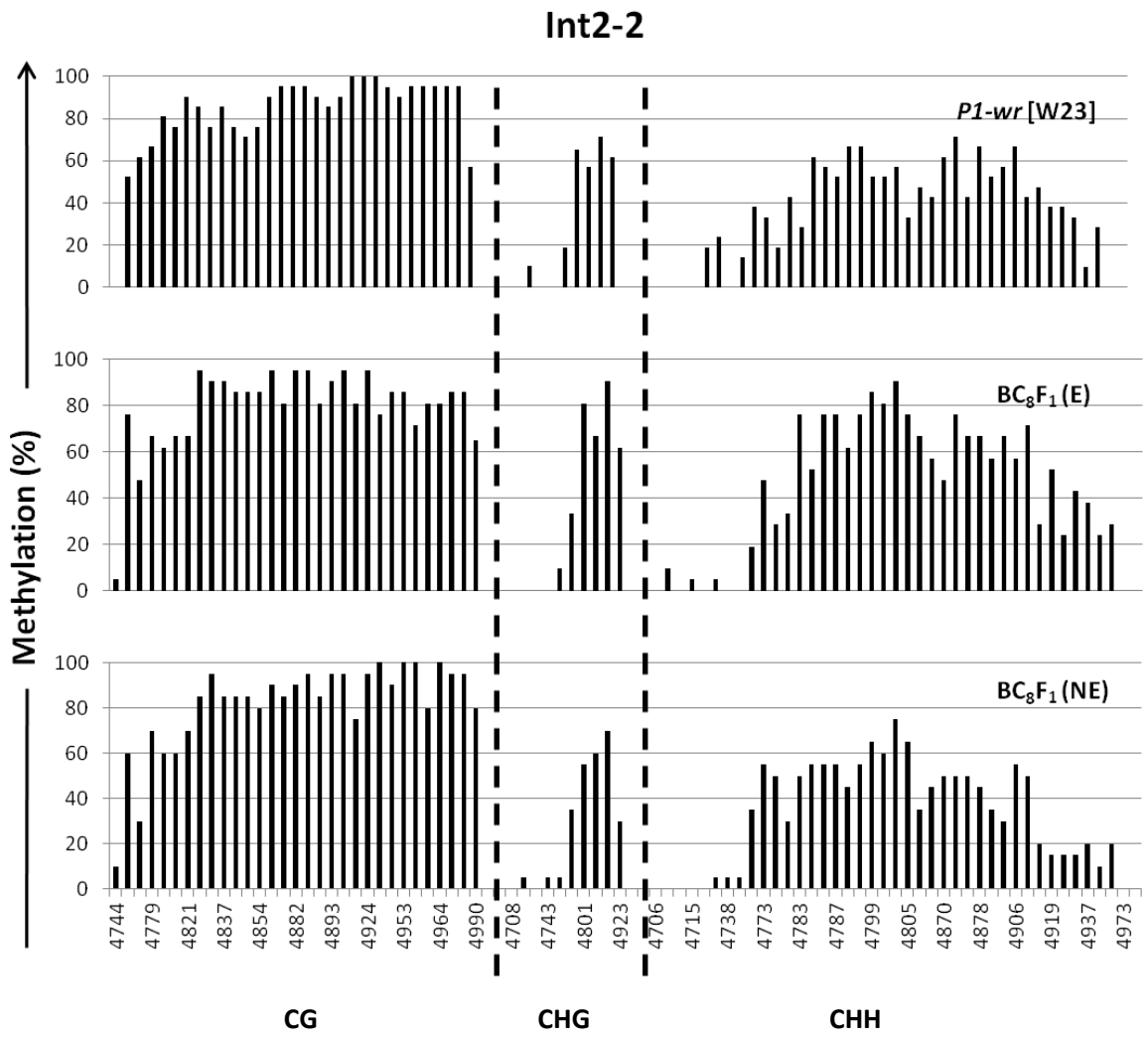
Appendix C: DNA methylation changes at *P1-wr; Ufo1-1* expresser and non-expressers plants. Genomic bisulfite sequencing was performed on two consecutive regions of intron 2 (A, Int2-1 and B, Int2-2), see Appendix A for coordinates of these two regions). Bisulfite sequencing assays were performed on cob tissues of BC_8F_1 *P1-wr; Ufo1-1* Expressers (E) & Non-Expressers (NE). Overall DNA methylation in CG, CHG and CHH (H is A, T, or G) contexts was analyzed and compared between *P1-wr* [W23], *P1-wr; Ufo1-1* Expressers (E) & Non-Expressers (NE). Overall methylation in each context was calculated by dividing the number of methylated cytosines with total cytosines in that context in all the clones. At least two plants were analyzed from each category and the averages are reported here.

Appendix D



Appendix D: Site-specific methylation profile of the 196-bp Int2-1 region obtained by bisulfite sequencing assay on cob tissues of *P1-wr* [B73] and BC_8F_1 *P1-wr; Ufo1-1* Expressers (E) & Non-Expressers (NE). The percentage methylation of individual sites in CG, CHG, and CHH sequence contexts is shown on the y-axis and the coordinates of each site are shown on the x-axis.

Appendix E



Appendix E: Site-specific methylation profile of the 291-bp Int2-2 region obtained by bisulfite sequencing assay on cob tissues of *P1-wr* [B73] and BC_8F_1 *P1-wr; Ufo1-1* Expressers (E) & Non-Expressers (NE). The percentage methylation of individual sites in CG, CHG, and CHH sequence contexts is shown on the y-axis and the coordinates of each site are shown on the x-axis.