Transposon Insertions in the Promoter of the *Zea mays a1* Gene Differentially Affect Transcription by the Myb Factors P and C1

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ABSTRACT

The understanding of control of gene regulation in higher eukaryotes relies heavily on results derived from non-*in vivo* studies, but rarely can the significance of these approximations be established *in vivo*. Here, we investigated the effect of *Mutator* and *Spm* insertions on the expression of the flavonoid biosynthetic gene *a1*, independently regulated by the transcription factors C1 and P. The *a1-mum2* and *a1-m2* alleles carry *Mu1* and *Spm* insertions, respectively, in a *cis*-element (ARE) of unknown function located between the P- and C1-binding sites. We show that the insertions of *Mu1* and *Spm* similarly influence the expression of *a1* controlled by C1 or P. The P-controlled *a1* expression in *a1-m2* is *Spm* dependent, and the mutant phenotype of *a1-mum2* is suppressed in the pericarp in the absence of the autonomous *MuDR* element. Footprints within the ARE affect the regulation of *a1* by C1 and P differently, providing evidence that these factors control *a1* expression using distinct *cis*-acting regulatory elements. Together, our findings contribute significantly to one of the best-described plant regulatory systems, while stressing the need to complement with *in vivo* experiments current approaches used for the study of control of gene expression.

THE maize *a1* gene encodes the enzyme dihydroflavonol reductase (DFR; O'Reilly *et al.* 1985; Schwarz-Sommer *et al.* 1987), which participates in the formation of the red phlobaphene and purple anthocyanin pigments. Loss of *a1* function results in no anthocyanin or phlobaphene pigmentation. While plant and aleurone tissues remain green or colorless, respectively, in the absence of *a1* function, the pericarp accumulates a brown pigment of unknown composition if the other genes in the pathway are expressed (Styles and Ceska 1989).

In maize, the accumulation of the anthocyanin and phlobaphene pigments is regulated independently by the transcription factors C1 and P, respectively (reviewed in Mol *et al.* 1998). *C1* and *P* are members of the large maize *R2R3 Myb* family of regulatory genes (Rabinowicz *et al.* 1999). *C1* is responsible for the anthocyanin pigmentation of the aleurone, while its close relative *Pl* (Cone *et al.* 1993) leads to pigmentation with anthocyanins in other plant organs such as green tissues and the pericarp (Coe and Neuffer 1989). The regulatory activity of C1 is modulated by the physical interaction of the Myb domain of C1 with members of the R/B class of helix-loop-helix factors (Goff *et al.* 1992; Grotewold *et al.* 2000). Thus, C1 activates *a1* expression and anthocyanin accumulation only when expressed together with R or B. In contrast, P activates transcription independently of the R/B proteins (Grotewold *et al.* 1994), and no coactivators necessary for P function have yet been identified. The *a1* promoter has a modular organization (Figure 1) with a proximal *cis*-acting regulatory element bound *in vitro* with moderate high affinity by P (^{ha}PBS) and by a distal element bound by P with low affinity (^{la}PBS). Between the ^{ha}PBS and ^{la}PBS, the *a1* promoter contains the anthocyanin regulatory element (ARE; Figure 1), which is present in the promoters of other anthocyanin biosynthetic genes (Tuerck and Fromm 1994; Lesnick and Chandler 1998). *In vitro,* C1 binds both the ^{ha}PBS and ^{la}PBS with low affinity (Sainz *et al.* 1997). In transient expression experiments using maize suspension cells, either C1 and R (C1/R) or P can direct high levels of expression from promoters containing the ^{ha}PBS (Grotewold *et al.* 1994) or the ^{la}PBS together with the ARE (Tuerck and Fromm 1994). However, mutations in any one of these three *cis*-acting regulatory regions (^{ha}PBS, ARE, or ^{la}PBS) have modest effects on the activation of *a1* by P or by C1/R in transient expression experiments. For example, mutations in the ^{ha}PBS reduce the P-regulated expression of *a1* to 15% of wild-type levels, while the activation by C1/R is reduced to only 35% (Grotewold *et al.* 1994). Similarly, mutations of the ^{la}PBS or the ARE reduce *a1* expression to 20% by P and to 35% by C1/R (Sainz *et al.* 1997), and mutations of the ARE site (in a promoter lacking the ^{ha}PBS) reduce *a1* expression to ~30% by P and to 10% by C1/R (Tuerck and Fromm 1994). Only mutations in both the ^{ha}PBS and ^{la}PBS resulted in >95% inhibition of *a1* activation by P and in >80% inhibition by C1/R (Sainz *et al.* 1997). These findings indicate that the *cis*-acting regulatory elements

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in the *a1* promoter can largely compensate for each other in these assays, suggesting a high level of redundancy in the regulation of *a1*.

The results derived from the transient expression and *in vitro* binding experiments described above are contrasted by the striking effect upon aleurone pigmentation in *a1-m2* and *a1-mum2* alleles of *a1*. *a1-m2* alleles, originally isolated and described by McClintock (1961), carry the autonomous *Spm* transposon or nonautonomous *dSpm* derivatives inserted in the ARE element (Masson *et al.* 1987; Figure 1). *a1-m2* belongs to the *Spm*-dependent class of alleles (Masson *et al.* 1987), in which the expression of the *a1* gene (evidenced by anthocyanin accumulation) happens only in the presence of a *trans*-acting nondefective *Spm*. The *a1-mum2* allele, originally isolated by Robertson (described in Chomet *et al.* 1991), carries the nonautonomous *Mu1* transposable element inserted in the ARE element, two nucleotides 5′ of the site of *Spm* insertion in the *a1-m2* allele (Figure 1). *a1-mum2* belongs to the *Mu*-suppressible mutant class (reviewed in Martienssen 1996), in which the mutant phenotype (*i.e.*, no anthocyanin pigmentation) is suppressed (*i.e.*, results in pigmentation) in the absence of the autonomous *MuDR* element. In the presence of *MuDR*, excision of *Mu1* in the aleurone results in the formation of frequent revertant sectors on a colorless background, because of the inhibitory effect of *MuDR* on the expression of *a1*. In this regard, *a1-mum2* is similar to the *hcf106-mum1* allele, a well-described suppressible mutation in maize (Barkan and Martienssen 1991; Settles *et al.* 2001). In *hcf106-mum1*, transcription in phenotypically suppressed plants (*i.e.*, no *MuDR*) initiates within the *Mu1* element inserted in the promoter of the *hcf106* gene. The presence of *MuDR* is associated with an extensive hypomethylation of *Mu1* and adjacent *hcf106* promoter sequences (reviewed in Martienssen 1996) and may interfere with the activation of a regulatory element present in *Mu1* that is responsible for the expression of *hcf106-mum1*. However, the suppression of the mutant *a1-mum2* phenotype in the absence of *MuDR* appears to be tissue specific, as it was reported to occur in the plant body, but not in the aleurone (Martienssen 1996).

The dramatic effects on anthocyanin pigmentation caused by the *Mu1* and *Spm* insertions in the promoter of the *a1* gene are in striking contrast to transient expression and *in vitro* DNA-binding experiments results, in which no single *cis*-regulatory element completely abolished *a1* activity when mutated (Grotewold *et al.* 1994; Tuerck and Fromm 1994; Sainz *et al.* 1997). Here, we investigated the effect that these transposon insertions have on the phlobaphene pigmentation specified by the *P* gene and compared it with the effect that they have on the anthocyanin pigmentation regulated by C1/R. We show that the *a1-m2* allele is *Spm* dependent for the pericarp pigmentation specified by *P*, and that *a1-mum2* mutant phenotype is partially suppressible in the pericarp. In contrast with previous findings, our studies suggest that the *a1-mum2* mutant phenotype is also suppressible in the aleurone, although this suppression takes more than one generation after the removal of *MuDR* function. The study of somatic and germinal excisions of *Mu1* and *Spm* from the *a1-m2* and *a1-mum2* alleles, respectively, provides the first evidence that specific *cis*-acting regulatory elements in the *a1* promoter important for the regulation of *a1* by P or C1/R differ. Together, our findings suggest that these two transposon insertions provide a unique opportunity to correlate results derived from transient expression and *in vitro* DNA-binding experiments with the *in vivo* control of gene expression.

TABLE 1

***a1* alleles used in this study**

Allele name	Maize Coop accession	Abbreviation used (if different from allele name)
a1-m2-7991A::Spm-s	317K	
a1-m2-8010A::Spm-s	317M	
a1-m2-7977B::dSpm	309J	
a1-m2-7991A-p2	315L	*a1-315L*
a1-m2-7991A-p4	315N	*a1-315N*
a1-m2-7991A-p5	315P	*a1-315P*
a1-mum2	330I and 330J	
a1::r-dt		*a1*

MATERIALS AND METHODS

Genetic stocks: Unless otherwise noted, all the stocks used in this study contain the dominant functional alleles for all the structural and regulatory genes necessary for anthocyanin pigment formation (Table 1). Alleles of the *P1* gene (indicated as *P* in this study to avoid confusion with the *Pl* regulator of anthocyanin biosynthesis) are named according to the red phlobaphene pigmentation in the pericarp and cob glumes. For example, *P-rr* specifies red pericarp and red cob, *P-wr* white pericarp and red cob, and *P-ww* white pericarp and white cob. In the pericarp, *P-rr* is dominant over *P-wr* and *P-ww*. The *P-rr* allele used in this study corresponds to the *P-rr-4B2* allele previously described (Grotewold *et al.* 1991), unless otherwise indicated. The *P-ww* allele corresponds to *P-ww-1112* resulting from a deletion of the entire transcribed region of *P* (Athma and Peterson 1991). The *a1-mum2* alleles used in this study were obtained from the Maize Cooperation Stock Center (Maize Coop, Urbana, IL) and correspond to accessions 330I (*a1-mum2; A2 C1 C2 MuDR R1*) and 330J (*a1-mum2; A2 C1 C2 R1*). The *a1-m2* and derivative alleles were obtained from the Maize Coop and correspond to *a1-m2-7991A::Spm-s* (accession 317K), *a1-m2-8010A::Spm-s* (accession 317M), *a1-m2-7991A-p2* (accession 315L), *a1-m2-7991A-p4* (accession 315N), and *a1-m2-7991A-p5* (accession 315P). These alleles were all homozygous and linked to the dominant *Sh2* allele. The *a1::r-dt sh2* stock was obtained from Dr. Patrick Schnable (Iowa State University).

Hand pollination was used for all genetic crosses in the field and greenhouse experiments. To create the *P-rr a1::r-dt sh2* stock (referred to in this study as *P-rr a1 sh2*), *a1::r-dt sh2*

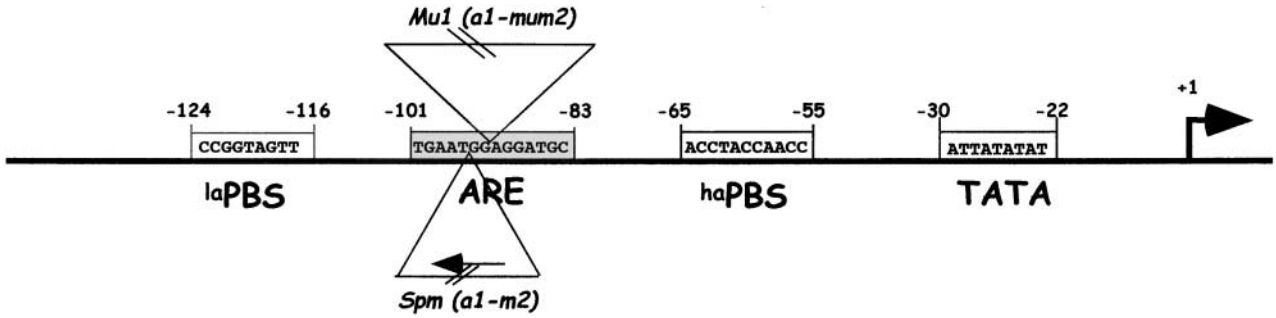

FIGURE 1.—Modular structure of the maize *A1* promoter. The high-affinity ([ha]PBS) and low-affinity P-binding sites ([la]PBS) and the anthocyanin regulatory element (ARE) and the TATA box are shown as boxes. The start of transcription in the wild-type *A1* allele (SCHWARZ-SOMMER *et al.* 1987) is indicated as +1. The positions of the *Spm* transposon in the *a1-m2* allele (SCHWARZ-SOMMER *et al.* 1987) and of the *Mu1* transposon in the *a1-mum2* allele are shown as triangles.

plants were crossed to *P-rr A1 Sh2* pollen. Seeds were planted and the resulting plants were self-pollinated. Kernels carrying the recessive *sh2* allele providing the typical shrunken phenotype in the endosperm were planted and backcrossed as females to the original *a1::r-dt sh2* stock to score for pericarp pigmentation.

Determination of the site of *Mu1* insertion in the *a1-mum2* allele: The 5′ and 3′ regions flanking the *Mu1* element in the *a1-mum2* allele were PCR-amplified using *a1*-specific primers based on the published sequence of *A1* (SCHWARZ-SOMMER *et al.* 1987) and the LIR50 *Mutator* primer previously described (DAS and MARTIENSSEN 1995). PCR products were cloned into the pT-AdvanTAge vector (CLONTECH, Palo Alto, CA) and sequenced.

Analysis of somatic and germinal excision products: To characterize the stable footprint alleles derived from *a1-m2*, maize genomic DNA was extracted from 2- to 3-week-old seedlings (CONE 1989). PCR was performed using two independent genomic DNAs with high-fidelity Platinum Taq polymerase (Bethesda Research Laboratories, Gaithersburg, MD) and the *a1* promoter-specific primers, AP-FG-1 and AP1. AP-FG-1 corresponds to the sequence 5′-CACTGAGTACTGCTGGTTG TTCAA-3′ located between positions −262 and −239 (Figure 1) and AP1 to the sequence 5′-TCTCAGCTGCTCCAGTTC CG-3′ located between positions +13 and −7 (Figure 1). The 275-bp PCR products were excised from agarose gel and purified using a Qiaquick PCR purification kit (QIAGEN, Germany). The PCR products were directly sequenced.

Analysis of pericarp pigments by spectrophotometric methods: The extraction method was modified from DAS *et al.* (1994). A total of six kernels were soaked in water for at least 2 hr. Pericarps were peeled manually, ground with liquid nitrogen, and placed in a tared tube. Tissue was dried by lyophilization overnight and its dried weight was measured (∼50 mg). The dried ground pericarps were extracted with 0.1 ml concentrated HCl and 0.4 ml dimethylsulfoxide (DMSO) sequentially with vigorous vortexing after each addition. Extracts were vortexed for another 20 min, after which tubes were centrifuged for 1 min to clarify the suspension. Clear extracts were diluted with methanol (20% final concentration) and centrifuged briefly to clarify the suspension. Absorbance spectra of the clear supernatants were determined within the 380–650 nm range using a CALY3E spectrophotometer (Varian, San Fernando, CA). The λ-max of phlobaphene pigment was determined at 510 nm.

Analysis of aleurone pigments by spectrophotometric methods: A total of 8–10 kernels were soaked in water for at least 2 hr. Pericarps and embryos were removed manually. The aleurone and endosperm were ground in liquid nitrogen and placed in a tared tube. Tissue was dried by lyophilization overnight and its dried weight was measured (∼80 mg). The dried ground tissues were extracted with 2 ml of acidic (1% HCl) methanol by shaking at room temperature for at least 3 hr. The tube was centrifuged for 1 min to clarify the suspension. Clear extracts were diluted with methanol (50% final concentration) and the absorbance spectra were determined within the 380–650 nm range using a CALY3E spectrophotometer (Varian). The λ-max of anthocyanin pigment was determined at 520 nm.

RESULTS

Transposon insertions in the *a1-mum2* and *a1-m2* alleles have similar effects upon P- or C1/R-regulated *a1* expression: Aleurones expressing dominant alleles of *C1* and *R* accumulate purple anthocyanins (Figure 2A) that are absent in the presence of the homozygous recessive mutant *a1* allele (Figure 2B). *P-rr A1* pericarps (Figure 2F) accumulate the brick-red phlobaphene pigments, while *P-rr* pericarps are brown in the presence of the recessive mutant *a1* allele (Figure 2G). To investigate the effect of *Mu1* and *Spm* insertions (Figure 1) on the regulation of *A1* by P, we crossed plants carrying the *P-rr* allele of *P* with plants carrying the *a1-mum2* or *a1-m2* alleles. Because the pericarp is a maternal tissue (modified ovary wall), pigmentation in the pericarp was scored in the subsequent generation. Figure 2 shows that the *Spm* insertion present in the *a1-m2-7991A::Spm-s* allele has a similar effect upon pigmentation in the pericarp (Figure 2H), controlled by *P*, and in the aleurone (Figure 2C), controlled by C1 and R. *P-rr a1-m2-7991A::Spm-s* pericarps display frequent variegation with some large red revertant sectors (see Figure 2H, inset) as expected from the excision properties of these *Spm* elements from the aleurone tissue (MASSON *et al.* 1987; FEDOROFF 1989). Pericarps carrying the *a1-m2-7991A::Spm-s* allele also have significant red pigmentation (compare background color between Figures 2G and 2H), suggesting that as for the aleurone pigmentation, phlobaphene accumulation is also *Spm* dependent. Indeed, *P-rr* pericarps containing a defective *Spm* (*a1-m2-7977B::*

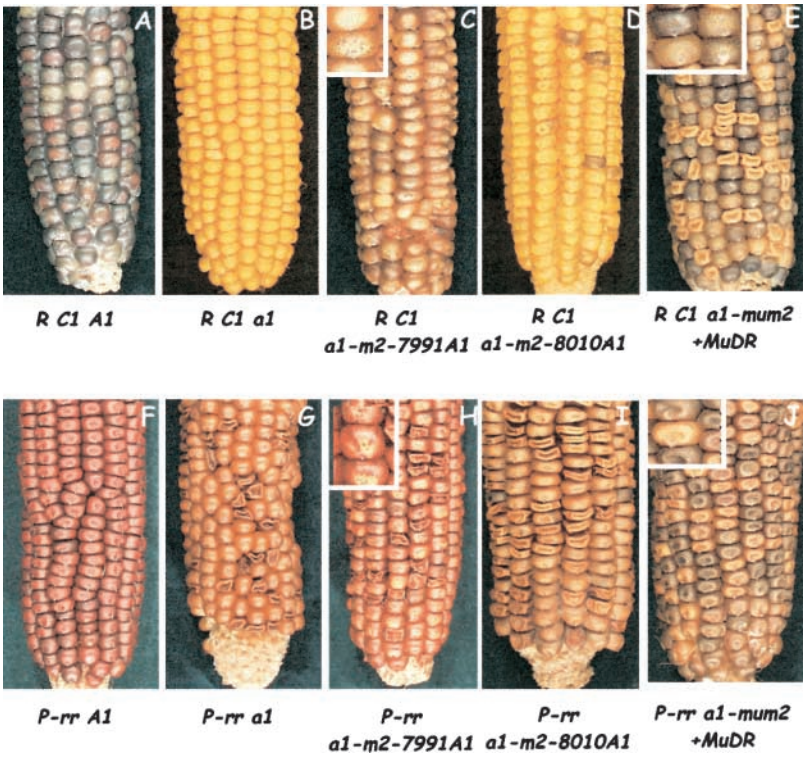

FIGURE 2.—Pigmentation provided by various *a1* alleles in pericarp and aleurone tissues. All the top ears (A–E) express the *C1* and *R* (*R-g*) genes, but lack pericarp pigmentation because they contain either the *P-wr* or *P-ww P* alleles. (A) Anthocyanin accumulation in the presence of the wild-type *A1* allele. The presence of purple and pink kernels reflects the segregation of the recessive *pr* allele (COE and NEUFFER 1989). (B) Absence of *a1* function in the aleurone results in no anthocyanin pigmentation. (C) The *a1-m2-7991A::Spm-s* allele provides frequent spotting as well as pink background anthocyanin pigmentation. (D) The *a1-m2-8010A::Spm-s* allele shows less frequent aleurone spotting and no background pigmentation. (E) Ear resulting from the self-pollination of a *a1-mum2 Sh2/a1 sh2, MuDR* plant. The frequent spotting in the aleurone reflects the somatic excision of *Mu1* from the *a1-mum2* allele. The shrunken kernels lack anthocyanin pigmentation because of the close linkage between the recessive *sh2* and *a1* alleles. All the bottom ears (F–J) express the *P-rr* allele in the pericarp that specifies phlobaphene pigmentation, but unless otherwise specified, lack aleurone anthocyanins because the functional regulators C1 or R are not expressed. (F) Phlobaphene accumulation in the *A1* pericarps. (G) Brown pigments accumulate in *a1* pericarps. The segregation of shrunken kernels reflects the fact that this ear was obtained from a self-pollination of a *a1 Sh2/a1 sh2* plant. (H) Pericarps carrying the *a1-m2-7991A::Spm-s* allele are variegated and show intense red background pigmentation. The segregation of shrunken kernels reflects that this ear was obtained from a self-pollination of a *a1-m2-7991A::Spm-s Sh2/a1 sh2* plant. Darker kernels correspond to aleurones expressing R and C1 to verify *Spm* activity. (I) Pericarps carrying the *a1-m2-8010A::Spm-s* allele show no evident variegation and brown pigmentation. The segregation of shrunken kernels reflects that this ear was obtained from a self-pollination of a *a1-m2-8010A::Spm-s Sh2/a1 sh2* plant. Darker kernels correspond to aleurones expressing R and C1. (J) Pericarps carrying the *a1-mum2* allele are brown and display almost no variegation (see text). The segregation of darker kernels reflects that the underlying aleurone segregates for the *a1-mum2* allele displaying the frequent purple spotting.

dSpm, Table 1) show, in the absence of the autonomous *Spm* element, a pigmentation indistinguishable from *P-rr a1* pericarps (data not shown). Unlike *a1-m2-7991A::Spm-s*, the *a1-m2-8010A::Spm-s* allele shows infrequent aleurone spotting and colorless background pigmentation (Figure 2D; MASSON *et al.* 1987) in the presence of C1/R. Similarly, almost no variegation was detected in *a1-m2-8010A::Spm-s* pericarps, which accumulate only brown pigments (Figure 2I) in the presence of *P-rr*. Thus, the *Spm* insertions present in the different *a1-m2* alleles affect similarly aleurone pigmentation controlled by *C1* and *R* and pericarp pigmentation specified by *P*.

In the presence of the autonomous *MuDR* element, *a1-mum2* aleurones display very frequent small revertant spots on a colorless background (Figure 2E), characteristic of the frequent and late excision of *Mutator* (CHANDLER and HARDEMAN 1992; BENNETZEN *et al.* 1993). In contrast, pericarps with the same *a1* allele are brown in color and display none or very little of the variegation expected for the excision of *Mu1* (WANG and GROTEWOLD 1999; Figure 2J). Thus, like the aleurone pigmentation specified by *C1* and *R*, the *Mu1* insertion interferes with the normal expression of *a1* in response to *P*, in the presence of *MuDR*.

The mutant phenotype associated with *a1-mum2* is partially suppressible in the pericarp: To investigate whether the mutant phenotype of *P-rr a1-mum2* pericarps (Figure 2J) is suppressed by the absence of *MuDR*, we carried out the genetic crosses shown in Figure 3A. The presence of shrunken kernels, a phenotype provided by the recessive mutant *sh2* allele closely linked to the recessive mutant *a1* allele, allowed us to distinguish ears containing the *a1-mum2* allele from sibling ears carrying the mutant *a1* allele. In the absence of *MuDR*, the *a1-mum2* allele confers a reddish color upon the pericarp and cob glumes (Figure 3B, left ear) absent in *a1 P-rr* pericarps, which are solid brown (Figure 3B, right ear). However, the reddish color of the *P-rr a1-mum2* pericarp is weaker than the red color provided by the dominant *A1* allele in *P-rr* pericarps (Figure 2F). The degree of suppression of the mutant phenotype was investigated by comparing the accumulation of the pigments present in *P-rr A1*, *P-rr a1*, and *P-rr a1-mum2* pericarps (Figure 3C). Quantification of the absorption peak at 510 nm, characteristic of the red phlobaphene pigments (DAS *et al.* 1994) and absent in *P-rr a1* pericarps (data not shown), indicated that *P-rr a1-mum2* accumulate ~14% of the level of phlobaphenes present in *P-rr*

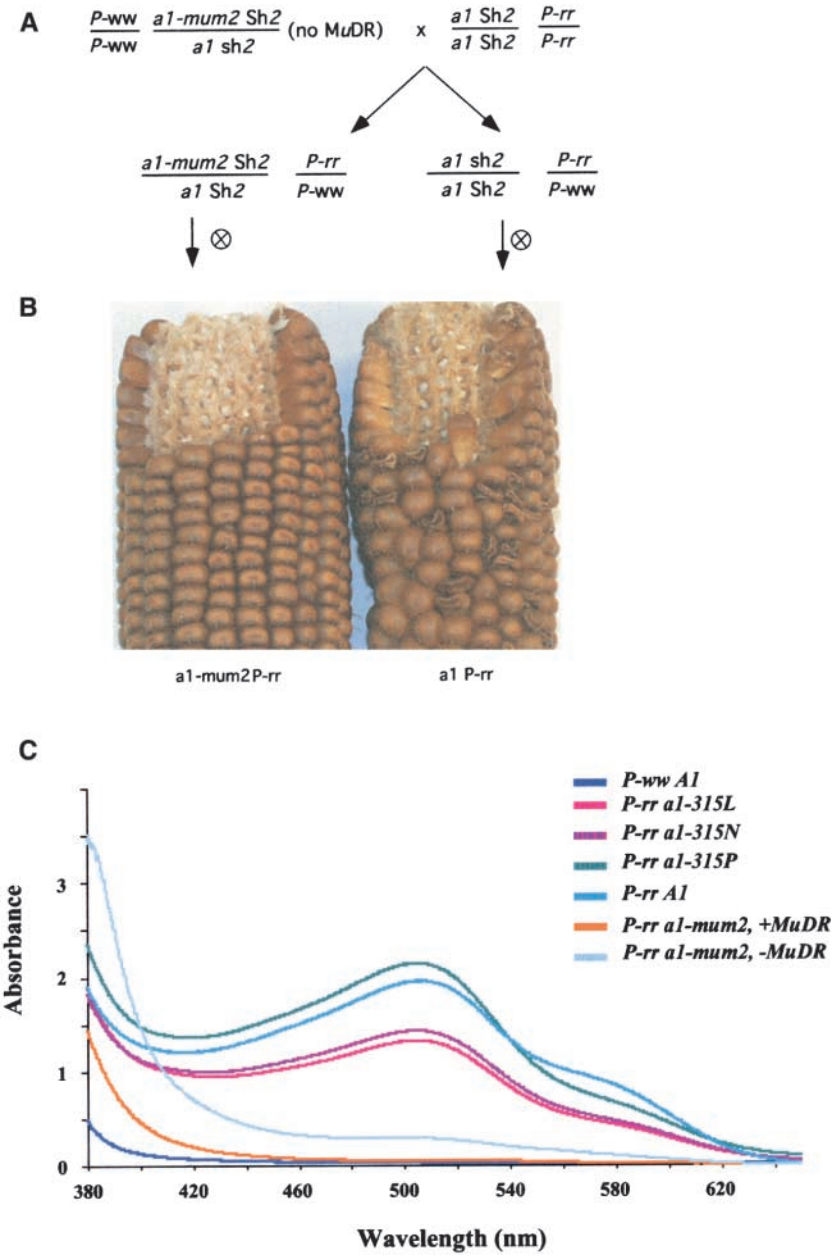

FIGURE 3.—The mutant phenotype of *a1-mum2* is partially suppressible in the pericarp. (A) Pedigree outlining the genetic cross that allows the direct comparison of *P-rr a1* and *P-rr a1-mum2* (−*MuDR*) ears. (B) Phenotypes of *a1-mum2/a1 P-rr* (left ear) and *a1/a1 P-rr* pericarps (right ear). (C) Absorption spectra of the pericarp pigments extracted in the conditions described (DAS *et al.* 1994) from ears carrying several different *a1* alleles.

A1 pericarps. In the presence of *MuDR*, *P-rr a1-mum2* pericarps are completely brown (Figure 2J), with no detectable phlobaphene accumulation (Figure 3C). Thus, as previously described for other vegetative plant tissues (MARTIENSSEN 1996), the mutant phenotype of the *a1-mum2* allele is partially suppressible in the pericarp.

Suppression of the mutation *a1-mum2* in the aleurone: The reported differences in the suppression of the *a1-mum2* mutant phenotype in vegetative tissues (suppressible) and in the aleurone (not suppressible; MARTIENSSEN 1996) prompted us to investigate in more detail the behavior of this allele in the aleurone. Self-pollination of *a1-mum2 Sh2/a1 sh2, MuDR* plants resulted in an ear with 244/426 (57%) plump spotted kernels, 71/426 (17%) plump colorless kernels, and 111/426 (26%) shrunken colorless kernels (Figure 4A). This segregation is consistent with a single *MuDR* element unlinked to the *a1* locus, lost in the plump colorless kernels containing the *a1-mum2* allele and the linked dominant *Sh2* allele. Plump colorless kernels were planted and the ears resulting from the self cross showed variable amounts of aleurone pigmentation (Figure 4B). In contrast to *a1-mum2* aleurones containing *MuDR* (Figure 4A), the *a1-mum2* (−*MuDR*) aleurones are not spotted; rather they display a variable amount of pigmentation at the top of the kernel (Figure 4B). The pigmentation in the aleurone increases even further in the next generation (data not shown). Interestingly, however, not all the aleurones are equally pigmented (Figure 4B), a phenomenon found in many ears derived from similar experiments (not shown). Similarly, ears derived from

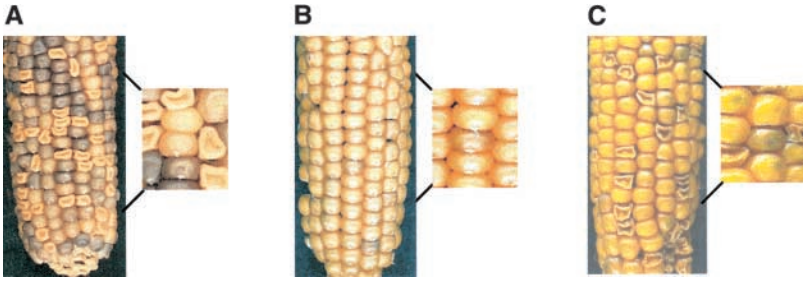

FIGURE 4.—The suppression of the mutant phenotype of *a1-mum2* aleurones takes several generations. (A) Ear resulting from self-pollinating a *a1-mum2 Sh2/a1 sh2*, + *MuDR* plant. Colorless plump kernels have lost *MuDR* but retain the *a1-mum2* allele. Such kernels were planted to give a plant that, when self-pollinated, gave a *a1-mum2 Sh2/a1-mum2 Sh2* ear shown in B, which displays weak purple pigmentation in the aleurone, but no spotting indicative of the absence of *MuDR*. (C) Ear obtained by self-pollination of a *a1-mum2 Sh2/a1 sh2* (no *MuDR*) plant showing the appearance of pigmentation in the aleurone after *MuDR* has been lost for several generations.

planting seeds obtained from the Maize Coop carrying the *a1-mum2* allele but no *MuDR* (stock 330J) show, after self-pollination, a variable pigmentation in the top region of the kernels (Figure 4C). Similar findings with different *Mutator* stocks are reported elsewhere (WALBOT and RUDENKO 2002). Thus, contrary to previous reports (MARTIENSSEN 1996), the mutant phenotype of *a1-mum2* is also suppressible in the aleurone, although this suppression is also partial and requires at least an additional generation after the removal of *MuDR*.

Stable derivative alleles derived from *a1-m2* affect P- and C1/R-specified pigmentation differently: Several stable *a1* alleles obtained by excision of *Spm* from the unstable *a1-m2-7991A::Spm-s* were analyzed (see MATERIALS AND METHODS). The footprints left by *Spm* excision in these alleles were determined by PCR amplification and direct sequencing. The stable alleles were also introduced into *P-rr* maize stocks (see MATERIALS AND METHODS), and the phenotype of the pericarp (specified by *P-rr*) and aleurone (specified by *C1* and *R*) were compared (Figure 5). Derivative alleles from *a1-m2-7991A::Spm-s* frequently have pale pigmentation in the aleurone, as previously reported (MASSON *et al.* 1987). Allele *a1-315L* contains the three-nucleotide target site duplication characteristic of *Spm* (AAT), as well as the insertion of the ATT trinucleotide sequence, present in the original *a1-m2-7991A::Spm-s* allele flanking the right end of the transposable element (MASSON *et al.* 1987). The *a1-315L* allele shows pale pigmentation in the aleurone (~7% of wild-type levels), yet the pigmentation of the pericarp is visually indistinguishable from wild-type *A1* alleles, with ~67% of wild-type levels of phlobaphenes (Figure 5). Thus, the six-nucleotide insertion that distinguishes *a1-315L* from the wild-type *A1* allele dramatically affects *a1* activation by C1/R, but not by P. The excision of *Spm* that resulted in the *a1-315M* allele left behind a footprint, which contains just the ATT insertion (Figure 5). Like *a1-315L*, this allele shows pale aleurone pigmentation (36% of wild-type levels), but pigmentation in the pericarp is only minimally affected by this insertion (Figure 5). The *a1-315P* allele contains a four-nucleotide insertion when compared with wild-type *A1* alleles (Figure 5). This footprint results in normal aleurone and pericarp pigmentation (Figure 5), and the anthocyanin and phlobaphene levels are above wild-type levels (160 and 110%, respectively). Together, these findings indicate that the footprints left in the ARE by *Spm* excision affect the pigmentation specified by P or C1/R in different ways.

DISCUSSION

Study of control of gene expression is largely dependent upon the results of *in vitro* (*e.g.*, DNA binding) and semi-*in vivo* (*e.g.*, transient expression) studies to investigate the participation of *trans*-acting factors and *cis*-acting elements in gene regulation. Few cases are available to directly compare results derived from these approximations with the relevance that particular *cis*-acting regulatory elements have for gene expression *in vivo*. We have utilized two previously described transposon insertions in the promoter of the maize *a1* gene to highlight the *in vivo* significance of a *cis*-acting element central in the regulation of *a1* by the related R2R3 Myb-domain transcription factors, P and C1.

Previously, we showed that the ^{ha}PBS *cis*-element (Figure 1) fused to a minimal *CaMV* 35S promoter is sufficient to drive transcription by P and C1/R in maize transient expression experiments (GROTEWOLD *et al.* 1994). Thus, the more distally located *Mu1* and *Spm* insertions present in the *a1-mum2* and *a1-m2* alleles, respectively (Figure 1), would not have been predicted to affect the expression of *a1* by these regulators. In contrast, we show here (Figure 2) that the phlobaphene pigmentation specified by *P* in the pericarp and the anthocyanin pigmentation specified by *C1* and *R* in the aleurone are dramatically affected by these insertions.

a1-m2-7991A::Spm-s aleurones are spotted due to the frequent excision of *Spm* and *a1-m2-7991A::Spm-s* pericarps are variegated (Figure 2). As previously shown for the aleurone anthocyanin pigmentation (MASSON *et al.* 1987), the *a1-m2-7991A::Spm-s* allele is *Spm* dependent for phlobaphene pigmentation in the pericarp. The mechanism by which transcription of *a1* becomes de-

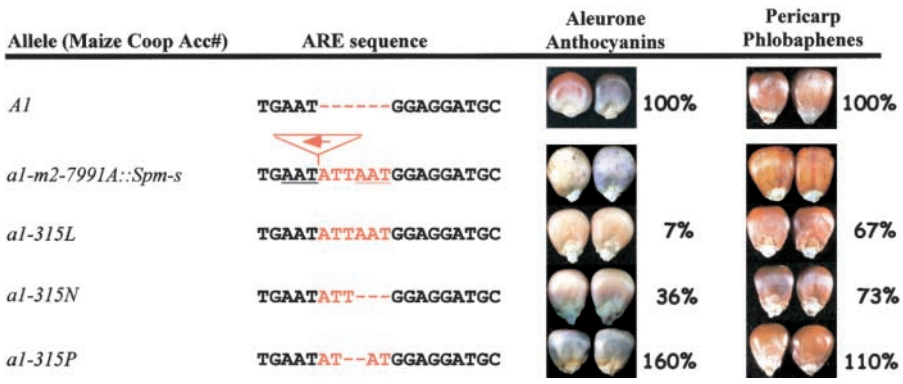

FIGURE 5.—Stable excision alleles derived from *a1-m2*. The sequence of the footprints left by the excision of *Spm* from *a1-m2-7991A::Spm-s* is shown, with the phenotype of the corresponding kernels in the presence of the *C1* and *R* (aleurone) or *P-rr* (pericarp) regulatory alleles. The approximate amount of phlobaphenes in the pericarps is derived from Figure 3C. The quantitation of anthocyanins is described in MATERIALS AND METHODS. The black text denotes the sequence of the wild-type *A1* allele in the region of the ARE element, and the red text indicates extra nucleotides left as footprints by the excision of *Spm*. The red triangle indicates the position of the Spm transposon in the *a1-m2-7991A::Spm-s* allele.

pendent on a gene product encoded by the autonomous *Spm* element is not yet understood. However, our results provide evidence that this phenomenon is not restricted to the control of *a1* by the C1/R regulators of anthocyanin biosynthesis, but that the participation of components of the *Spm* transposon on the regulation of *a1* extends as well to the independent *P* regulatory system. The insertion of *Spm* into the *a1* promoter in the *a1-m2-7991A::Spm-s* is characterized by the 3-bp target site duplication characteristic of *Spm* (AAT, Figure 5). In addition, the *a1-m2-7991A::Spm-s* has another three-nucleotide insertion that probably arose during the selection of novel *a1-m2* alleles, derived from the original *a1-m2* identified by McClintock (MASSON *et al.* 1987). In contrast, the *a1-m2-8010A::Spm-s* allele contains only the three-nucleotide insertion from the target site duplication (MASSON *et al.* 1987). Unlike *a1-m2-7991A::Spm-s*, *a1-m2-8010A::Spm-s* shows much less frequent aleurone spotting and no obvious pericarp variegation (Figure 2). Moreover, *a1-m2-8010A::Spm-s* does not display the *Spm*-dependent background pigmentation in the aleurone (MASSON *et al.* 1987) or in the pericarp tissues (Figure 2). The frequent generation of unpigmented derivative excision alleles from the *a1-m2-8010A::Spm-s* allele (MASSON *et al.* 1987) suggests that the three-nucleotide ATT insertion present in *a1-m2-7991A::Spm-s* is responsible for the different properties of these two *a1-m2* alleles. The insertion of *Mu1* in the *a1-mum2* allele is 2 bp closer to the transcription start site from the *Spm* insertion site in *a1-m2* (Figure 1). In spite of the proximity of these insertion sites, the *Mu1* and *Spm* insertions affect transcription of *a1* in very different ways. The *a1-mum2* allele is a member of the growing class of suppressible mutations, in which the mutant phenotype, in this case lack of pigmentation, is evidenced only in the presence of the autonomous *MuDR* element (MARTIENSSEN 1996). We show here that the lack of phlobaphene pigmentation in the pericarp is partially suppressible (Figure 3B), suggesting that the presence of *MuDR* interferes with the activation of *a1* by *P*. This is similar to what is described for other vegetative tissues (CHOMET *et al.* 1991; MARTIENSSEN 1996), although we observe only a reduced (14% of wild type) accumulation of phlobaphenes in *P-rr a1-mum2* pericarps (Figure 3B). Thus, even in the absence of *MuDR*, the *Mu1* insertion present in *a1-mum2* impairs the activation of *a1* by P, further indicating that *in vivo*, the ^{ha}PBS are not sufficient for high-level activation of *a1* by P or that the presence of *Mu1* inhibits the interaction of P with the ^{ha}PBS. In contrast to previous studies that indicated that the mutant phenotype is not suppressible in the aleurone (MARTIENSSEN 1996), we observed significant accumulation of anthocyanin pigments in this tissue in the absence of *MuDR*. Interestingly, however, this suppression of the mutant aleurone phenotype does not occur immediately after the removal of *MuDR*, but takes at least one additional generation (Figure 4). Different from the *hcf106-mum1 Mu*-suppressible allele (BARKAN and MARTIENSSEN 1991), in which *Mu1* is inserted 3′ of the regulatory elements that control normal *hcf106* expression, the location of *Mu1* in the *a1-mum2* allele is upstream of the ^{ha}PBS *cis*-regulatory elements required for the control of *a1* by P and C1 (Figure 1). Thus, *MuDR* must be interfering with the control of *a1* by C1/R and P in different ways than proposed for *hcf106-mum1* (MARTIENSSEN 1996).

The unexpected pigmentation patterns provided by the *Mu1* and *Spm* insertions could be a consequence of the disruption by the transposons of *cis*-regulatory elements important for the regulation of *a1*. Alternatively, the insertion of the transposons could be interfering with the activities of the regulatory factors controlling *a1* in an indirect fashion. To distinguish between these two possibilities, we examined the effect of germinal footprints left by the excision of the transposons on the P- and C1-specified pigmentation. Three germinal

revertant alleles from *a1-m2-7991A::Spm-s* were molecularly characterized and their effects on anthocyanin and phlobaphene pigmentation were compared (Figure 5). Interestingly, while *a1-315P* has normal C1- and P-regulated pigmentation, it does contain an insertion of four nucleotides when compared with wild-type *a1* alleles. In contrast, both *a1-315N* and *a1-315L* dramatically reduce anthocyanin accumulation, without significantly affecting phlobaphene pigmentation. In previous studies, promoter mutations that impaired *a1* transcription controlled by P also affected transcription by C1/R (Grotewold *et al.* 1994; Tuerck and Fromm 1994; Sainz *et al.* 1997), consistent with the very similar DNA-binding preferences of P and C1 (Sainz *et al.* 1997). Thus, the footprints present in *a1-315N* and *a1-315L* provide the first evidence indicating that promoter elements important for the control of *a1* by C1/R are not important for the activation of *a1* by P *in vivo*.

P-rr a1-mum2 pericarps almost completely lack variegation (Wang and Grotewold 1999), compared with the frequent spotting of underlying aleurones (Figure 2). Although we cannot conclusively rule out the possibility that *Mu1* does not transpose at a high frequency from *a1-mum2* pericarps, it is more likely that the very low frequency of variegation is a consequence of the footprints left by the excision of *Mu1* from this allele. These footprints would rarely result in sequences that allow the *a1* promoter to be activated by P. In contrast, the frequent spotting of *a1-mum2* aleurones suggests that similar footprints are not affecting transcription of *a1* by C1/R. While *Mu1* somatic footprints largely resemble those left by other transposons, larger insertions and deletions that may result from recombinational events have been identified as a result of the somatic excision of *Mu1* from the *bz-mum9* allele (Doseff *et al.* 1991). Together with the results obtained from the *Spm* germinal excision alleles, we conclude that the ARE element plays a much more important role in the regulation of *a1* by P or C1/R than predicted from previous studies. Moreover, within the ARE there appear to be subelements that can be physically separated, which are important for the control of *a1* by each regulator, P or C1/R.

The absolute *in vivo* requirement of the ARE for the regulation of *a1* is strikingly different from previous transient expression studies indicating that the ^{ha}PBS element is sufficient for activation by P and C1/R in maize cells (Grotewold *et al.* 1994). Although a potential Myb consensus binding site is present slightly upstream of the defined ARE site (Tuerck and Fromm 1994), neither P nor C1 recognizes this site on the basis of *in vitro* DNA-binding experiments (Grotewold *et al.* 1994; Sainz *et al.* 1997). Thus, it is possible that other factor(s) involved in the regulation of *a1* by C1/R and P recognize this element that is present in the promoters of several flavonoid biosynthetic genes. Alternatively, the presence of the ARE element in multiple flavonoid biosynthetic genes could reflect the participation of this element in other aspects of gene regulation, such as maintaining a particular chromatin structure. Such a participation of the ARE would explain some of the inconsistencies between the transient expression experiments (probably using nonchromatin templates) and the *in vivo* effects of mutations in this element.

Together, the findings described here stress the importance of the ARE element in the control of *a1* by the P and C1/R regulatory systems. The importance of this element in the regulation of *a1* by two different regulators was determined only because of the presence of transposable element insertions. These studies emphasize the limitations associated with *in vitro* DNA-binding and transient expression experiments and indicate that transposable elements provide a powerful tool to probe promoter function *in vivo*.

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