The Plant Cell, Vol. 8, 1149–1158, July 1996

Alleles of the Maize *P* Gene with Distinct Tissue Specificities Encode Myb-Homologous Proteins with C-Terminal Replacements

Surinder Chopra,[a] Prasanna Athma,[b] and Thomas Peterson[a,1]

[a] Department of Zoology and Genetics and Department of Agronomy, Iowa State University, Ames, Iowa 50011
[b] Institute for the Genetic Analysis of Common Diseases, New York Medical College, 4 Skyline Drive, Hawthorne, New York 10532

The maize *P* gene is a transcriptional regulator of genes encoding enzymes for flavonoid biosynthesis in the pathway leading to the production of a red phlobaphene pigment. Multiple alleles of the *P* gene confer distinct patterns of pigmentation to specific floral organs, such as the kernel pericarp and cob tissues. To determine the basis of allele-specific pigmentation, we have characterized the gene products and transcript accumulation patterns of the *P-wr* allele, which specifies colorless pericarps and red cob tissues. RNA transcripts of *P-wr* are present in colorless pericarps as well as in the colored cob tissues; however, the expression of *P-wr* in pericarp does not induce the accumulation of transcripts from the *C2* and *A1* genes, which encode enzymes for flavonoid pigment biosynthesis. The coding sequences of *P-wr* were compared with the *P-rr* allele, which specifies red pericarp and red cob. The *P-wr* and *P-rr* cDNA sequences are very similar in their 5′ regions. There are only two nucleotide changes that result in amino acid differences; both are outside of the Myb-homologous DNA binding domain. In contrast, the 3′ coding region of *P-rr* is replaced by a unique 210-bp sequence in *P-wr*. The predicted P-wr protein has a C-terminal sequence resembling a cysteine-containing metal binding domain that is not present in the P-rr protein. These results indicate that the differential pericarp pigmentation specified by the *P-rr* and *P-wr* alleles does not result from an absence of *P-wr* transcripts in pericarps. Rather, the allele-specific patterns of *P-rr* and *P-wr* pigmentation may be associated with structural differences in the proteins encoded by each allele.

INTRODUCTION

Flavonoid compounds are secondary metabolites (Stafford, 1991) involved in a number of functions, including response to pathogens (Snyder and Nicholson, 1990), protection from UV radiation (Wingender et al., 1990; Li et al., 1993; Kootstra, 1994; Stapleton and Walbot, 1994), and germination of pollen tubes (Mo et al., 1992; Ylstra et al., 1992). Anthocyanins (derived from 3-hydroxy flavonoids) and phlobaphenes (derived from 3-deoxy flavonoids) are two major maize flavonoid pigments. Flavonoid pigmentation patterns in plants have offered a convenient phenotype to study genetics of gene regulation (Styles and Ceska, 1972, 1989; Dooner, 1983; Ludwig et al., 1989; Roth et al., 1991). Recently, plant genes encoding transcription factors that regulate pigment biosynthesis have been analyzed in Antirrhinum, maize, bean, and petunia (reviewed in Ludwig and Wessler, 1990; Dooner et al., 1991; Goodrich et al., 1992; van der Meer et al., 1993). Although the general action of some of these regulatory transcription factors has been described, in very few instances has the mechanism by which regulatory factors elicit distinct patterns of pigmentation been elucidated.

The pattern of accumulation of phlobaphene pigments in maize floral organs has been the subject of our analysis of the molecular basis of organ- and cell-specific regulation of gene expression. Three genes, namely, *C2*, *Chi1*, and *A1* (Figure 1; Styles and Ceska, 1989) are required for synthesis of 3-deoxy flavonoids and phlobaphenes and are under the transcriptional control of the maize *P* gene (Grotewold et al., 1991a). The *P* gene encodes a Myb-homologous protein that binds to the promoter of the *A1* gene and activates transcription (Grotewold et al., 1994). The Myb-homologous DNA binding domain of the P protein is very similar (70% identical) to the Myb domain of the maize C1 protein, which regulates anthocyanin biosynthesis in the kernel aleurone (Cone et al., 1986; Paz-Ares et al., 1987). In maize, the *C1* gene (or its homolog *Pl*) does not function alone but requires a basic helix-loop-helix (bHLH) protein product of the *R/B* genes to activate transcriptionally the genes for anthocyanin biosynthesis (Goff et al., 1992). In contrast to *C1*, the *P* gene product does not require

[1] To whom correspondence should be addressed.

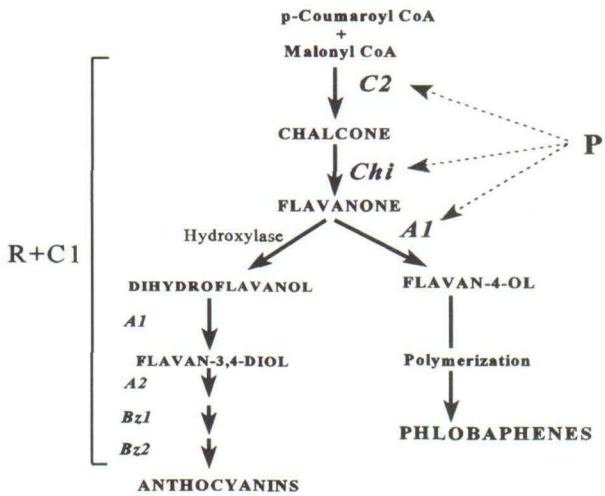


Figure 1. Biosynthetic Pathway for Anthocyanin and Phlobaphene Pigments in Maize.

The *C2*, *Chi*, and *A1* genes encode enzymes for chalcone synthase, chalcone isomerase, and dihydroflavonol reductase, respectively. The *A2* (*Anthocyaninless2*), *Bz1* (*Bronze1*), and *Bz2* (*Bronze2*) genes are required for biosynthesis of anythocyanins but not of phlobaphenes. This figure was modeled after one in Styles and Ceska (1989). CoA, coenzyme A.

an *R/B* coactivator to activate transcription (Grotewold et al., 1994).

Although the *P* gene can induce pigmentation in a variety of floral organs, the most striking coloration is observed in the kernel pericarp (the outermost layer of the kernel derived from the ovary wall) and the cob tissues (the palea and lema, floral bracts that subtend the kernel and compose the chaff of the mature cob). Alleles of the *P* gene are generally identified by a suffix that indicates their expression in pericarp and cob tissues. Thus, *P-rr* specifies red pericarp and red cob, *P-wr* specifies white (colorless) pericarp and red cob, *P-rw* specifies red pericarp and white (colorless) cob, and *P-ww* specifies white (colorless) pericarp and cob (Figure 2). The existence of allelomorphs that confer pigmentation independently to pericarp or cob, such as *P-wr* and *P-rw*, has suggested the possibility that the *P* locus contains duplicate functions capable of pigmenting pericarp and cob independently; however, early genetic tests for recombination between such hypothetical multiple elements were negative (Anderson, 1924). More recent molecular characterization of the *P-rr* allele (Lechelt et al., 1989; Grotewold et al., 1991a), together with mutational studies (Athma et al., 1992; Moreno et al., 1992), indicates that the *P-rr* allele contains a single gene required for pigmentation of both pericarp and cob.

Little is known about the mechanism(s) by which multiple alleles of a single plant gene confer distinct patterns of gene expression. Alleles of the maize *B* gene, which condition distinct patterns of anthocyanin pigmentation, encode nearly identical proteins; however, the 5′ leader and promoter sequences differ significantly (Radicella et al., 1992). For two other maize genes, DNA hypermethylation is associated with altered patterns of transcription of specific alleles, *Pl-Bh* (the *Blotched* allele of *Pl*; Cocciolone and Cone, 1993) and *P-pr* (the patterned red allele of the *P* gene derived from *P-rr*; Das and Messing, 1994). These examples illustrate plant pigment patterns elicited by differential transcription of functionally equivalent protein coding sequences. In contrast, we report here that the *P-wr* and *P-rr* alleles of the maize *P* gene have similar transcription patterns; however, *P-wr* encodes a protein with a different C-terminal sequence relative to the protein encoded by the *P-rr* allele. The unique C-terminal region of the P-wr protein contains a cysteine-rich motif resembling a metal binding domain. Thus, the organ-specific pigmentation patterns determined by the *P-wr* and *P-rr* alleles are associated with a striking difference in the encoded proteins that may affect their function in a cell-specific manner.

RESULTS

P Transcripts Are Present in Colorless *P-wr* Pericarps

The *P-rr* allele has been shown to regulate the accumulation of transcripts from three genes (*C2*, *Chi1*, and *A1*) encoding enzymes for biosynthesis of flavan-4-ol, a precursor of the red phlobaphene pigment (Styles and Ceska, 1977; Grotewold et al., 1991a, 1994). To determine whether the colorless pericarp phenotype specified by the *P-wr* allele is due to lack of transcription of the *P* gene in pericarps, *P-rr* and *P-wr* message

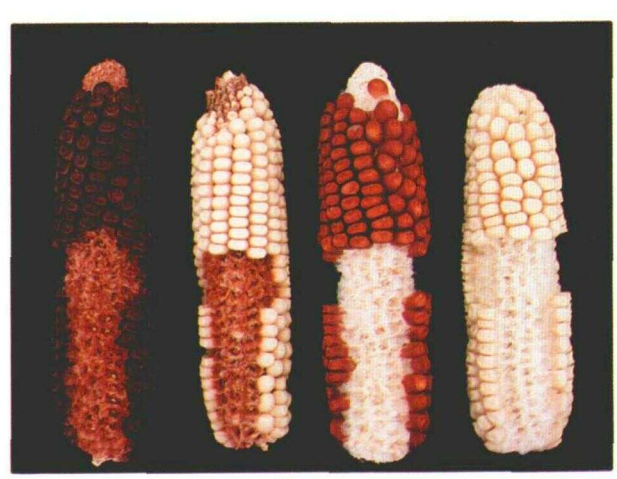


Figure 2. Pericarp- and Cob-Specific Pigmentation by *P* Gene Allelomorphs.

Shown from left to right are maize ears from genotypes *P-rr*, *P-wr*, *P-rw*, and *P-ww*.

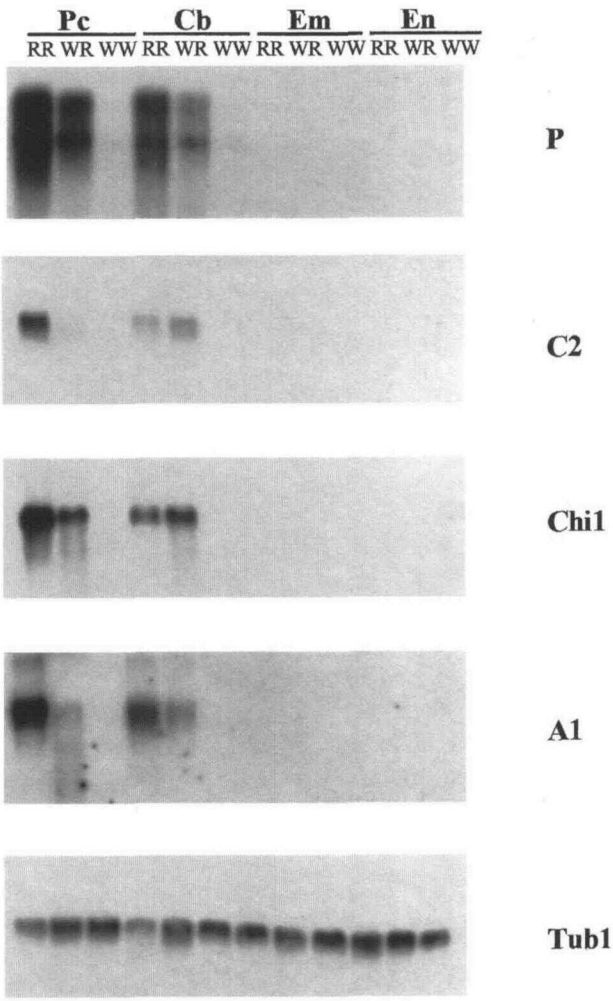


Figure 3. RNA Gel Blot Analysis of Tissue- and Genotype-Specific Steady State Levels of *P*, *C2*, *Chi1*, and *A1* Gene Transcripts.

Tissue sources of RNA indicated at top as Pc (pericarps), Cb (cob), Em (embryos), and En (endosperms) were from maize ears 22 DAP. RR, WR, and WW indicate the *P-rr*, *P-wr*, and *P-ww* genotypes, respectively. Hybridization probes are indicated at right. The *Tubulin1* (Tub1) cDNA probe was used as a control for RNA loading accuracy.

levels were compared by RNA gel blot analysis of RNA extracted from plant tissues of *P-wr* and *P-rr* genotypes (Figure 3). At 22 days after pollination (DAP), *P-wr* pericarps and cob tissues contained two transcripts of ~1.8 and 1.0 kb. The *P-wr* transcripts were similar in size or slightly smaller than the *P-rr* transcripts. The *P-wr* transcripts were present at ~30 and 15% of the level of *P-rr* transcripts in pericarps and cobs, respectively; the presence of *P-wr* transcripts was also confirmed by reverse transcriptase–polymerase chain reaction (RT-PCR) experiments (see below). Similar levels of *P-wr* transcripts were detected in other maize inbred lines carrying the *P-wr* allele (data not shown). In contrast, no *P*-specific transcripts were detected in pericarp and cob tissues of the *P-ww* mutant, in which the *P* gene is deleted (Athma and Peterson, 1991).

RNA gel blots were also hybridized with *C2*, *Chi1*, and *A1* gene probes to test whether P-wr acts as a transcriptional regulator of the genes encoding enzymes for flavonoid biosyntheses. The results (Figure 3) show that in 22-DAP pericarps, these three genes are expressed at levels lower in *P-wr* than in *P-rr*. Levels of *C2* and *A1* gene transcripts are much reduced in *P-wr* compared with *P-rr* pericarps, whereas *Chi1* gene transcripts are moderately reduced in *P-wr* pericarps. In cob tissues 22 DAP, the levels of *C2* and *Chi1* transcripts are similar in *P-wr* and *P-rr* genotypes, whereas *A1* message is reduced in *P-wr* compared with *P-rr*. Transcripts from *C2*, *Chi1*, and *A1* are much reduced or absent in the *P-ww* deletion mutation (*P-ww-1112*; Figure 3). Figure 3 also shows that *P-rr* and *P-wr* expression is restricted to pericarp and cob glumes; transcripts of the *P*, *C2*, *Chi1*, and *A1* genes were not detected in embryo or endosperm cells, which do not synthesize flavonoid pigments in these genotypes.

P and *P*-Controlled Structural Genes Are Developmentally Regulated

Additional experiments were performed to compare the kinetics of accumulation of *P-rr* and *P-wr* messages and the messages of the *P*-regulated genes *C2*, *Chi1*, and *A1* during development of pericarp and cob (Figure 4). The *P-rr* and *P-wr* message levels were assessed by RNA gel blot (data not shown) and RT-PCR assay (Figure 4A). In the early stages of ear development (0 and 5 DAP), the *P-wr* and *P-rr* transcript levels were similar. From 11 to 22 DAP, the *P-wr* pericarps and cobs showed somewhat lower levels of *P* message compared with those of *P-rr* pericarps and cobs. RNA gel blot experiments showed that the transcripts from the *P*-regulated genes *C2*, *Chi1*, and *A1* were present at a low level at 0 DAP and at a moderately higher level at 5 DAP (Figure 4B). Accumulation of these transcripts requires a functional *P* gene, as indicated by the lack of message in the *P-ww* control; however, the levels are similar in *P-rr* and *P-wr* genotypes at these stages. At later stages of development (11 to 18 DAP; Figure 4B), *P-wr* pericarp and cobs have little or no message corresponding to *C2*, *Chi1*, and *A1*, whereas these genes are strongly expressed in *P-rr* pericarps and to a lesser extent in *P-rr* cobs. At 22 DAP, transcripts of *C2*, *Chi1*, and *A1* were detected in *P-wr* pericarps and cobs (Figure 3), but the levels for *A1* and *C2* gene transcripts remained low in *P-wr* pericarps compared with *P-rr* pericarps.

P-wr Encodes a Myb-like Transcriptional Regulator with a Unique C Terminus

P-wr cDNAs were isolated from both pericarp and cob RNA by PCR methods; the *P-wr* pericarp and cob cDNA sequences were compared and found to be identical. In addition, the *P-wr*

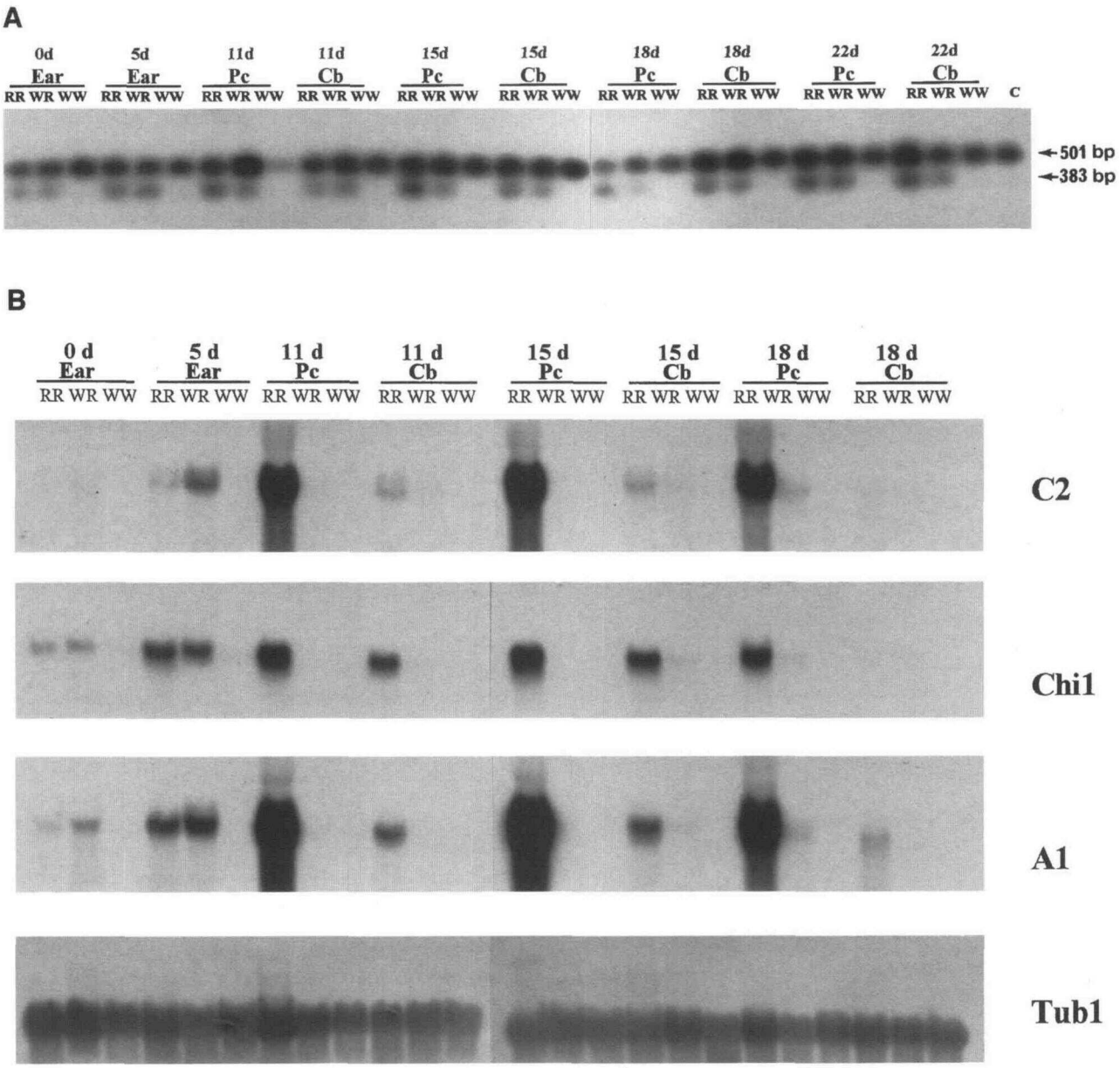


Figure 4. Accumulation of RNA in *P-rr*, *P-wr*, and *P-ww* Genotypes during Maize Ear Development.

Transcripts were detected by RT-PCR **(A)** and RNA gel blot analysis **(B)** performed on poly(A)$^+$ RNA extracted from maize ear (Ear), pericarp (Pc), and cob (Cb) tissues at the indicated number of DAP (d). RR, WR, and WW represent maize genotypes carrying *P-rr*, *P-wr*, and *P-ww* alleles, respectively.

(A) RT-PCR products were fractionated on 1.5% agarose gels, blotted, and hybridized with a genomic fragment of *P-wr*. The amplified band corresponding to *P* transcripts is 383 bp; the 501-bp band is an internal control for PCR reactions. Lane C is the PCR reaction performed with the internal control alone.

(B) Gel blot of poly(A)$^+$ RNA was hybridized to *C2*, *Chi1*, *A1*, and *Tubulin1* (Tub1) probes.

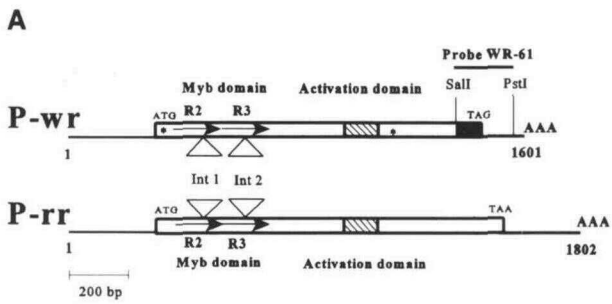
```
P-wr                                                    MGRAPCCEKVG  11
P-rr                                                    ...T.......  11
                                    Myb R2
                    ----------------------------------------------->
P-wr LKRGRWTAEEDQLLANYIAEHGEGSWRSLPKNAGLLRCGKSCRLRWINYLRAD  64
P-rr ..........................................................  64
                                    Myb R3
                    ----------------------------------------------->
P-wr VKRGNISKEEEDIIIKLHATLG NRWSLIASHLP GRTDNEIKNYWNSHLSRQ 115
P-rr ..................... .......... ..................... 115

P-wr IHTYRRKYTAGPDDTAIAIDMSKLQSADRRRGGRTPGRPPKASASRTKQADAD 168
P-rr ..........................................................  168

P-wr QPGGEAKGPAAAASSPRHSDVVNPGPNQPNSSSGSTGTAEEEGPSSEDASGPW 221
P-rr ..........................................................  221
                                    Activation domain

P-wr VLEPIELGDLVWGEADSEMDALMPIGPGGTTRLPSKGLARSAERPRWTTCSTW 274
P-rr ..........................................A.............  274

P-wr TGMASRPICGAGRSRTSTARSCGRPPSRWKLLLLLLLRRRPAPRTIASWRRSR 327
P-rr ..........................................................  327

P-wr LGSCPTRSDGSGHRTDQTDQPRWPGHMVDAASRRCSCVQFFFLFCRFCS      376
P-rr ....................IIGSRVLARSLPS.G.WFRWPNNWEKNSTARAV 380

P-rr KPPPCAPDVDACRVELLRI                                    399
```

Figure 5. Alignment of Deduced Amino Acid Sequences of the *P-wr*– and *P-rr*–Encoded Proteins.

The P-wr sequence (upper) is identical to that of P-rr (lower) except where shown; dots indicate sequence identity. Arrowheads indicate the Myb R2 and Myb R3 semiconserved tryptophan repeats of the c-Myb DNA binding domain (Saikumar et al., 1990). Gaps were inserted in Myb R3 to align it with Myb R2. The single underline indicates the putative transcriptional activation domain. The unique C terminus of P-wr protein is indicated in boldface letters, and the putative zinc finger domain is indicated by the double underline. The GenBank accession numbers for P-wr and P-rr cDNA sequences are U75002 and M62878, respectively.

cDNA sequence is identical to coding regions of the *P-wr* genomic sequence (data not shown). Although the RNA gel blots showed two transcripts (1.8 and 1.0 kb) hybridizing with the *P* probe, only one *P-wr* cDNA (corresponding to 1.8 kb) was isolated. The 1.0-kb *P-wr* transcript may be a nonfunctional product of alternate splicing, as shown for the *P-rr* allele (Grotewold et al., 1991a).

To compare *P-wr* and *P-rr* cDNAs, their deduced amino acid sequences and physical maps are aligned in Figures 5 and 6, respectively. The transcription start and 5′ leader sequences (319 bp upstream of first ATG) are conserved between *P-wr* and *P-rr*, as are the positions of two introns. Like *P-rr*, the 5′ region of *P-wr* encodes a protein homologous with the DNA binding domain of Myb-like transcriptional regulators. The *P-wr* and *P-rr* transcripts have two single nucleotide differences in the region outside the Myb domain: one change in codon 4 converts a threonine in *P-rr* to an alanine in *P-wr*, and a second difference at codon 264 changes an alanine in *P-rr* to a glutamate in *P-wr*. In addition to these single nucleotide differences, the 3′ end of the *P-wr* message is 201 bp shorter and completely different from *P-rr* due to a sequence divergence at nucleotide position 1361. The deduced P-wr protein contains 376 amino acids, whereas the deduced P-rr protein contains 399 amino acids. The unique 3′ end of *P-wr* encodes a cysteine-rich sequence resembling zinc finger or metal binding domains of the type $CX_1CX_7CX_2C$ (where X indicates any amino acid).



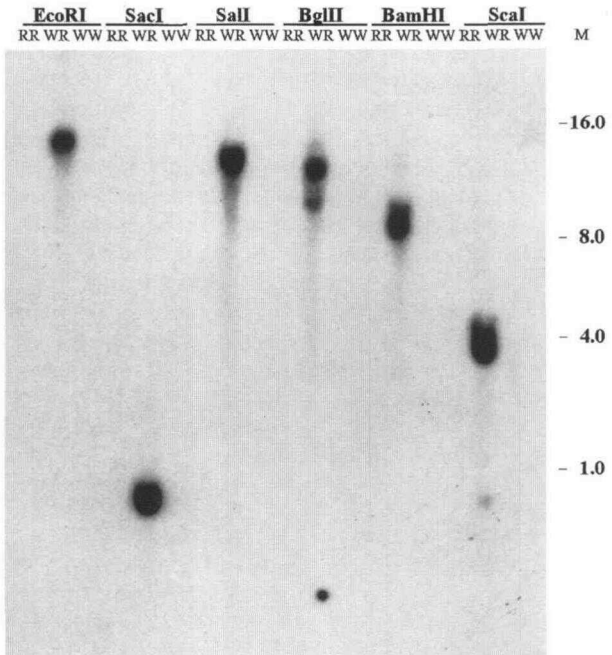


Figure 6. The *P-wr* and *P-rr* cDNAs Have Different 3′ Ends.

(A) Schematic alignment of *P-wr* and *P-rr* cDNA maps. The transcription start site is at position 1. Triangles represent introns (Int 1 and Int 2), and AAA indicates the poly(A) tail. ATG indicates the translation start codons, and TAG and TAA are translation stop codons in *P-wr* and *P-rr*, respectively. Arrows indicate the R2 and R3 tryptophan Myb repeats, and the hatched box represents the putative transcriptional activation domain. Asterisks show the positions of two single nucleotide differences between *P-rr* and *P-wr* cDNAs. Probe WR-61 (solid line) indicates the DNA fragment containing the unique DNA sequence of *P-wr*, and the black box indicates the P-wr zinc finger motif.
(B) Gel blot of *P-rr* (RR), *P-wr* (WR), and *P-ww* (WW) genomic DNAs digested with the indicated restriction enzymes and hybridized with probe WR-61. Size markers (M) are indicated at right in kilobases.

When used as a probe in stringent DNA gel blot hybridizations, the unique 3′ end of *P-wr* (probe WR-61; Figure 6A) did not detect any hybridizing bands in genomic DNA of *P-rr* or *P-ww* lines derived from the progenitor *P-vv* allele (Figure 6B). Additional DNA gel blot hybridizations showed that the 3′ end of the *P-wr* allele is present in the *P-wr* locus of eight other maize inbred lines of *P-wr* genotype (data not shown). Thus, the 3′ end of *P-wr* is uniquely associated with alleles conferring the *P-wr* phenotype.

DISCUSSION

The striking feature of the *P-wr* allele is its ability to produce a distinct pigmentation pattern in which kernel pericarps are colorless and cobs are strongly colored (Figure 2). In contrast, *P-rr* conditions red pigmentation of both the kernel pericarps and cob tissues. Genetic studies (Anderson, 1924) have demonstrated that the colorless pericarp phenotype specified by *P-wr* is recessive (*P-rr*/*P-wr* heterozygous plants produce red pericarps) and not caused by unlinked or loosely linked modifying factors (the colorless pericarp, red cob phenotype segregates as an allele of the *P* locus in testcrosses). Moreover, *P-wr* pericarps lack the intermediates of flavonoid biosynthesis, suggesting that the pathway for flavonoid biosynthesis is blocked at an early stage in *P-wr* pericarps (Styles and Ceska, 1989).

Transcriptional Regulation of *P-wr*

RNA gel blot analysis showed that the lack of pigmentation of *P-wr* pericarps is not due to the absence of *P-wr* transcripts in pericarps. Rather, *P-wr* transcripts are present throughout development of the pericarp, albeit at lower levels than *P-rr* transcripts. Transcriptional run-on experiments need to be performed to show whether the observed reduction in *P-wr* transcript levels is due to differences in transcription rate, transcript stability, or both. Interestingly, the *P-wr* allele contains approximately five complete copies of *P-wr* coding sequence within tandem repeats of a 12.6-kb sequence (S. Chopra and T. Peterson, unpublished results). Assuming that all five copies are equally transcribed and given the ~30% reduction in *P-wr* transcript level relative to *P-rr*, it follows that the expression of each of the five *P-wr* copies is reduced ~15-fold relative to the single *P-rr* gene. This reduced level of *P-wr* transcript may be due to different strengths of the *P-rr* and *P-wr* promoters; however, the *P-wr* and *P-rr* promoter regions are 99% identical within a 5-kb region upstream of the transcription start sites (X. Li, S. Chopra, and T. Peterson, unpublished results). Alternatively, *P-wr* transcription may be downregulated by homology-dependent gene silencing, such as occurs in transgenic plants (Matzke and Matzke, 1995). A similar phenomenon termed reduced expression of endogenous duplications has been described for the duplicated *R* and *Sn* (*Scutellar node*) genes, which regulate anythocyanin synthesis in maize (Ronchi et al., 1995).

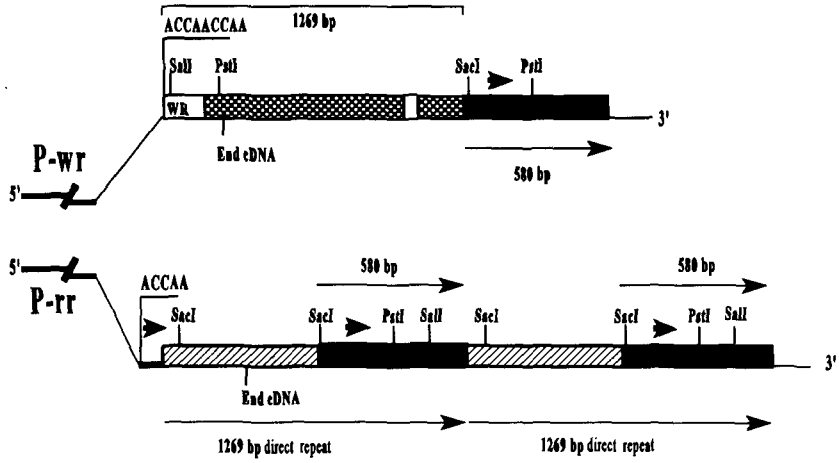


Figure 7. DNA Rearrangements at the 3′ Ends of the *P-wr* and *P-rr* Genes.

The *P-wr* and *P-rr* coding sequences are similar until position 1360. Downstream of this point, *P-wr* contains a unique 210-bp sequence (WR) encoding the C-terminal zinc finger motif. The checkered box in *P-wr* indicates a 1-kb region homologous with a sequence in *P-rr* located 8 kb 5′ of the start of transcription; a small white box within the checkered box indicates an 80-bp insertion present in the *P-wr* sequence. Together, the hatched and black boxes in *P-rr* make up a 1269-bp sequence, which is present as a tandem direct repeat in *P-rr*. The 1269-bp repeats are bounded by repeats of the 8-bp sequence TCGATGCC (not shown). The *P-wr* allele contains a 580-bp sequence (black box) homologous with the 3′ region of the 1269-bp repeat (black boxes). Arrowheads indicate a 72-bp (11/50/11-bp) repeat sequence found in both *P-wr* and *P-rr*. The sequence ACCAACCAA present in *P-wr* is a putative P binding site.

Another possible explanation for the reduced transcript level of *P-wr* is negative transcriptional autoregulation caused by binding of the P-wr protein to a site within the unique *P-wr* 3′ sequence. The Myb domain of the P-rr protein previously was shown to bind to the consensus sequence CC(T/A)ACC in vitro. The P-wr and P-rr proteins probably bind to the same sites, because their Myb-homologous DNA binding domains are identical. Transient expression experiments have indicated that the P-rr protein can activate transcription of promoters containing a P binding site (Grotewold et al., 1994). The 3′ transcribed region of the *P-wr* gene contains the sequence ACCAACCAA (Figure 7). This sequence not only matches the *P-rr* consensus binding site, but it is also contained within an oligonucleotide selected in a P-rr protein binding selection assay (Grotewold et al., 1994). Because of the multiple repeats of the *P-wr* coding sequence, this putative P binding site would be present approximately five times in the *P-wr* locus. Binding of P-wr protein to these sites in the 3′ end of *P-wr* might interfere with *P-wr* transcription, causing reduced transcript levels. Alternatively, binding of P-wr protein to these sites may cause a net decrease in the amount of P-wr protein available to activate transcription from the target genes *C2*, *Chi1*, and *A1*, whose expression is required for phlobaphene pigment biosynthesis.

P-wr Protein Contains a Putative Metal Binding Domain

The *P-wr*– and *P-rr*–encoded proteins contain identical Myb-homologous DNA binding domains and identical negatively charged putative transcriptional activation domains. In fact, the proteins encoded by the *P-wr* and *P-rr* alleles have only two differences in the first 347 amino acids. The 3′ end of *P-wr*, however, is completely different from that of *P-rr*, leading to the replacement of the C-terminal region of *P-rr* with a new sequence in P-wr. The unique 3′ end of *P-wr* encodes a cysteine-rich sequence similar to zinc finger or metal binding domains of the type $CX_1CX_7CX_2C$ (Figure 5). The *P-wr* unique sequence also contains five phenylalanine residues and one leucine residue, which are commonly found in the loop region of zinc finger domains (Coleman, 1992).

Similar cysteine motifs (consensus: $CX_{1-3}CX_{7-8}HX_2C$) are found in type I retrotransposons in *Bombyx mori* (Xiong and Eickbush, 1988) and Drosophila (Jakubczak et al., 1990) and in some non–long-terminal-repeat retrotransposons (Leeton and Smyth, 1993). The function of the cysteine motifs of retrotransposons is not well known, although they may be involved in nucleic acid binding during retroviral integration. Most zinc finger proteins identified to date contain two or more individual zinc fingers (Coleman, 1992). The P-wr protein contains a single zinc finger motif, as do a number of other DNA binding proteins, including the E1A protein of adenovirus type 5 (Culp et al., 1988), the terminus factor of Drosophila (Baldarelli et al., 1988), the ABF1 protein of yeast (Diffley and Stillman, 1989), the early B cell factor (Hagman et al., 1995), and the MNB1a protein of maize (Yanagisawa, 1995).

The P-wr protein contains both a Myb DNA binding domain and a putative zinc finger domain. Several proteins have been identified previously which contain more than one putative DNA binding domain. Examples include POU domain transcription factors (Herr et al., 1988), glycine-rich protein (GRP2) from Arabidopsis and *Nicotiana sylvestris* (Kingsley and Palis, 1994), and the product of the Arabidopsis *APETALA2* gene (Weigel, 1995). However, to our knowledge, there are no reports of other proteins containing both a Myb DNA binding domain and a zinc finger domain.

Models for the Tissue-Specific Phenotype of *P-wr*

The results of RNA gel blot experiments (Figures 3 and 4) show that there is considerable variation in the proportional levels of the *C2*, *Chi1*, and *A1* transcripts when measured in different genotypes (*P-rr* and *P-wr*), different tissues (pericarp and cob), and at different developmental stages (0 to 22 DAP). This variation in proportional levels indicates that expression levels of the *C2*, *Chi1*, and *A1* genes are subject to some degree of independent regulation. Nevertheless, it is clear that the *P-wr* gene is required for accumulation of *C2*, *Chi1*, and *A1* gene transcripts in cob tissues (Figure 3; compare *P-wr* and *P-ww* lanes). However, the *C2*, *Chi1*, and *A1* gene transcripts do not accumulate to high levels in *P-wr* pericarps, despite the presence of significant levels of *P-wr* transcripts. Therefore, the effective block to *P-wr* function in pericarps appears to be post-transcriptional. We have not yet shown that the P-wr protein is present in pericarps, but such a determination will be made as soon as P-wr–specific antibodies are available. If P-wr protein is indeed present at significant levels in pericarps, then it seems likely that the unique C-terminal region of the P-wr protein is responsible for the *P-wr* phenotype. For example, the zinc finger domain of the P-wr protein may interact with an accessory transcription factor that is differentially expressed in pericarp and cob glume cells. Alternatively, the C-terminal region of P-wr protein may be the target of post-translational modifications that affect its function in a cell-specific manner. Domain swap experiments, in which regions of P-rr and P-wr proteins are exchanged and then reintroduced into transgenic plants, can localize the protein regions responsible for the tissue-specific *P-wr* phenotype.

Another hypothesis is that *P-wr* does not pigment cob glumes specifically but rather specifies the pigmentation of cells along the margins of floral organs. Supporting this hypothesis is the observation that *P-wr*–specified pigmentation is found not only in cob tissues but also in the thin margins of tassel glumes and along the thin edges of husks. Moreover, when *P-wr* expression is examined in genotypes carrying the dominant *Tunicate* factor, which causes increased growth of cob glumes, *P-wr*–specified pigments accumulate along the margins of the enlarged glumes and not in the multicellular interior regions of the glumes (T. Peterson, unpublished data). In contrast, *P-rr* induces pigmentation throughout the enlarged *Tunicate* glumes

and in all parts of the husk. Thus, the *P-wr* phenotype may not be due to cob-specific expression but rather may reflect a pigmentation pattern specific to the edges of floral organs. By using this reasoning, we concluded that *P-wr* pericarps lack pigmentation because they have no edge or margin. Why pigmentation develops only in marginal cells of *P-wr* plants remains speculative. The cells at the margins of organs are probably in a different physiological state than cells in the interior of the same organ, due to differences in desiccation and maturity. In this altered physiological state, the presumed post-transcriptional block to *P-wr* function may be lifted, resulting in induction of the genes for flavonoid biosynthesis and pigmentation of the cells along floral organ margins.

Gene Rearrangements in *P-wr* and *P-rr* Alleles

The *P-wr* and *P-rr* alleles have substantially rearranged 3′ regions (Figure 7). Immediately downstream of the WR-61 sequence, which encodes the putative zinc finger motif, *P-wr* has a 1-kb region homologous with a sequence found 8 kb 5′ of the transcription start site in *P-rr*. Following this, *P-wr* contains a 580-bp region (Figure 7, black box) that is homologous with part of a 1269-bp tandem direct repeat in *P-rr*. The *P-wr* 3′ end also contains a 72-bp sequence (Figure 7, black arrowheads) composed of two 11-bp imperfect direct repeats flanking a central 50-bp core. This 11/50/11-bp complex is present three times in *P-rr*: once at the point of divergence with *P-wr*, and once in each of the two 1269-bp direct repeats. Remarkably, the length of the region in *P-wr*, which is nonhomologous with the 3′ end of *P-rr*, is exactly the same length as the tandem direct repeat of *P-rr*, that is, 1269 bp (Figure 7).

We propose that the *P-rr* allele was derived from a progenitor allele that had a 3′ structure similar to that of *P-wr*. A deletion or recombination event could have removed the *P-wr*–specific sequences, including the region encoding the C-terminal zinc finger motif. The deletion could have been filled in to generate a single 1269-bp sequence, which was then duplicated to give the 1269-bp tandem repeats in the *P-rr* 3′ region. The hypothesis that *P-rr* was derived from a progenitor resembling *P-wr* is supported by the fact that the sequence unique to *P-wr* (WR-61), which encodes the putative zinc finger motif, is not found elsewhere in the genome of *P-wr* (Figure 6B). It is more likely that the unique *P-wr* sequence was deleted from *P-rr* rather than acquired de novo in *P-wr*. In contrast, the 5′ region of the 1269-bp tandem repeats in *P-rr* (hatched boxes in Figure 7 and identified as fragment 14 in Lechelt et al. [1989]) is present in several copies in all maize genotypes examined, including *P-wr* (data not shown). This sequence may represent filler DNA incorporated at the time of deletion. Previous analysis has shown that deletions in the maize *Waxy* gene are associated with insertions of filler DNA, which may be derived from other genomic sequences (Wessler et al., 1990). Furthermore, there are two other sites in the *P-rr* allele with short sequence duplications that are present in a single copy in the *P-wr* allele: one in the second intron (Grotewold et al., 1991b) and another located 5′ of the transcription start (data not shown). These short sequence duplications may represent transposon footprints and would be additional evidence that the *P-rr* allele was derived from an ancestral allele with a protein coding sequence resembling that of *P-wr*. In conclusion, our model predicts that the expanded pigmentation potential of the *P-rr* allele results from loss of the C-terminal zinc finger motif from the *P*-encoded protein.

METHODS

Maize Stocks

Maize stocks used in this study carry *P-rr* (*P-rr-4B2*, derived as a revertant from *P-vv-1114* by excision of the transposable element *Activator*; Grotewold et al., 1991b), *P-wr* (from inbred line W23), or *P-ww* (*P-ww-1112*, in which most of the *P* gene is deleted; Athma and Peterson, 1991).

RNA and DNA Isolation and Gel Blot Hybridizations

Tissue samples were collected from self-pollinated homozygous plants of *P-wr*, *P-rr*, or *P-ww* genotypes. Tissue samples were ground in liquid nitrogen, and total RNA was extracted by using the method of Verwoerd et al. (1989). Poly(A)$^+$ RNA was purified by using the Poly AT tract kit (Promega), fractionated in formaldehyde-containing agarose gels (Sambrook et al., 1989), blotted to a nylon membrane, and probed with α-^{32}P-labeled fragments of the *P-rr* cDNA (using the region similar to *P-wr*), the maize *C2* cDNA (Wienand et al., 1986), the maize *Chi1* genomic fragment (Grotewold and Peterson, 1994), the maize *A1* cDNA (Schwarz-Sommer et al., 1987), and maize *Tubulin1* cDNA (Villemur et al., 1992). Plant genomic DNA was isolated by using the method of Dellaporta et al. (1983). All hybridizations were performed for 24 hr at 43°C with a hybridization mixture containing 50% formamide, 0.25 M $NaPO_4$, pH 7.2, 0.25 M NaCl, 1 mM EDTA, 7% SDS, and 0.05 mg/mL salmon sperm DNA. Filters were washed in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, twice at 50°C for 15 min, and once at 65°C for 15 to 30 min. Filters were exposed to X-OMAT:AR film (Kodak) for 1 to 7 days.

cDNA Cloning and Sequencing

DNA blot hybridizations indicated that *P-wr* and *P-rr* are highly homologous (data not shown); therefore, oligonucleotide primers homologous with *P-rr* cDNA sequences were used to reverse transcribe and polymerase chain reaction (PCR) amplify *P-wr* cDNA. Sequences of primers EP5-2, EP5-4, EP1PE, EP5-8, and EP5-16 were presented previously (Grotewold et al., 1991a). Additional primers used here are EP3-13 (position 511 to 489; 5′-AGGAATTCCGCCCGAAGGTAGTTGATCC-3′) and SC0-02 (position 1395 to 1378; 5′-CTAGCGGCGTCGACCATA-3′). One microgram of poly(A)$^+$ RNA isolated from pericarps or cob tissues of *P-wr* ears 22 days after pollination (DAP) was reverse transcribed to synthesize first-strand cDNA (Sambrook et al., 1989). The 3′ and 5′ ends of the cDNAs were amplified by the rapid amplification of cDNA ends protocol (Frohman et al., 1988) by using an oligo (dT) adapter

primer and an adapter primer along with either the *P* gene–specific primer EP5-16 for the 3′ end or EP3-13 for the 5′ end. The overlapping internal cDNA fragment was amplified by using primers EP5-8 and SC0-02, and reaction conditions were as described by Grotewold et al. (1991a). PCR reactions were repeated at least four times, and the products were cloned in either pBluescriptII SK– (Stratagene) or pT7-Blue plasmids (Novagen, Madison, WI). At least four clones corresponding to each PCR reaction were sequenced from both strands. DNA sequencing was done using a Sequenase kit (U.S. Biochemical Corp.), and/or at the Iowa State University DNA sequencing and synthesis facility.

Reverse Transcriptase-PCR Assay to Detect *P* Transcripts

Reverse transcriptase (RT)–PCR was used to detect *P* gene transcripts as follows: 1 μg of poly(A)$^+$ RNA was reverse transcribed using an oligo(dT) primer. One-tenth (by volume) of the first-strand cDNA was then amplified by PCR with primers EP5-8 and EP3-13. As an internal control, the PCR reactions also included 1 ng of a plasmid DNA containing a *P-wr* genomic fragment spanning oligonucleotide primers EP5-8 and EP3-13. The *P-wr* genomic fragment contains a 118-bp intron (data not shown) giving a PCR product of 501 bp, whereas the *P-wr* cDNA yields a 383-bp product. PCR products were resolved by agarose gel electrophoresis and hybridized with the 501-bp *P-wr* genomic fragment probe. RT-PCR reactions were repeated at least five times to demonstrate reproducibility of the results.

ACKNOWLEDGMENTS

We thank Jo Dieter and Karen Price for excellent technical assistance, Jackeline Alverio and ChenWen Zhu for assistance with RNA purifications, Bryan Regan and Roberto Morales for field assistance, Udo Wienand and Peter Snustad for plasmids, Erich Grotewold and Xianggan Li for oligonucleotide primers and helpful discussions, and Suzy Cocciolone and Dan Voytas for comments on the manuscript. This work was supported by Grant No. DE-FG02-93ER20124 from the U.S. Department of Energy. This is journal paper No. J-16697 of the Iowa Agriculture and Home Economics Experiment Station (Ames, IA) Project No. 3297 and was supported by Hatch Act and State of Iowa funds.

Received March 15, 1996; accepted May 20, 1996.

REFERENCES

Anderson, E.G. (1924). Pericarp studies in maize. II. The allelomorphism of a series of factors for pericarp color. Genetics **9,** 442–453.

Athma, P., and Peterson, T. (1991). *Ac* induces homologous recombination at the maize *P* locus. Genetics **128,** 163–173.

Athma, P., Grotewold, E., and Peterson, T. (1992). Insertional mutagenesis of the maize *P* gene by intragenic transposition of *Ac*. Genetics **131,** 199–209.

Baldarelli, R.M., Mahoney, P.A., Salas, F., Gustavson, E., Boyer, P.D., Chang, M.F., Roark, M., and Lengyel, J.A. (1988). Transcripts of the Drosophila blastoderm-specific locus, terminus, are concentrated posteriorly and encode a potential DNA-binding finger. Dev. Biol. **125,** 85–95.

Cocciolone, S.M., and Cone, K.C. (1993). *Pl-Bh*, an anthocyanin regulatory gene of maize that leads to variegated pigmentation. Genetics **135,** 575–588.

Coleman, J.E. (1992). Zinc proteins: Enzymes, storage proteins, transcription factors, and replication proteins. Annu. Rev. Biochem. **61,** 897–946.

Cone, K.C., Burr, F.A., and Burr, B. (1986). Molecular analysis of the maize anthocyanin regulatory locus *C1*. Proc. Natl. Acad. Sci. USA **83,** 9631–9635.

Culp, J.S., Webster, L.C., Friedman, D.J., Smith, C.L., Huang, W.-J., Wu, F.Y.-H., Rosenberg, M., and Ricciardi, R.P. (1988). The 289-amino acid E1A protein of adenovirus binds zinc in a region that is important for trans-activation. Proc. Natl. Acad. Sci. USA **85,** 6450–6454.

Das, O.P., and Messing, J. (1994). Variegated phenotype and developmental methylation changes of a maize allele originating from epimutation. Genetics **136,** 1121–1141.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. **1,** 19–21.

Diffley, J.F., and Stillman, B. (1989). Similarity between the transcriptional silencer binding proteins ABF1 and RAP1. Science **246,** 1034–1038.

Dooner, H.K. (1983). Coordinated genetic regulation of flavonoid biosynthetic enzymes in maize. Mol. Gen. Genet. **189,** 136–141.

Dooner, H.K., Robbins, T.P., and Jorgensen, R.A. (1991). Genetic and developmental control of anthocyanin biosynthesis. Annu. Rev. Genet. **25,** 173–199.

Frohman, M.A., Dush, M.K., and Martin, G.R. (1988). Rapid production of full length cDNAs from rare transcripts: Amplification using a single gene specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85,** 8998–9002.

Goff, S.A., Cone, K.C., and Chandler, V.L. (1992). Functional analysis of the transcriptional activator encoded by the maize *B* gene: Evidence for a direct functional interaction between two classes of regulatory proteins. Genes Dev. **6,** 864–875.

Goodrich, J., Carpenter, R., and Coen, E. (1992). A common gene regulates pigmentation patterns in diverse plant species. Cell **68,** 955–964.

Grotewold, E., and Peterson, T. (1994). Isolation and characterization of a maize gene encoding chalcone flavonone isomerase. Mol. Gen. Genet. **242,** 1–8.

Grotewold, E., Athma, P., and Peterson, T. (1991a). Alternatively spliced products of the maize *P* gene encode proteins with homology to the DNA binding domain of Myb-like transcription factors. Proc. Natl. Acad. Sci. USA **88,** 4587–4591.

Grotewold, E., Athma, P., and Peterson, T. (1991b). A possible hotspot for *Ac* insertion in the maize *P* gene. Mol. Gen. Genet. **230,** 329–331.

Grotewold, E., Drummond, B., Bowen, B., and Peterson, T. (1994). The *myb*-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell **76,** 543–553.

Hagman, J., Gutch, M.J., Lin, H., and Grosschedl, R. (1995). EBF contains a novel zinc coordination motif and multiple dimerization and transcriptional activation domains. EMBO J. **14,** 2907–2916.

Herr, W., Sturm, R.A., Clerc, R.G., Corcoran, L.M., Baltimore, D., Sharp, P.A., Ingraham, H.A., Rosenfeld, M.G., Finney, M., Ruvkun, G., and Horvitz, H.R. (1988). The POU domain: A large conserved region in the mammalian *pit-1, oct-1, oct-2,* and *Caenorhabditis elegans unc-86* gene products. Genes Dev. **2,** 1513–1516.

Jakubczak, J.L., Xiong, Y., and Eickbush, T.H. (1990). Type I (R1) and type II (R2) ribosomal DNA insertions of *Drosophila melanogaster* are retrotransposable elements closely related to those of *Bombyx mori.* J. Mol. Biol. **212,** 37–52.

Kingsley, P.D., and Palis, J. (1994). GRP2 proteins contain both CCHC zinc fingers and a cold shock domain. Plant Cell **6,** 1522–1523.

Kootstra, A. (1994). Protection from UV-B–induced DNA damage by flavonoids. Plant Mol. Biol. **26,** 771–774.

Lechelt, C., Peterson, T., Laird, A., Chen, J., Dellaporta, S., Dennis, E., Peacock, W.J., and Starlinger, P. (1989). Isolation and molecular analysis of the maize *P* locus. Mol. Gen. Genet. **219,** 225–234.

Leeton, P.R.J., and Smyth, D.R. (1993). An abundant LINE-like element amplified in the genome of *Lilium speciosum.* Mol. Gen. Genet. **237,** 97–104.

Li, J., Ou-Lee, T.-M., Raba, R., Amundson, R.G., and Last, R.L. (1993). Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell **5,** 171–179.

Ludwig, S.E., and Wessler, S.R. (1990). Maize *R* gene family: Tissue-specific helix-loop-helix proteins. Cell **62,** 849–851.

Ludwig, S., Habera, L., Dellaporta, S., and Wessler, S.R. (1989). *Lc,* a member of the maize *R* family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc-homology region. Proc. Natl. Acad. Sci. USA **86,** 7092–7096.

Matzke, M.A., and Matzke, J.M. (1995). How and why do plants inactivate homologous (trans)genes? Plant Physiol. **107,** 679–685.

Mo, Y., Nagel., C., and Taylor, L.P. (1992). Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. Proc. Natl. Acad. Sci. USA **89,** 7213–7217.

Moreno, M.A., Chen, J., Greenblatt, I., and Dellaporta, S.L. (1992). Reconstitutional mutagenesis of the maize *P* gene by short-range *Ac* transpositions. Genetics **131,** 939–956.

Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P.A., and Saedler, H. (1987). The regulatory *c1* locus of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. EMBO J. **6,** 3553–3558.

Radicella, P.J., Brown, D., Tolar, L.T., and Chandler, V.L. (1992). Allelic diversity of the maize *B* regulatory gene: Different leader and promoter sequences of two *B* alleles determine distinct tissue specificities of anthocyanin production. Genes Dev. **6,** 2152–2164.

Ronchi, A., Petroni, K., and Tonelli, C. (1995). The reduced expression of endogenous duplications (REED) in the maize *R* gene family is mediated by DNA methylation. EMBO J. **14,** 5318–5328.

Roth, B.A., Goff, S.A., Klein, T.M., and Fromm, M.E. (1991). *C1*- and *R*-dependent expression of the maize *Bz1* gene requires sequences with homology to mammalian myb and myc binding sites. Plant Cell **3,** 317–325.

Saikumar, P., Murali, R., and Reddy, P. (1990). Role of tryptophan repeats and flanking amino acids in Myb–DNA interactions. Proc. Natl. Acad. Sci. USA **87,** 8452–8456.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Schwarz-Sommer, Z., Shepherd, N., Tacke, E., Gierl, A., Rohde, W., Leclercq, L., Mattes, M., Berndtgen, R., Peterson, P.A., and Saedler, H. (1987). Influence of transposable elements on the structure and function of the *A1* gene of *Zea mays.* EMBO J. **6,** 287–294.

Snyder, B.A., and Nicholson, R.L. (1990). Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. Science **248,** 1637–1639.

Stafford, H.A. (1991). Flavonoid evolution: An enzymic approach. Plant Physiol. **96,** 680–685.

Stapleton, A.E., and Walbot, V. (1994). Flavonoids can protect maize DNA from the induction of ultraviolet radiation damage. Plant Physiol. **105,** 881–889.

Styles, E.D., and Ceska, O. (1972). Flavonoid pigments in genetic strains of maize. Phytochemistry **11,** 3019–3021.

Styles, E.D., and Ceska, O. (1977). The genetic control of flavonoid synthesis in maize. Can. J. Genet. Cytol. **19,** 289–302.

Styles, E.D., and Ceska, O. (1989). Pericarp flavonoids in genetic strains of *Zea mays.* Maydica **34,** 227–237.

van der Meer, I.M., Stuitje, A.R., and Mol, J.N.M. (1993). Regulation of general phenylpropanoid and flavonoid gene expression. In Control of Plant Gene Expression, D.P.S. Verma, ed (Boca Raton, FL: CRC Press), pp. 125–155.

Verwoerd, T.C., Dekker, B.M.M., and Hoekema, A. (1989). A small scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. **17,** 2362.

Villemur, R., Joyce, M., Haas, N.A., Goddard, R.H., Kopczak, S.D., Hussey, P.J., Snustad, D.P., and Silflow, C. (1992). α-Tubulin gene family of maize (*Zea mays* L.). Evidence for two ancient α-tubulin genes in plants. J. Mol. Biol. **227,** 81–96.

Weigel, D. (1995). The APETALA2 domain is related to a novel type of DNA binding domain. Plant Cell **7,** 388–389.

Wessler, S., Tarpley, A., Purugganan, M., Spell, M., and Okagaki, R. (1990). Filler DNA is associated with spontaneous deletions in maize. Proc. Natl. Acad. Sci. USA **87,** 8731–8735.

Wienand, U., Weydemann, U., Niesbach-Klosgen, U., Peterson, P.A., and Saedler, H. (1986). Molecular cloning of the *C2* locus of *Zea mays,* the gene coding for chalcone synthase. Mol. Gen. Genet. **203,** 202–207.

Wingender, R., Rohrig, H., Horicke, C., and Schell, J. (1990). *cis*-Regulatory elements involved in ultraviolet light regulation and plant defense. Plant Cell **2,** 1019–1026.

Xiong, Y., and Eickbush, T.H. (1988). The site-specific ribosomal DNA insertion element R1Bm belongs to a class of non-long-terminal-repeat retrotransposons. Mol. Cell. Biol. **8,** 114–123.

Yanagisawa, S. (1995). A novel DNA-binding domain that may form a single zinc finger motif. Nucleic Acids Res. **23,** 3403–3410.

Ylstra, B., Touraev, A., Moreno, R.M.B., Stoger, E., van Tunen, A.J., Vicentie, O., Mol, N.N.M., and Heberle-Bors, E. (1992). Flavonols stimulate development, germination, and tube growth of tobacco pollen. Plant Physiol. **100,** 902–907.