The Plant Journal (2001) **27**(5), 467–478

Tissue-specific patterns of a maize *Myb* transcription factor are epigenetically regulated

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Summary

The maize *p1* gene encodes a *Myb*-homologous regulator of red pigment biosynthesis. To investigate the tissue-specific regulation of the *p1* gene, maize plants were transformed with constructs combining promoter and cDNA sequences of two alleles which differ in pigmentation patterns: *P1-wr* (white pericarp/red cob) and *P1-rr* (red pericarp/red cob). Surprisingly, all promoter/cDNA combinations produced transgenic plants with red pericarp and red cob (RR pattern), indicating that the *P1-wr* promoter and encoded protein can function in pericarp. Some of the RR patterned transgenic plants produced progeny plants with white pericarp and red cob (WR pattern), and this switch in tissue-specificity correlated with increased transgene methylation. A similar inverse correlation between pericarp pigmentation and DNA methylation was observed for certain natural *p1* alleles, which have a gene structure characteristic of standard *P1-wr* alleles, but which confer red pericarp pigmentation and are consistently less methylated than standard *P1-wr* alleles. Although we cannot rule out the possible existence of tissue-specific regulatory elements within the *p1* non-coding sequences or flanking regions, the data from transgenic and natural alleles suggest that the tissue-specific pigmentation pattern characteristic of the *P1-wr* phenotype is epigenetically controlled.

Keywords: epigenetic, maize, transgenic plants, tissue-specific, pericarp, flavonoids.

Introduction

Multi-cellular organisms are composed of a wide variety of cell types with distinctive patterns of gene expression. In plants the striking tissue-specific production of red and purple flavonoid pigments is controlled by differential expression of regulatory genes, which in turn activate genes encoding the biosynthetic enzymes of the pathway. Moreover, alleles of individual regulatory genes can have different tissue-specificities. The maize *p1* gene encodes a *Myb*-homologous regulatory protein required for synthesis of the red phlobaphene pigments (Grotewold *et al.*, 1991). Alleles of the *p1* gene confer a variety of spatial pigmentation patterns, which are most conspicuous in the pericarp (the outer layer of the kernel) and the cob glumes (the floral bracts subtending the kernel). A two-letter suffix in the allele designation denotes the presence or absence of pigmentation in pericarp and cob, respectively. Figure 1a shows the ear phenotypes of four common *p1* alleles: *P1-wr* (white pericarp/red cob), *P1-rr* (red pericarp/red cob), *P1-rw* (red pericarp/white cob), and *P1-ww* (white pericarp/white cob).

The *P1-rr* and the *P1-wr* alleles have been cloned and sequenced (Athma *et al.*, 1992; Chopra *et al.*, 1996; Chopra *et al.*, 1998; Grotewold *et al.*, 1991). Sequence comparison shows that the upstream regulatory regions of *P1-rr* and *P1-wr* are highly homologous, sharing 99% similarity for 5.2 kb 5′ of the transcription start site (Figure 2a). Upstream of this region the sequences differ: *P1-rr* has a 1.2-kb sequence that is not present in the upstream regulatory region of *P1-wr*, while *P1-wr* has a 1.1-kb sequence that is 96% homologous to a more distal region

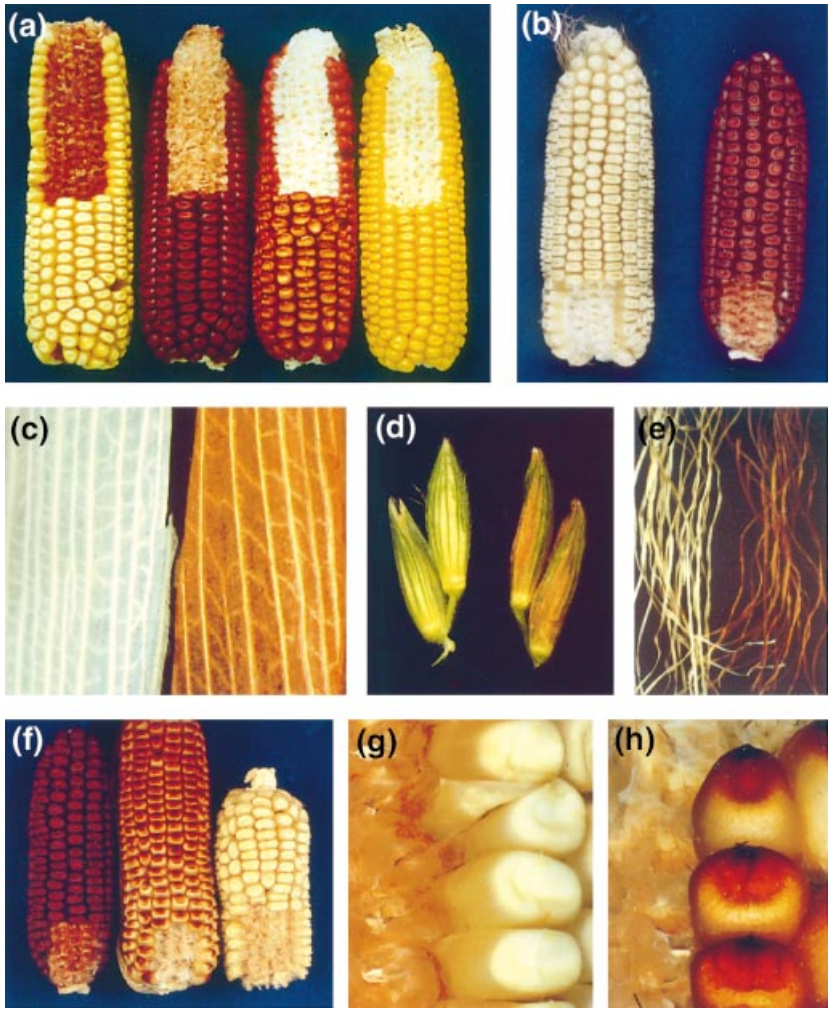

Figure 1. P::P transgene expression.
(a) Ear pigmentation patterns of standard *p1* alleles, from left to right, *P1-wr*, *P1-rr*, *P1-rw* and *P1-ww*.
(b) Ear phenotype of transformation recipient line Hi II (left) and pWRWR transgenic line SC12–8-2 (right).
(c–e) Phenotypes of a non-transformed plant (left) and pWRWR transgenic line SC12–8-2 (right) in dried husks (c), fresh tassel glumes (d), and dried silks (e).
(f–h) Patterns of ear pigmentation among T_2 plants of transgenic line SC12–8-2 (f) left to right, RR pattern, singed pattern, and WR pattern. Close-ups of WR pattern (g) and singed pattern (h).

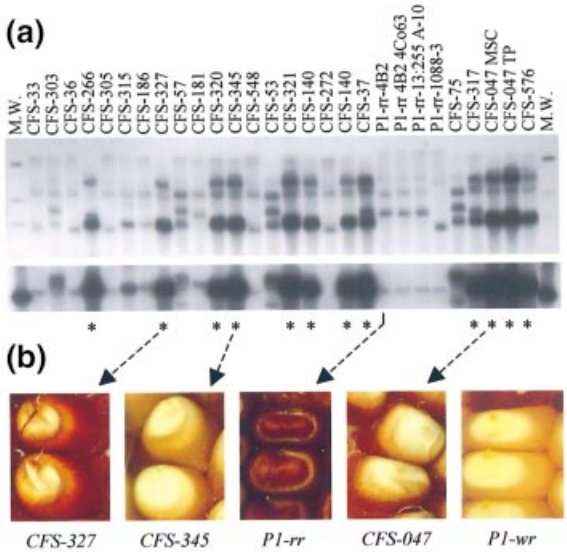

Figure 4. Identification of *p1* alleles with a *P1-wr* gene structure that confer red pericarp.
(a) Red pericarp and cob alleles from the Brink and Styles collection (CFS alleles) were analyzed by DNA gel blot analysis. The top panel shows *Bam*HI-digested DNA hybridized with *P1* genomic probe fragment 12, which hybridizes to exon 3 of both *P1-rr and P1-wr*. The bottom panel shows a *Hin*dIII digestion hybridized with probe pWRP62, which is present at the 3′ end of the *P1-wr* coding region (wr box in Figure 1). Previously characterized *P1-rr* alleles (*P-rr-4B2*, *P-rr-4B2* introgressed into a 4Co63 background, *P-rr-1088–3* and *P-rr-255 A-10*) were included for comparison. The single copy band detected with probe pWRP62 in the standard *P1-rr* samples reflects cross-hybridization with the recently identified *p2* gene (Zhang *et al.*, 2000). Asterisks indicate alleles that have a red gown phenotype.
(b) Pericarp phenotype of *p1* alleles (left to right) CFS-327, CFS-345, *P1-rr*, CFS-047, and *P1-wr* 4C063.

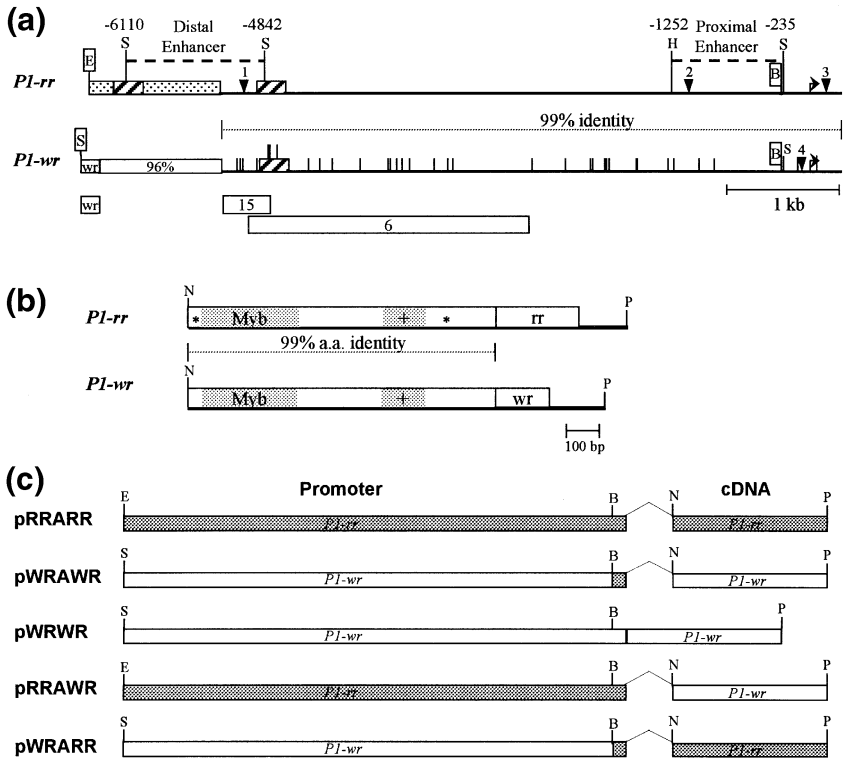

Figure 2. Comparison of *P1-rr* and *P1-wr* upstream regulatory and cDNA sequences utilized in the P::P transgene constructs.
(a) Maps of the upstream regulatory regions of *P1-rr* (upper) and *P1-wr* (lower). Numbers represent distances from transcription start site. Dashed lines indicate proximal and distal enhancer regions of *P1-rr*. On the *P1-rr* map, dotted boxes represent sequences from the 1.2 kb sequence unique to the *P1-rr* promoter and hatched boxes indicate a region of homology between *P1-wr* and *P1-rr* that is duplicated in *P1-rr*. The dotted line indicates a 5.2-kb region of 99% sequence identity between *P1-rr* and *P1-wr*. Within this region, short vertical lines on the *P1-wr* map demarcate sequence differences of 1–3 bp and solid triangles represent larger sequence differences. Triangles 1, 2 and 3 mark sequences of 6 bp, 15 bp and 6 bp, respectively, that are duplicated in *P1-rr*. Triangle 4 indicates a 19-bp insertion present in *P1-wr*. On the *P1-wr* map, the box labeled wr represents a *P1-wr*-specific sequence. The box labeled 96% indicates homology to sequences in *P1-rr* located 1.1 kb upstream of the EcoRI site, but not included in the *P1-rr* transgene constructs. Bent arrows show the transcription start sites. Boxed letters indicate restriction enzyme sites used in making transgene constructs. The positions of hybridization probes wr, 15, and 6 are as shown.
(b) Schematic maps of *P1-rr* and *P1-wr* cDNA sequences. The boxed regions represent protein coding sequences with shaded areas indicating putative functional domains (Myb) Myb DNA binding domain and (+) acidic activation domain. Asterisks represent DNA sequence differences that result in amino acid changes. The dotted line between the maps marks the region encoding 99% amino acid identity for the P1-rr and P1-wr proteins. The carboxy-terminal domains of P1-rr (rr) and P1-wr (wr) and the 3′ untranslated regions are completely different. The wr box represents the same sequence as the wr box in the *P1-wr* promoter diagram in Figure 2(a).
(c) Schematic drawings of the P::P constructs. Promoter and cDNA sequences included in the P::P constructs are indicated by gray boxes for *P1-rr* and white boxes for *P1-wr*. Angled lines indicate presence of the maize *adh1* gene intron 1.
B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; and S, *Sal*I.

of *P1-rr*. Functional analysis of the *P1-rr* promoter has identified three fragments containing regulatory elements: a 561-bp basal promoter region, a 1.0-kb proximal enhancer region and a 1.2-kb distal enhancer region (Sidorenko *et al.*, 2000; Sidorenko *et al.*, 1999). Similar analysis of the *P1-wr* promoter has not been performed; however, the basal promoter and proximal enhancer regions of *P1-rr* are located within the 5.2 kb region of 99% homology (Figure 2a).

The predicted P1-rr and P1-wr proteins contain two domains indicative of transcriptional activator function: a *Myb*-R2R3 DNA binding domain and a putative acidic transcriptional activation domain (Figure 2b). The first 347 amino acids of the P1-rr and P1-wr proteins are nearly identical, except for two amino acid differences outside of the predicted functional domains. In contrast, the carboxy-terminal regions of the P1-rr and P1-wr proteins are entirely different, producing a predicted zinc finger or metal binding domain in P1-wr that is not present in P1-rr (Chopra *et al.*, 1996).

Chopra *et al.* (1996) have proposed that the unique carboxy terminal domain of the P1-wr protein may mediate the *P1-wr* pattern of pigmentation through a post-transcriptional regulatory mechanism. This idea was supported by the detection of *p1* transcript in colorless *P1-wr* pericarp – albeit at 30% of the level in red *P1-rr* pericarp (Chopra *et al.*, 1996). However, the observation of *p1* transcript in *P1-wr* pericarp does not exclude the idea that tissue-specific expression of *P1-wr* may be transcriptionally regulated, e.g. reduced transcript levels in *P1-wr*

pericarp may produce insufficient protein to activate the phlobaphene pathway. Differential transcription of *P1-rr* and *P1-wr* promoters could be due to small sequence differences in the proximal 5.2 kb region of 99% homology, or from larger polymorphisms located greater than 5.2 kb from the transcription start site. Indeed, allele-specific differences in expression caused by promoter sequence differences have been reported for alleles of the maize anthocyanin regulatory genes *b1* and *r1* (Ludwig *et al.*, 1989; Radicella *et al.*, 1992).

Functional analyses of the anthocyanin regulatory genes *r1* (Ludwig *et al.*, 1990), *b1* (Goff *et al.*, 1990) or *c1* (Goff *et al.*, 1991) have been performed using microprojectile bombardment of maize aleurone cells, which resulted in activation of the entire anthocyanin biosynthesis pathway and production of red pigmented cells. Similar attempts to utilize microprojectile bombardment to test the function of *P1-rr* in maize pericarp did not induce phlobaphene biosynthesis (Grotewold *et al.*, 1998). The lack of phlobaphene pigment production in such transient assays may be due to the inability to reproduce a cell maturation process, which is thought to be required for polymerization of colorless flavan-4-ol to red phlobaphenes (Styles and Ceska, 1989). Use of a *P1-rr* promoter linked to a GUS reporter gene in transient assays were also not highly specific in reproducing the tissue-specificity observed *in planta* (Sidorenko *et al.*, 1999). These difficulties with transient assays prompted us to utilize stable transformation of maize to study the tissue-specific regulation of *P1-wr.*

To determine whether the tissue-specificity of P1-wr was conferred by sequence differences in the promoter or the protein coding region, we transformed maize plants with constructs containing approximately 6.2 kb of upstream regulatory sequences from *P1-wr* or *P1-rr* combined with the cDNA sequences of each allele (Figure 2c). All promoter/cDNA combinations produced plants with the red pericarp and red cob phenotype characteristic of *P1-rr*, or the red pericarp and white cob phenotype characteristic of *P1-rw*. None of the initial transgenic plants exhibited the white pericarp and red cob phenotype of *P1-wr*; however, some of the transgenic plants, which were initially of a *P1-rr* phenotype, produced progeny plants with a *P1-wr* phenotype. Thus, a single transgene construct could confer pigmentation patterns resembling three standard *p1* alleles: *P1-rr*, *P1-rw* and *P1-wr*. Further analyses of transgenic and natural *p1* alleles indicate that epigenetic effects play a role in determining the tissue-specific expression pattern of the *P1-wr* allele. Our results also demonstrate that isolating a gene and transforming it back into the plant may release the gene from the epigenetic controls that formerly governed its expression. Thus, plant transformation may be a potential tool for uncovering natural epialleles.

Results

All P1-wr *and* P1-rr *promoter/cDNA combinations produced transgenic plants with red pericarp and red cob*

The *P1-wr* and *P1-rr* promoter/cDNA constructs were transformed into maize by microprojectile bombardment. The 'parental' constructs (pWRAWR and pRRARR) contain the promoter of each allele with its respective cDNA, while the 'recombinant' constructs combine the promoter of one allele with the cDNA of the other allele (pWRARR and pRRAWR). The 'A' in each construct name indicates presence of the first intron of the maize *adh1* gene, which was introduced to increase transgene expression levels (Callis *et al.*, 1987). An additional construct, pWRWR, combines the promoter and cDNA of *P1-wr* but lacks the a*dh1* intron. Collectively, these will be referred to as P::P constructs. The transformation experiments were performed using the maize Hi-II line, which is phenotypically *P1-ww* and allows detection of transgene-promoted pigmentation in the initial transformants (T_0 generation). All P::P transgenes, including pWRWR and pWRAWR, conditioned uniform red pericarp and cob color that was indistinguishable from a standard *P1-rr* allele (Figure 1b). In addition to the *P1-rr*-like phenotype (RR pattern), considerable variability in pigmentation patterns and color intensities was observed among independent transgenic lines. Differential pigmentation was observed in the top and sides of the kernel, commonly referred to as crown and gown, respectively (Schwartz, 1982). Spatial patterns produced in the kernel pericarp included a colored gown with a white crown, a spot of color only at the silk attachment region, and an uneven blush on the kernels. Pigment intensities ranged from light orange to dark red in the pericarp and from white to dark red in the cob (not shown). To score expression of the P::P transgenes, presence of any visible pigmentation in pericarp and cob was given a positive (+) value and absence of pigmentation was given a negative (–) value. Based on this analysis, three classes of tissue-specific patterns were identified among initial P::P transformants: pigmentation in both pericarp and cob (+/+); pigmentation in pericarp, but not cob (+/–); and no pigmentation in pericarp or cob (–/–) (Table 1). Strikingly, none of the plants from the 62 independent transgenic T_0 lines exhibited colorless pericarp and colored cob (–/+), which would represent a *P1-wr* phenotype (Table 1).

P::P transgene expression in other tissues

In addition to pericarp and cob, the *p1* gene promotes pigmentation in husks, tassel glumes and silks. Husks and tassel glumes are differentially pigmented by *P1-rr* and *P1-wr*, being uniformly red in *P1-rr* plants and having red

Table 1. Pericarp and cob pigmentation patterns of P::P transgenic events[a]

Construct	Pigmentation patterns (pericarp/cob)[b]				Total events[c]
	+/+	+ –	–/+	–/–	
pWRAWR	5	6	0	9	18
pRRARR	5	4	0	6	15
pWRARR	5	3	0	8	12
pRRAWR	8	7	0	5	15
Total events	23	20	0	28	62

[a]T0 generation
[b]Given as the number of independent transformation events containing plants with the indicated pigmentation patterns. Patterns are given as the presence (+) or absence (–) of any visible pigmentation in pericarp and cob, respectively.
[c]The sum of events across pigmentation categories is greater than the total number of actual events because more than one pattern was observed among the progeny of some events.

margins in *P1-wr* plants. *P1-rr* and *P1-wr* both condition browning at the ends of freshly cut silks, which is caused by the oxidation of flavones (Levings and Stuber, 1971). The P::P transgenic plants were scored in the T_1 generation for transgene expression in husks, tassel glumes and silks. All of the transgene constructs produced some plants with uniformly pigmented husks and tassel glumes similar to that conferred by a *P1-rr* allele (Figure 1c,d). Plants with tassel glume margin pigmentation were also observed (not shown). In silks, each of the constructs produced plants exhibiting dark silk browning that was quite distinct from the very slight silk browning of non-transformed Hi-II plants. Red pigmentation could be observed in the silks of transgenic plants upon drying (Figure 1e). Of the 28 expressing lines scored in the T_1 generation, 11 had red phlobaphene pigmentation in all five organs – pericarp, cob, husks, silks and tassel glumes. The remaining 17 transgenic lines exhibited pigmentation in various subsets of these organs. Similar variation in spatial patterns was reported for maize plants transformed with P::GUS constructs, in which case the patterns appeared to conform to a developmental hierarchy (Cocciolone *et al.*, 2000).

We also observed transgene-conferred pigmentation of several tissues in which *p1*-regulated pigmentation had not previously been reported, including the sheath, auricle, ligule, and midrib of mature leaves, the pith of the stalk and the lateral veins of the coleoptile (not shown). The P::GUS constructs were also expressed in these tissues (Cocciolone *et al.*, 2000). Subsequent RT–PCR and RNA blot analysis of these vegetative tissues from non-transgenic *P1-wr* and *P1-rr* plants detected low levels of *p1* transcript (S. M. Cocciolone and L. V. Sidorenko, unpublished data).

RR pattern spontaneously switched to a WR pattern

Notably, the WR pattern of pigmentation was not observed among more than 500 T_0 plants from 62 independent lines transformed with the P::P constructs. However in two independent lines, plants with a RR pattern produced progeny plants with a WR pattern, having white pericarp, light to medium red pigmented cob, and red tassel glume margins. One of the lines, P2P10–36 contained the pWRAWR construct; in this line one out of four plants switched to a WR pattern in the T_1 generation. The other transgenic line, SC12–8-2, contained the pWRWR construct. All six of the T_0 transgenic plants of this line displayed uniform pigmentation in tassel and ear tissues similar to a standard *P1-rr* allele (Figure 1c–e), although the pigmentation tended to be darker and develop earlier (not shown). The switch from a *P1-rr* pattern to a *P1-wr* pattern occurred twice in this transgenic line: once among T_1 progeny derived from T_0 plant #6, and once among T_2 progeny derived from T_0 plant #10 (Figure 1f,g). In the latter case, an additional novel pigmentation pattern was also observed. This novel pattern had intense pericarp pigmentation concentrated at the silk attachment region that diminished to colorless or nearly colorless in the gown, giving the ear a 'singed' appearance; the cob was a uniform light red color (Figure 1f,h). To determine the heritability of the transgene phenotypes, plants with WR and singed patterns and four sibling plants with RR patterns were outcrossed with a *P1-ww* inbred line. As expected, approximately half of the T_3 progeny from this cross lacked the transgene and were herbicide sensitive. The remaining resistant plants were scored for transgene expression. The T_3 progeny plants generally inherited the RR, WR and singed patterns of their parents; although at a low frequency, the RR pattern switched to singed and inactive (WW) phenotypes and the singed pattern reverted to the original RR phenotype (Table 2). In contrast, the WR pattern neither reverted to a RR pattern, nor converted to a singed pattern in the 52 progeny plants grown; however, a few ears developed light red sectors in the pericarp gown (not shown). To summarize these observations, transformants that initially exhibit a *P1-rr* phenotype can produce progeny plants with WR or singed patterns, and these patterns are heritable but somewhat unstable.

Switch from RR pattern to WR pattern correlates with increased transgene methylation

The pWRWR line SC12–8-2 contained approximately 12 copies of the transgene construct, of which eight were truncated and up to four may be intact, based on DNA gel blot analysis (data not shown). Such multiple copy transgene insertions are a common outcome of the biolistic transformation method. Most often, the transgene

Table 2. Inheritance of transgene expression patterns[a]

Patterns[b] in T_2 generation	Patterns exhibited by T_3 plants				
	RR	Singed	WR	WW	Total
RR (4)	45	3	0	2	50
Singed (1)	1	44	0	0	45
WR (1)	0	0	52	0	52
Total	46	47	52	2	147

[a]Pericarp and cob pigmentation patterns given are for the progeny of the pWRWR transgenic line SC12–8-2, which were derived from plant #10 of the T_0 generation.
[b]The number of T_2 plants with each expression pattern are indicated in parenthesis.

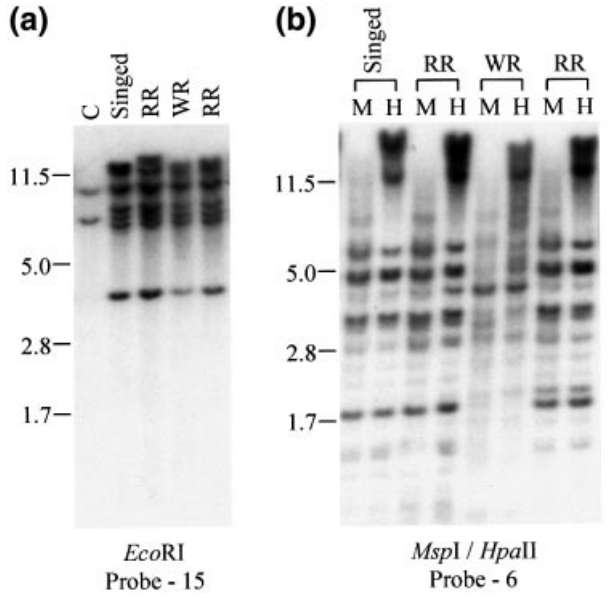

Figure 3. Gel blot analysis of DNA from plants with RR, WR, and singed patterns.
(a) Genomic DNA was isolated from sibling plants with RR, WR, and singed patterns (pWRWR transgenic line SC12–8-2; T_2 generation). DNA was digested with *Eco*RI and hybridized with *P1*-locus probe 15. Leaf DNA was used because the methylation state of endogenous *p1* alleles is generally conserved among different tissues (Chopra *et al.*, 1998; Das and Messing, 1994). The phenotypes of the transgenic plants are indicated above the lanes. Lane C contains DNA from a sibling plant that lacks the transgene. Positions of probe fragments are shown in Figure 1. Molecular size markers are indicated in kilobases.
(b) Genomic DNA samples as in (a) digested with *Msp*I (M) and *Hpa*II (H) and hybridized with *P1*-locus probe 6.

copies insert within or around a single chromosomal location and segregate as a single insertion event. However, since the transgene copies can also insert at unlinked sites within a genome, the three patterns observed for the pWRWR line SC12–8-2—RR, WR, and singed—could result from the segregation of independent transgene insertions. Alternatively, the different patterns may be produced by altered expression states of a single transgene insertion event. To distinguish between these possibilities, leaf DNA was isolated from sibling T_2 plants with RR, WR and singed patterns and digested with the methylation insensitive restriction enzyme *Eco*RI. Gel blot analysis and hybridization with *p1*-specific probe detected nearly identical banding patterns for the DNA samples, regardless of pigmentation pattern (Figure 3a). The same banding pattern was also observed for two additional RR patterned sibling plants (not shown). The only detectable difference was a shift in the upper band for one of the RR patterned plants (Figure 5a; lane 3), which is likely due to a spontaneous rearrangement of the transgene copies. Thus, the RR, WR and singed patterns represent different expression states of the same transgene insertion event.

Differences in gene expression in the absence of DNA sequence changes are commonly attributed to epigenetic regulatory mechanisms, which cause a change in gene expression without changing the DNA sequence of the gene. Epigenetic regulation in plants is often associated with alterations in DNA methylation, histone acetylation and/or chromatin structure. To determine if the switch from a RR pattern to WR and singed patterns correlated with changes in transgene methylation, DNA gel blot analysis was performed using the methylation sensitive enzymes *Msp*I and *Hpa*II. *Msp*I and *Hpa*II are isoschizomers that recognize the sequence CCGG but differ in sensitivity to DNA methylation, that is a methylation of either cytosine residue inhibits *Hpa*II cleavage, while *Msp*I is insensitive to methylation of the internal cytosine residue. Figure 3(b) shows that transgene DNA from the WR patterned plant was hypermethylated for both *Msp*I and *Hpa*II relative to the RR and singed patterns. This result was repeatable and observed with two probe fragments (fragments 6 and 15), both located in the promoter region; probe fragments within the coding region were not tested. The sizes of the hybridizing *Msp*I/*Hpa*II fragments were not the same as those predicted from the sequence of the transgene construct, likely because of the complexity of the transgene insertion and from methylation of HpaII sites within the transgene conferring the RR pattern of pigmentation.

In addition, progeny plants derived from each of the plants analyzed in Figure 3 were grown and subjected to similar DNA gel blot analysis. Each of the progeny plants reproduced the phenotypes of the parental plants. Out of seven RR patterned, four WR pattern and two singed plants, only the four plants with a WR patterned showed hypermethylation of the transgene DNA when digested with HpaII (not shown). This result indicates that the methylation patterns of the transgenes are maintained through meiosis.

Natural alleles with P1-wr *gene structure confer red pericarp phenotype*

In order to investigate the relationship of gene structure and expression in diverse *p1* alleles, we performed DNA gel blot analysis on 24 *P1-rr* lines originally collected and described by R. A. Brink and E. D. Styles (Brink and Styles, 1966). Historically, *p1* allelic designations are based on pigmentation patterns in the pericarp and cob (Figure 1a). More recently, molecular analyses have identified differences in the genomic structure of standard *P1-rr* and *P1-wr* alleles: *P1-rr* is a single copy gene, while *P1-wr* has six tandem gene copies (Chopra *et al.*, 1998). Interestingly, 12 of the lines classified as *P1-rr* by Brink and Styles had intensely hybridizing bands indicative of the amplified gene structure normally associated with *P1-wr* alleles (Figure 4a; top panel). These lines also showed intense hybridization with a probe specific for *P1-wr* (wr box, Figure 2b and Figure 4a, bottom). Extensive DNA gel blot analyses with probe fragments spanning the *p1* gene determined that the restriction maps of the 12 alleles with multicopy gene structures are identical to or only slightly modified from that of a standard *P1-wr* allele (not shown). Given the similarity of these alleles to *P1-wr* in gene structure, and to distinguish them from standard *P1-rr* alleles, we will refer to them as *P1-rr(wr)* alleles.

The *P1-rr(wr)* alleles all condition similar pigmentation patterns that differ from the uniform deep red color produced by standard *P1-rr*. Kernel pericarp pigmentation is lighter in intensity, ranging from medium red to light orange in the gown, and very light or colorless in the crown (Figure 4b). This red pericarp phenotype segregates with the *p1* locus, indicating that pericarp color is not specified by an unlinked gene (data not shown). In addition, the pigmentation pattern in other tissues is more similar to that conferred by *P1-wr*, with dark red cob color and pigmentation predominantly in the margin regions of husks and tassel glumes (not shown). Taken together, these results indicate that the *P1-rr(wr)* alleles are more similar to *P1-wr* alleles both in gene structure and plant phenotype, suggesting that the *P1-rr(wr)* alleles are actually *P1-wr* alleles that have either acquired or maintained competency to confer pericarp pigmentation

P1-rr(wr) *alleles are less methylated than standard* P1-wr *alleles*

To determine whether the red pericarp phenotype of the *P1-rr(wr)* alleles was associated with decreased DNA methylation relative to a standard *P1-wr* allele, we examined the methylation of 10 *P1-rr(wr)* alleles by DNA gel blot analysis. The standard *P1-wr* allele has six tandem copies of a 12.6-kb repeat unit (Chopra *et al.*, 1998). Each repeat unit contains three unmethylated *Hpa*II sites; two are located close together in exons 1 and 2, whereas the third is located near the beginning of exon 3. Hybridization of *Hpa*II-digested *P1-wr* DNA with a probe to the second intron detects a 4062-bp fragment, while a probe to the upstream regulatory region detects a 8105-bp fragment (Chopra *et al.*, 1998). All 10 of the *P1-rr(wr)* alleles exhibited some degree of hypomethylation relative to *P1-wr* (not shown); representative methylation patterns of three *P1-rr(wr)* alleles are shown in Figure 5. *P1-rr(wr)* alleles CFS-047, CFS-345 and CFS-327 produced the same 4062 bp *Hpa*II fragment present in *P1-wr* (Figure 5b); however, hybridization with probes to the upstream regulatory region (probes 15 and 6) and to exon 3 (probe 13) detected not only the 8105 bp amplified fragment observed for *P1-wr*, but also additional smaller fragments (Figure 5a). Most of the additional fragments could be mapped to *Hpa*II sites present within the *P1-wr* sequence (Figure 5b). CFS-047 and CFS-317 each produced a novel fragment that could not be mapped to the existing *Hpa*II sites of the standard *P1-wr* allele; these novel fragments probably result from heterogeneity of the methylation pattern or sequence polymorphism among *P1-wr* gene copies. The hypomethylated *Hpa*II sites of the *P1-rr(wr)* alleles were primarily localized to the upstream regulatory region, and in intron 2 of one of the alleles.

The analysis of *P1-wr* methylation was originally performed using inbred line W23 (Chopra *et al.*, 1998). To determine whether *P1-wr* alleles in different genetic backgrounds have different methylation patterns, we examined the methylation status of *P1-wr* alleles in three genetic backgrounds: inbred line W23, inbred line C123, and *P1-wr* from W23 introgressed into inbred line 4Co63. The latter was included because the *P1-rr(wr)* alleles are in a 4Co63 genetic background. For all three *P1-wr* lines, cleavage with *Hpa*II produced only the hypermethylated fragments detected for *P1-wr* W23 (Figure 5a), that is genetic background did not alter the pattern of *P1-wr* methylation. Hence, the detection of hypomethylated fragments in *Hpa*II digestions of the *P1-rr(wr)* alleles cannot be attributed to effects of genetic background. We conclude that, analogous to the results from the P::P transgene studies, the natural *P1-wr* gene may exist in alternate tissue-specific expression states which are associated with patterns of DNA methylation

Discussion

The white kernel/red cob phenotype common to Midwestern corn varieties is conditioned by the *P1-wr* allele. To determine whether the *P1-wr* promoter or encoded protein confers this tissue-specific phenotype, maize plants were transformed with constructs containing *P1-wr* and *P1-rr* promoter and cDNA sequences in various combinations. We predicted that constructs combining the

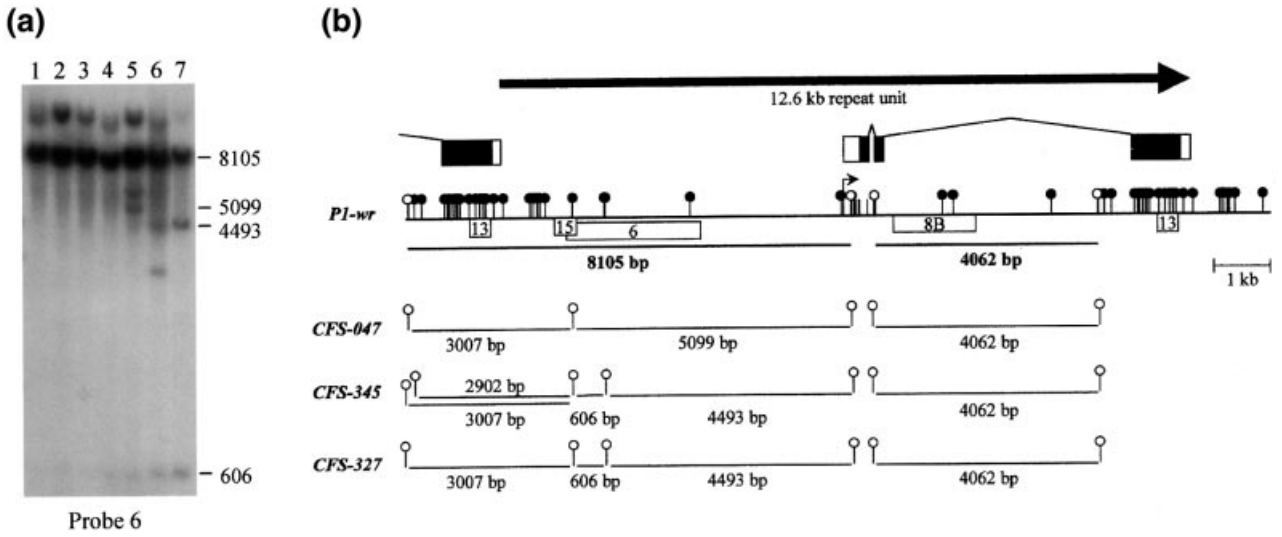

Figure 5. *P1-rr(wr)* alleles are hypomethylated relative to *P1-wr*.
(a) Methylation states of *P-wr* and *P-rr(wr)* alleles. Genomic DNA from leaves of *P-wr* and *P-rr(wr)* plants was digested with *Hpa*II and hybridized with *P1* genomic probe fragment 6. Lanes 1 through 4 contain *P1-wr* genotypes in four different genetic backgrounds (lane 1, W23; lane 2, W22; lane 3, C123; and lane 4, *P1-wr* allele from W23 introgressed into 4Co63 background). Lanes 5 through 7 contain *P1-rr(wr)* genotypes (lane 5, CFS-047; lane 6, CFS-345; and lane 7, CFS-327). The sizes of DNA bands corresponding to *P1-wr* fragments were deduced from the genomic sequence. CFS-047 and CFS-317 each produced a fragment that did not correspond to known *Hpa*II sites of *P1-wr*: CFS-047 has a band of approximately 5.6 kb that hybridizes with probe fragments 13, 15, and 6, and CFS-317 has a band of about 3.6 kb that hybridizes only to probe 6.
(b) Methylation maps of *P-wr* and *P-rr(wr)* alleles. The arrow delineates a single 12.6 kb *P1-wr* repeat. Boxes (exons) joined by bent lines (introns) represent transcribed sequences. Open boxes indicate 5′ and 3′ untranslated regions and the solid boxes indicated coding regions. Exon 3 of the upstream repeat, as well as the complete transcribed region of the 12.6 kb repeat, are indicated. The bent arrow shows the transcription start site. Vertical lines indicate the positions of *Hpa*II sites: open circles represent unmethylated sites; closed circles represent methylated sites; and vertical lines without circles represent sites of undetermined methylation status. Numbered boxes represent hybridization probes. Digestion of *P1-wr* with *Hpa*II produces 8105 and 4062 bp fragments as indicated below the *P1-wr* map (Chopra *et al.*, 1998). *Hpa*II digestion of *P1-rr(wr)* alleles CFS-047, CFS-345, and CFS-327 produces additional fragments; those corresponding to the *P1-wr* restriction map are shown as horizontal lines with fragment sizes given in base pairs.

promoter and cDNA of a single allele would produce plants with the phenotype of the respective endogenous allele, while the plant phenotypes produced by the chimeric promoter/cDNA constructs would indicate whether the promoter or the protein determined the *P1-wr* pattern of pigmentation. Surprisingly, all promoter/cDNA combinations produced plants with red pericarp and red cob similar to a *P1-rr* allele, while none of the transgene constructs produced T_0 plants with the white pericarp and red cob pattern of a *P1-wr* allele. Thus the *P1-wr* promoter, when incorporated in a transgene, is capable of inducing adequate transcription levels to fully activate the phlobaphene biosynthetic pathway in pericarp. Likewise, the *P1-wr* encoded protein can function in the pericarp despite the presence of a unique carboxy-terminal domain. This result indicates that neither the promoter nor the encoded protein is sufficient to specify a *P1-wr* pattern of pigmentation. Hence, the regulatory elements required for tissue-specific regulation of *P1-wr* may either be missing from the transgene construct or were not reproduced in the transgenic experiment.

Since the P::P transgenes contained the cDNA sequence of *P1-wr*, potential regulatory elements could be located within intron or 3′ flanking regions. The *P1-wr* and *P1-rr* alleles have two conserved introns of 118 bp and 4.6 kb that are 100% and 99.5% homologous, respectively, while the 3′ flanking regions of the two alleles differ considerably due to complex gene rearrangements (Chopra *et al.*, 1996). Transformation experiments are in progress to determine if these genomic sequences perform a regulatory role. The regulatory elements might also be located outside of the *P1-wr* complex. Alternatively, a specific DNA modification state or a chromatin conformation that was not reproduced in the initial transgenic plants may be required for correct tissue-specific regulation of *P1-wr*. Indeed, the *P1-wr* allele differs considerably from *P1-rr* with respect to gene structure and DNA methylation. *P1-rr* is a single copy gene, while *P1-wr* contains six head-to-tail tandem repeats that each includes 6.3 kb of genic sequence and 6.3 kb of upstream sequence. In addition, *P1-wr* sequences are heavily methylated relative to *P1-rr*, with each repeat unit having identical patterns of methylation (Chopra *et al.*, 1998). Repetitive sequences and DNA methylation have both been implicated in a number of epigenetic phenomena, including: repeat-induced point mutation (RIP; Selker, 1990), methylation induced premeiotically (MIP; Rhouonim *et al.*, 1992), paramutation (reviewed in Hollick *et al.*, 1997), and homology-dependent transgene

silencing (reviewed in Matzke and Matzke, 1998). A model for epigenetic regulation of *P1-wr* has been proposed by Chopra *et al.* (1998), in which association of the tandem repeat units via intra-allelic pairing in somatic tissues modulates the tissue-specific expression of the *P1-wr* gene copies.

Our analysis of P::P transgene expression and heritability lend support to the idea that *P1-wr* is epigenetically regulated. Although none of the initial P::P transgenic lines produced plants with a WR pattern of pigmentation, in two independent transgenic lines the RR pattern switched to a WR pattern. In the best studied line, pWRWR transformant SC-12–8-2, the RR pattern is metastable – switching not only to a WR pattern, but also to a completely inactive state or to a novel pattern (singed) that has dark pigmentation at the crown of the kernel and a light or colorless gown. Conceptually, the singed pattern could represent an intermediate of the RR and WR patterns; however, the origin and phenotypic behavior of the WR pattern does not support this idea. The WR pattern originated directly from transgenic plants with the RR pattern. In addition, plants with the WR pattern occasionally produced ears with light sectors of pericarp pigmentation, but this pigmentation was found in the pericarp gown, and not at the silk attachment region. Hence, the WR and singed expression patterns likely arise by different regulatory mechanisms or represent alternative expression states.

When related transgenic plants with different expression patterns were analyzed by DNA gel blot analysis, the presence of similar transgene bands indicated that the various tissue-specific patterns were produced by differential expression of the same transgene insertion. In addition, digestion with methylation sensitive enzymes showed that the transgene was hypermethylated in plants expressing a WR pattern relative to those with RR or singed patterns. The observed changes in transgene expression occurred spontaneously and sporadically and do not appear to fit the general models for homology dependent gene silencing, including paramutation or cosuppression. The *P1-ww* allele carried by the transgenic lines is not known to be involved in inducing paramutation, neither does it suppress the expression of other functional *p1* alleles. Also, the transgenes were maintained in a hemizygous condition to avoid silencing effects that can be associated with homozygosity (de Carvalho *et al.*, 1992).

The inverse correlation between methylation and pericarp pigmentation observed for the P::P transgenes was also seen in plants carrying *P1-pr*, a spontaneous epiallele of *P1-rr*. *P1-rr* and *P1-pr* are identical in DNA sequence, but differ in DNA methylation and chromatin structure (Das and Messing, 1994; Lund *et al.*, 1995). Hypermethylated *P1-pr* alleles confer a variegated or nearly colorless pericarp phenotype and reduced cob pigmentation. Heritable suppression of *P1-rr* expression can also be induced by exposure to a P::GUS transgene containing the 1.2 kb distal upstream enhancer region of *P1-rr* (Sidorenko and Peterson, 2001). This suppressed state of *P1-rr* resembles paramutation and is associated with increased methylation and decreased *p1* transcript levels. Here, we also report a similar inverse correlation between pericarp pigmentation and DNA methylation for natural *P1-wr* alleles. We identified a class of *p1* alleles that confer red pericarp pigmentation, but have the multicopy gene structure of a standard *P1-wr* allele. All of the *P1-rr(wr)* alleles exhibited less DNA methylation than a standard *P1-wr* allele; however, the lower level of methylation did not occur in all gene copies, but instead occurred in either a subset of gene copies within the tandem repeat, or all of the gene copies in a subset of tissues. *Hpa*II digestion products from the hypomethylated region of *P1-rr(wr)* alleles were evident as discrete bands that consisted of adjacent fragments within a gene copy. This observation suggests that the hypomethylation occurred coordinately at specific sites located primarily in the upstream regulatory regions within particular gene copies. Taken together, these results suggest that the *P1-rr(wr)* alleles represent *P1-wr* alleles competent to condition pericarp pigmentation and that this competency is associated with decreased DNA methylation.

Several lines of evidence have mechanistically linked DNA methylation with histone deacetylation and chromatin remodeling. Genetic screens to detect Arabidopsis mutants defective in DNA methylation identified *ddm1*, which causes a 70% reduction in genomic DNA methylation and progressive development of morphological abnormalities (Kakutani *et al.*, 1996; Vongs *et al.*, 1993). The DDM1 protein is a member of the SWI2/SNF2 gene family of chromatin-remodeling proteins, and is required for maintenance of DNA methylation (Jeddeloh *et al.*, 1999). In animal systems, some of the methyl-CpG-binding proteins are physically associated in complexes with histone deacetylases. Histone deacetylases are integral components of the cellular machinery involved in establishing repressive chromatin (reviewed in Bird and Wolffe, 1999). Interestingly, the pattern of *P1-wr* methylation is similar for both pericarp and cob tissues (Chopra *et al.*, 1998). Hence, the tissue-specific pattern of *P1-wr* may not be controlled directly by DNA methylation, but rather at the level of chromatin structure. Chromatin structure has been proposed to be the primary determinant of the epigenetic state of *Pl1-Blotched*, an epiallele of the maize anthocyanin regulatory gene *Pl1-Rhoades*. Both alleles exhibit changes in DNA methylation during development; however, the chromatin structure of *Pl-Blotched* is consistently more nuclease-resistant than *Pl1-Rhoades* in both juvenile and adult tissues (Hoekenga *et al.*, 2000).

Natural *p1* alleles exhibit a high degree of phenotypic variability. Over 100 *p1* alleles have been collected and introgressed into a common genetic background; in this collection, pericarp color forms a continuum from dark red to colorless (Brink and Styles, 1966). An example of the variation in *p1* allele pigmentation intensity can be seen in Figure 4(b). Similarily, the P::P transgenes produced a wide variety of tissue-specific expression patterns and pigmentation intensities in pericarp and cob, irrespective of whether the sequences were derived from *P1-wr* or *P1-rr*. This phenotypic variability was observed among plants transformed with the same transgene construct, and a single construct could produce pigmentation patterns resembling three standard alleles with distinct expression patterns in pericarp and cob: *P1-rr*, *P1-rw*, and *P1-wr*. When pigmentation of other organs such as husks, silks, and tassel glumes is considered, the variety of spatial pigmentation patterns observed in both transgenic and natural genetic stocks is greatly increased. These observations suggest that differences in epigenetic regulation, rather than DNA sequence polymorphism, can be a major contributor to diversity in gene expression patterns in plants.

Experimental procedures

Maize stocks

Unless otherwise noted, the *P1-rr* allele used in this study was *P1-rr-4B2* (Grotewold *et al.*, 1991) and the *P1-wr* allele was from inbred line W23 (obtained from the Maize Genetics Cooperation Stock Center, Urbana, IL, USA). Inbred line 4Co63 (genotype *P1-ww*) was obtained from the National Seed Storage Laboratory, Fort Collins, CO, USA. Inbred lines W22 and C123 (*P1-wr* genotypes) were provided by Jerry Kermicle (University of Wisconsin, Madison, WI, USA) and Benjamin Burr (Brookhaven National Laboratory, Upjon, NY, USA), respectively. *P1-rr-1088–3* differs from *P-rr-4B2* in the absence of a 1.6-kb transposable element located 6.3 kb upstream from the transcription start site (Sidorenko *et al.*, 1999). *P1-rr-255 A-10* was a *P-rr* revertant derived by excision of the *Ac* transposable element from *P-vv9D42B* (Athma *et al.*, 1992). Seed of a large number of distinct *p1* alleles, collected and described by Brink and Styles (1966), was obtained from the National Seed Storage Laboratory.

Plasmid construction

The upstream regulatory regions of the P::P constructs consisted of either the *Eco*RI-*Bgl*II fragment of *P1-rr* (–6414 to –261) or the *Sal*I-*Bgl*II fragment of *P1-wr* (– 6495 to –282). The *P1-rr* cDNA and Nos terminator used in pWRARR and pRRARR constructs were obtained from the p35SARR plasmid. p35SARR is identical to pPHI1962 (Grotewold *et al.*, 1994), except that the *P1-rr* cDNA sequence was truncated at the *Pst*I site (position 1689) in the 3′ untranslated region, and the polyadenylation sequence from the potato proteinase inhibitor II (PinII) gene was replaced with the nopaline synthase polyadenylation sequence. A *Nco*I site was introduced at the translation start site of the *P1-wr* cDNA by PCR-mediated site-directed mutagenesis utilizing the mutagenesis primer SC1217 (5′-GGCGCGCCATGGGGAGGGC-3′) and downstream primer EP3–12 (5′-AAGCTTGAATTCGAGTTCCAGTAGTTCTTGATC-3′). The *P1-wr* cDNA sequence from the introduced *Nco*I site to a *Pst*I site (position 1578) in the 3′ untranslated region was included in pWRAWR and pRRAWR. The maize *adh1* first intron was incorporated into the constructs as a *Bgl*II-*Nco*I fragment from P1.0b::GUS (Sidorenko *et al.*, 1999), which also included the 5′ untranslated leader and proximal promoter of *P1-rr* (–235 to + 326). For the pWRWR construct, the upstream *Sal*I-*Bgl*II fragment of *P1-wr* (– 6495 to –282) was joined to the full-length *P1-wr* cDNA (Chopra *et al.*, 1996) by a *Bgl*II-*Pvu*II genomic fragment (– 282 to + 142), and included the PinII terminator from pPHI1962.

Tissue culture, transformation and transgenic plant handling

Transformation of maize plants with the pWRAWR, pRRARR, pWRARR, and pRRAWR constructs was performed by the Plant Transformation Facility at Iowa State University (Frame *et al.*, 2000). Type II callus was cobombarded with the plasmid of interest and the plasmid pBAR184(–) (Frame *et al.*, 2000), which confers resistance to the herbicide Bialaphos. Bialaphos-resistant calli were screened for P::P transgene inserts using standard PCR procedures.

Transformation of the pWRWR construct was performed as follows. Ears of maize Hi II germplasm (Armstrong and Green, 1985) were harvested 9–10 days after pollination and sterilized in 70% ethanol for 1 min followed by 50% bleach (Clorox ^{TM}) for 10 min. After three rinses with sterile water, 1–1.5 mm immature embryos were excised and placed on medium with the scutellum exposed. Medium for callus induction and maintenance contained N6 salts and vitamins (Chu *et al.*, 1976), supplemented with 2.0 mg l^{-1} 2,4-D, 2.9 g l^{-1} L-proline, 100 mg l^{-1} casein hydrolysate, 8.5 mg l^{-1} $AgNO_3$ and 20 g l^{-1} sucrose. All media were adjusted to pH.5.8 and solidified with 2.4 g l^{-1} Gelrite® and cultures were kept in the dark at 28°C. Callus was maintained by subculturing weekly and pre-embryogenic callus lines were selected (Welter *et al.*, 1995). Prior to bombardment, callus pieces (3–4 mm) were placed on N6 medium with 0.69 g l^{-1} L-proline, 2.0 mg l^{-1} 2,4-D, 0.86 mg l^{-1} $AgNO_3$ and 120 g l^{-1} sucrose for osmotic pretreatment (3–4 h). Bombardments were as described by Klein *et al.*, 1988 and Bowen, 1992). The pWRWR construct was co-bombarded with the selectable-marker plasmid pPHI3528 that contains the BAR gene driven by the 35S promoter (De Block *et al.*, 1987). Following bombardment, callus was transferred to N6 medium containing 2.0 mg l^{-1} 2,4-D, 0.86 mg l^{-1} $AgNO_3$, 30 g l^{-1} sucrose and 0.69 g l^{-1} L-proline. Three to five days after bombardment, callus was transferred to N6 selection medium containing Bialaphos (3 mg l^{-1}), 2.0 mg l^{-1} 2,4-D, 0.86 mg l^{-1} $AgNO_3$ and 30 g l^{-1} sucrose. During the selection stage, callus was transferred to fresh medium biweekly. At 6–8 weeks after bombardment, Bialaphos-resistant calli were picked and transferred to fresh medium of the same composition for an additional 2 weeks. Actively growing calli were moved to embryo maturation medium containing MS salts (Murashige and Skoog, 1962) with 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} zeatin, 0.5 mg l^{-1} IAA, 0.5 mg l^{-1} ABA, 60 g l^{-1} sucrose and no selective agent. After 2 weeks mature somatic embryos were moved to the light on germination medium containing MS salts and vitamins, 100 mg l^{-1} myo-inositol, 40 g l^{-1} sucrose and no selective agent.

The regenerated plants (T_0) were grown to maturity in the greenhouse and outcrossed to inbred line 4Co63 (*P1-ww*). Transgenic plants were identified in segregating populations by resistance to foliar applications of the herbicide Liberty((AgrEvo, Wilmington, DE, USA) diluted to 1.6% of the active ingredient.

DNA gel blot analysis

Genomic DNA was isolated from either fresh or lyophilized leaf tissue by the CTAB method (Saghai-Maroof *et al.*, 1984) and digested with restriction enzymes according to the manufacturer's instructions. The fragments were separated by gel electrophoresis on 0.7% agarose gels and transferred to nylon membranes. Filters in Figure 4 were produced according to the UMC Maize RFLP Procedures Manual (University of Missouri, 1995) and hybridized as previously described (Byrne *et al.*, 1996). Filters in Figures 3 and 5 were produced and hybridized as described by Cone *et al.* (1986).

Acknowledgements

We would like to acknowledge Kan Wang, Bronwyn Frame and the staff of the Iowa State University Plant Transformation Facility for maize transformation. We thank Larry Darrah for providing comments on the manuscript. This research was supported by a grant from the National Science Foundation to T. P., Journal Paper No. J-19130 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project 3297, and supported by Hatch Act and State of Iowa funds.

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