Transgene-Induced Silencing Identifies Sequences Involved in the Establishment of Paramutation of the Maize p1 Gene

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A transgene carrying a distal enhancer element of the maize P1-rr promoter caused silencing of an endogenous P1-rr allele in the progeny of transgenic maize plants. Expression of both the transgene and the endogenous P1-rr allele was reduced in the affected plants. The silenced phenotype was observed in the progeny of seven of eight crosses involving three independent transgenic events tested (average frequency of 19%). This phenotype was associated with an induced epigenetic state of the P1-rr allele, termed P1-rr⁹, which is characterized by increased methylation of the P1-rr flanking regions and decreased levels of P1-rr transcript. The P1-rr⁹ epiallele is highly heritable in the absence of the inducing P1.2b::GUS transgene, and it can impose an equivalent state on a naive P1-rr allele in subsequent crosses (paramutation). In contrast, parallel experiments with two other P::GUS transgenes that contained the same basal P1-rr promoter fragment but different upstream sequences revealed no detectable silencing effect. Thus, transgenes carrying a specific enhancer fragment of the P1-rr gene promoter can trigger a paramutant state (P1-rr⁹) of the endogenous P1-rr gene that is maintained in the absence of the inducing transgene. We discuss the potential role of the P1-rr distal enhancer element in the establishment and propagation of a paramutation system in maize.

INTRODUCTION

Homology-dependent gene silencing can occur between two homologous transgenes (Meyer et al., 1992; Vaucheret, 1993; M.A. Matzke et al., 1994) or between a transgene and a homologous endogenous gene (Napoli et al., 1990; Vaucheret et al., 1995; Itoh et al., 1997). The mechanisms of homology-dependent gene silencing are in many respects still unclear, but they appear to involve at least two distinct epigenetic processes, one operating at the transcriptional level and one at post-transcriptional levels (reviewed in Stam et al., 1997; Vaucheret et al., 1998). In post-transcriptional silencing, the transgene and the endogenous gene share homology within the coding sequence (Hart et al., 1992; Lindbo et al., 1993). Both the transgene and the endogenous gene produce transcripts that are quickly degraded in the cytoplasm, and as a result, little or no protein is produced in affected cells. Post-transcriptional silencing requires the continued presence of the homologous coding sequences to maintain silencing. In contrast to post-transcriptional silencing, transcriptional gene silencing (TGS) is established via promoter homology and results in the reduction of transcription per se. Maintenance of the suppressed transcriptional state of genes affected by TGS does not require the continuous presence of the transgene, because reduced transcription states can be heritable for several generations after segregation of the homologous sequences (Hart et al., 1992; Assaad et al., 1993; Vaucheret, 1993). TGS has been associated with local chromatin compaction and is often correlated with increased methylation of the silenced gene (Meyer, 1995; Park et al., 1996; Ye and Signer, 1996).

A classic example of natural TGS, called paramutation, occurs when certain combinations of alleles are present together in heterozygous conditions. Paramutation was first described in maize for the r1 (Red1) gene (Brink, 1956) and later was shown to occur for two other maize genes, b1 (Booster [Coe, 1959]) and p1 (Purple plant [Hollick et al., 1995; Chandler et al., 2000]), each of which encode transcriptional regulators of anthocyanin biosynthesis. Paramutation produces a heritable change in the expression state of the affected allele, resulting in reduced tissue pigmentation. Paramutation of b1 and p1 was correlated with reduced transcription of the affected genes, without any detectable changes in their primary DNA sequence (Hollick et al., 1995; Chandler et al., 2000). In addition to these natural paramutation phenomena in maize, certain transgene loci in petunia (Meyer et al., 1992), tobacco (Vaucheret, 1993; A.J. Matzke et al., 1994), and rice (Itoh et al., 1997) are
reported to interact in a manner similar to that seen in para-mutation. These results indicate that trans-silencing effects resulting from homology-based interactions similar to para-mutation are likely to be widespread in plants.

The maize p1 gene encodes a myb-homologous transcriptional regulator of biosynthesis of a red phlobaphene pigment accumulating in husks and floral organs including silks, kernel pericarp, cob, and tassel glumes (Lechelt et al., 1989; Grotewold et al., 1991). Alleles of the p1 gene are designated by a two-letter suffix corresponding to their pigmentation in pericarp and cob. According to this classification, an allele giving red pericarp and red cob is termed P1-rr, an allele giving white pericarp and red cob is termed P1-wr, and an allele giving white pericarp and white cob is termed P1-ww. The P1-rr regulatory elements have been characterized by transposon mutagenesis (Athma et al., 1992; Moreno et al., 1992) and functional tests using transient expression assays (Sidorenko et al., 1999) and transgenic maize plants (Cocciolone et al., 2000; Sidorenko et al., 2000). The results indicate that the 5’ regulatory region of the P1-rr allele has a complex structure consisting of a basal promoter (Pb), including 235 bp of promoter and 326 bp of untranslated leader, and two enhancer elements. The proximal enhancer is contained within an ~1-kb fragment (P1.0) located 5’ adjacent to the basal promoter, whereas a distal enhancer is found in a 1.2-kb fragment (P1.2) located 5 to 6.2 kb upstream of the transcription start site (Figure 1).

In this study, we tested for epigenetic interactions between transgenes carrying either proximal (P1.0) or distal (P1.2) enhancer elements and the endogenous p1 gene alleles. The results indicate that plants carrying transgenes with the distal enhancer fragment (P1.2) exhibited strong suppression of both the transgene and the endogenous P1-rr gene. The phenotype of the suppressed P1-rr allele, termed P1-rr’, was highly heritable and correlated with increased DNA methylation and decreased transcript levels. Moreover, P1-rr’ exhibited paramutagenic effects on a naive P1-rr allele. These results demonstrate that an ectopic transgene carrying the P1.2 enhancer fragment established a paramutant state of the endogenous P1-rr allele that was maintained subsequently in the absence of the inducing transgene. In contrast, no heritable silencing effects were observed in parallel experiments using two other transgene constructs containing the proximal P1.0 enhancer fragment. These results link paramutation directly with transgene silencing and show how transgenic plants may be used to identify se-

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**Figure 1.** Map of the P1-rr Gene and Transgene Constructs.

*(Top)* The maize P1-rr gene coding sequence is indicated by small black boxes (exons) and thin lines (introns); the transcription start site is indicated by a bent arrow. The two 5.2-kb direct repeats flanking the coding sequence are shown as black bars, and the 1.2-kb direct repeats are shown as hatched boxes. The triangle indicates a 1.6-kb transposon present in standard P1-rr (P1-rr-4B2).

*(Bottom)* The P1.0b::GUS, P1.2b::GUS, and P2.0b::GUS transgene constructs used in this study. All constructs contain the basal fragment of the P1-rr promoter sequence (Pb; horizontally striped box), the AdhI gene intron I (bent line), the GUS gene coding region (dotted box), and the PinII gene transcription terminator (black box). P1.0b::GUS contains a 1-kb proximal enhancer fragment (open box); P1.2b::GUS contains an additional upstream-adjacent 1-kb HindIII fragment. P1.2b::GUS contains a 1.2-kb distal (hatched box) enhancer fragment in reverse orientation relative to its genomic position.
quences potentially involved in the establishment of paramutation.

RESULTS

The P1.2b::GUS Transgene Silences the Endogenous P1-rr Allele

To detect possible epigenetic interactions of the P::GUS (β-glucuronidase) transgenes and the endogenous p1 gene, we crossed plants homozygous for the P1-ww allele and hemizygous for the P1.2b::GUS or P1.0b::GUS transgene with a homozygous P1-rr tester line. Simple dominance of the P1-rr allele over the recessive P1-ww allele should produce F1 progeny with uniform red kernel pericarp and cob. However, ~20% of F1 progeny ears derived from crosses involving the P1.2b::GUS transgene exhibited a striking reduction of ear pigmentation and altered spatial distribution of pigment (Figure 2). This reduced pigmentation reflects a heritable suppression of expression of the P1-rr allele (see below), and the silenced state is identified as P1-rr’. The P1-rr’ ears had nonuniform pericarp pigmentation ranging from heavily striped to nearly colorless, with pigmentation only at the silk attachment region. Some P1-rr’ ears appeared variegated, with sharp-edged sectors that originated near the silk attachment region and stretched toward, but rarely reached, the base of the kernel. On some ears, large multiple-kernel sectors with different degrees of variegation were observed. These large sectors indicate that changes in the pattern of P1-rr expression occurred at early stages of ear development. Pigmentation of other organs, such as cob, husks, and tassel glumes, was also reduced in the affected plants. The color of cob glumes varied from light red for weakly suppressed P1-rr’ ears, to pink or colorless with light pink edges for strongly suppressed ears. Husks were striped or had colorless blades with lightly pigmented margins. The pigmentation of tassel glumes varied from light red to colorless with pink margins. Interestingly, the P1-rr’ ear and tassel pigmentation phenotype resembled that produced by P1-pr, an epiallele of P1-rr isolated previously by Das and Messing (1994).

The suppressed pigmentation characteristic of P1-rr’ was detected in the progeny of seven of eight crosses involving three independent P1.2b::GUS transgenic events tested. The frequency of silenced ears in individual crosses varied from 0 to 50%, with an overall average frequency of 19% (25 suppressed ears out of 126 total ears; Table 1). Significantly, 20 of 25 suppressed P1-rr’ ears were derived from plants that were resistant to herbicide. Because the particle bombardment transformation method used here results in frequent cointegration of the P::GUS and 35S::BAR constructs, most of the herbicide-resistant plants also carried the P1.2b::GUS transgene. Among the five plants that were herbicide sensitive and had a suppressed P1-rr’ phenotype, one plant was shown to carry a transgene insert by DNA gel blot analysis (the other four plants were not tested). Moreover, the frequency of herbicide-resistant plants in the progeny was less than expected (32% observed versus 50% expected). Thus, the four exceptional plants that were herbicide sensitive and produced suppressed P1-rr’ ears may have carried silenced transgene loci similar to those described for rice transgenic plants (Kumpatla et al., 1997). Together, these results indicate that the origin of the suppressed P1-rr’ phenotype is associated with the presence of the P1.2b::GUS transgene.

Importantly, parallel analyses conducted with the P1.0b::GUS transgene carrying the proximal enhancer fragment (Figure 1) revealed no significant effect on the phenotype of the endogenous P1-rr allele (Table 1). We also tested a third transgene (P2.0b::GUS) containing the P1.0 enhancer fragment plus 1 kb of 5’ adjacent sequence (Figure 1); this

![Figure 2. Representative Results Demonstrating the Silencing of P1-rr in the F1 Progeny of a Cross with a P1-ww Plant Containing the P1.2b::GUS Transgene (event 29-3).](image)
transgene also had no effect on P1-rr expression (Table 1). Therefore, suppression of P1-rr was observed only with a transgene that carried the 5-kb distal 1.2-kb enhancer-containing fragment.

### P1-rr and P1.2b::GUS Are Cosilenced

To determine whether transgene expression was affected in P1-rr plants, we examined F1 plants for GUS activity in floral organs of sibling plants from a family that included examples of both normal and suppressed expression of P1-rr. Comparison of the GUS expression patterns in immature pericarp and cob of ears that have phlobaphene pigmentation when mature showed that silenced P1-rr plants exhibited reduced levels of GUS activity (Figure 3A, ears 3, 4, and 6). Thus, the observed silencing of P1-rr is correlated with cosilencing of the P1.2b::GUS transgene.

### Cosilencing Correlates with Increased DNA Methylation and Decreased P1-rr Transcript Levels

We used DNA gel blot hybridization to determine whether phenotypic suppression of the endogenous P1-rr allele and the P1.2b::GUS transgene was correlated with changes in DNA methylation. When the genomic DNA was digested with KpnI, which is insensitive to cytosine methylation, the hybridization patterns for the endogenous P1-rr allele (probe P15) and the P1.2b::GUS transgene (probes P15 and GUS) were similar among siblings (Figure 3B). In contrast, when the DNA was digested with SalI, which is inhibited by cytosine methylation, silenced plants showed increased sizes of DNA fragments for both the endogenous P1-rr allele (Figure 3B, probe P15) and the P1.2b::GUS transgene (Figure 3B, probes GUS and P15). For the endogenous P1-rr locus, SalI digestion produced restriction fragments of 12, 11, 4.6, and 4.2 kb in silenced plants, in addition to the 3.4-, 3.0-, and 1.2-kb fragments produced in the control P1-rr plant (Figure 3B). These results indicate that SalI failed to cut at the sites delimiting both the upstream and downstream copies of the P1.2 fragments flanking the P1-rr gene (Figure 3C). Interestingly, this pattern of methylation of SalI sites in the P1-rr allele is very similar to that described for P1-pr, an epiallele of P1-rr with reduced pericarp pigmentation (Das and Messing, 1994). Digestion with two other methylation-sensitive enzymes, HpaII and PstI, also indicated increased methylation of both P1-rr and the P1.2b::GUS transgene (data not shown). Overall, 22 plants derived from two transgenic events of P1.2b::GUS were tested for DNA methylation by DNA gel blot analysis; nine of the 22 plants had the P1-rr phenotype, and the same nine plants showed increased methylation of both the P1-rr allele and the P1.2b::GUS transgene. This strict correlation between phenotypic suppression and DNA methylation suggests that DNA methylation is involved in either the establishment or

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**Table 1.** The P1.2b::GUS Transgene, but Not P1.0b::GUS or P2.0b::GUS Transgenes, Causes Suppression of the Endogenous P1-rr Allele

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Transgenic Events</th>
<th>Transgene Copy No.</th>
<th>Suppressed Ears /Total Ears</th>
<th>Suppressed Ears/Total Ears (total)</th>
<th>Average Frequency of Suppressed Ears (%)</th>
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</thead>
<tbody>
<tr>
<td>P1.2b::GUSa</td>
<td>29-2 8–10/5-6b</td>
<td>3/22c 11/25c</td>
<td>14/47 29</td>
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<td></td>
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<tr>
<td>P1-rr</td>
<td>29-3 6–8</td>
<td>4/28c 2/13d 1/13</td>
<td>7/54 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1.2b::GUS</td>
<td>29-4 10–15</td>
<td>3/6d 0/7e 1/12</td>
<td>4/25 19</td>
<td></td>
<td></td>
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<tr>
<td>Total</td>
<td>29-3 6–8</td>
<td>0/10 0/10 0/12</td>
<td>0/41 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1.0b::GUSd</td>
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<td>0/14 0/15 0/12</td>
<td>0/23 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAG-8 1–2/10–13e</td>
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<td>0/21 0</td>
<td></td>
<td></td>
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<tr>
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<td>0/95 0</td>
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<tr>
<td>PAG-6-16 12–18</td>
<td>0/11 0/10</td>
<td>0/185 0</td>
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<tr>
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<td>37-31-3 ntf</td>
<td>0/8a 0/8b 0/11c 0/11c 0/13c</td>
<td>0/51 0</td>
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<td>39-34-6 7–9</td>
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<td>0/54 0</td>
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</tr>
<tr>
<td>Total</td>
<td>0/185 0</td>
<td></td>
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a Genotype of transgenic plants was P1-ww/P1-ww; P1.2b::GUS/–, and they were crossed to homozygous P1-rr tester.
b Based on segregation data and DNA gel blot data, these transgenic plants contained two apparently unlinked transgene insertion events; the estimated copy numbers for each event are separated by a slash.
c Crosses in which transgenic plants were used as female parents. In the other crosses, transgenic plants were used as male parents.
d Genotype of transgenic plants was P1-ww/P1-ww; P1.0b::GUS/–, and they were crossed to homozygous P1-rr tester.
e Genotype of transgenic plants was P1-ww/P1-ww; P2.0b::GUS/–, and they were crossed to P1-rr/P1-wr tester. There was no observed effect on P1-rr expression; therefore, only the data for P1-rr/P1-wr progeny are presented.
f nt, not tested.
the maintenance of coordinated suppression of the P1.2b::GUS transgene and the endogenous P1-rr allele.

The levels of P1-rr transcript in silenced P1-rr− pericarp were monitored by RNA gel blot analysis. The results indicate that steady state levels of P1-rr transcript in developing P1-rr− pericarp were reduced significantly (67 to 8% of control P1-rr pericarp; Figure 4). Moreover, the degree of transcript reduction was approximately correlated with the degree of phenotypic suppression. The observation of reduced transcript levels, together with the increased DNA methylation and the heritability of the P1-rr− phenotype in the absence of the P1.2b::GUS transgene (see below), suggests that suppression is effected at the transcriptional level. However, a definitive conclusion will require the determination of P1-rr− transcription rates via run-on transcription assays.

The Silenced P1-rr− Phenotype Is Heritable in the Absence of the P1.2b::GUS Transgene

Is the silenced P1-rr− phenotype heritable, or is P1-rr expression restored after segregation of the P1.2b::GUS transgene? To answer this question, we examined ears of plants in which the P1-rr allele segregated away from the P1.2b::GUS transgene locus. A representative pedigree demonstrating the heritability of the P1-rr− phenotype is shown in Figure 5. Two sibling plants that were heterozygous at the p1 locus (P1-rr/P1-ww) and hemizygous for the P1.2b::GUS transgene were backcrossed with a homozygous P1-ww tester. If suppression of P1-rr requires the presence of the P1.2b::GUS transgene, then the P1-rr progeny plants lacking the P1.2b::GUS transgene should have normal red pigmentation. Alternatively, if maintenance of the suppression of P1-rr does not require the transgene, then all of the progeny plants carrying the P1-rr− allele should be silenced. In both families tested, the progeny exhibited only silenced P1-rr− ears, indicating that expression of the P1-rr− allele was not restored after removal of the P1.2b::GUS transgene. Overall, the progeny of strongly silenced plants exhibited high heritability of the suppressed P1-rr− phenotype in nine families derived from three independent transformation events of the P1.2b::GUS transgene (Table 2). Moreover, the strongly suppressed P1-rr− phenotype was inherited stably for two subsequent generations after transgene segregation (Table 2). In contrast, the progeny of four weakly suppressed P1-rr− ears displayed phenotypes ranging from strongly suppressed P1-rr− to normal red P1-rr pigmentation, indicating less heritability of the P1-rr− phenotype in the progeny of weakly affected P1-rr− ears.

Silenced P1-rr Is Paramutagenic to Naive P1-rr

Paramutation has been defined as an allelic interaction in which a paramutagenic allele heritably alters the expression of a paramutable allele upon their exposure to each other in a heterozygote (Brink et al., 1968; Hollick et al., 1997). This non-Mendelian behavior has been documented for three genes, r1, b1, and p1f, that control pigmentation in maize. We tested whether a similar effect could occur between the P1-rr− allele and a naive P1-rr allele. Plants homozygous for a normal P1-rr allele were crossed with plants heterozygous for P1-rr− and P1-ww and hemizygous for the P1.2b::GUS transgene (Figure 6). If the P1.2b::GUS transgene is required to trigger P1-rr suppression, then only plants carrying the transgene will exhibit the suppressed P1-rr− phenotype. However, if the P1-rr− allele had itself acquired the ability to silence P1-rr, then some of the progeny plants lacking the transgene also should exhibit a P1-rr− phenotype. The genotypes of 10 progeny plants were determined by DNA gel blot analysis. Four plants carried both the P1-rr− epiallele and the naive P1-rr allele and lacked the P1.2b::GUS transgene (Figure 6); two of these were strongly suppressed, one was weakly suppressed, and one was not visibly suppressed. One strongly suppressed plant was backcrossed with a naive P1-rr plant. In the progeny of this cross, 13 plants were strongly suppressed, two were weakly suppressed, and one had an ~20-kernel pericarp of slightly suppressed pericarp (Figure 6, bottom). We conclude that phenotypic suppression of the naive P1-rr allele occurred after exposure to the P1-rr− allele, in the absence of the P1.2b::GUS transgene.

To test whether the suppression of naive P1-rr described above was linked to the P1-rr− allele, we performed the crosses shown in Figure 7. A plant carrying P1-rr− heterozygous with P1-ww was crossed to a P1-ww homozygote. Approximately half of the F1 progeny carried the P1-rr− allele, whereas the other half carried the P1-ww allele, which had been heterozygous with P1-rr− in the previous generation. Both classes of F1 plants were crossed with naive P1-rr homozygotes, and the ears of the resulting progeny were scored for P1-rr expression. Among 13 progeny families derived from the P1-rr−/P1-ww parents, 11 families produced one or more suppressed ears. Within a family, the frequency of suppressed ears ranged from 6 to 50%. In contrast, no suppressed ears were found among a total of 74 ears in six families from P1-ww/P1-ww parents (Figure 7). Thus, suppression of naive P1-rr was linked with the P1-rr− allele and was not transmitted via P1-ww or other genomic sequences. These results indicate that the P1.2b::GUS transgene induced a heritable suppressed state of the endogenous P1-rr allele, which in turn was capable of silencing a naive P1-rr allele in a manner similar to paramutation. By analogy with other paramutation systems, P1-rr− is paramutagenic and P1-rr is paramutable.

We further characterized the paramutagenic activity of P1-rr− when homozygous, heterozygous with P1-ww, or in combination with the P1.2b::GUS transgene. The extent of paramutation, as measured by the frequency of affected ears within a progeny family, varied from 58 to 93% for crosses with homozygous P1-rr− and from 0 to 60% for crosses involving heterozygous P1-rr−/P1-ww (Table 3). Additionally, the degree of suppression, as indicated by the
Figure 3. Cosilencing of the P1.2b::GUS Transgene and the Endogenous P1-rr Allele Correlates with Increased DNA Methylation.

(A) The endogenous P1-rr allele and the P1.2b::GUS transgene (event 29-3) exhibit coordinated phenotypic suppression in the F1 progeny (ears 3, 4, and 6, asterisks).

(B) DNA gel blot analysis of plants shown in (A). The GUS probe is an ~1.8-kb Ncol-AlwNI fragment of the uidA gene coding sequence and de-
reduction in pigmentation, varied somewhat among ears (Figure 6). The absence of suppressed ears among the progeny of two of the 14 crosses may reflect a low degree of paramutation that was undetectable in the small number (~10 to 20) of progeny ears examined. Alternatively, some states of P1-rr may be suppressed significantly but have little or no paramutagenic activity. In several cases in which crosses of P1-rr' with P1-rr produced normal red-pigmented ears, analysis of the next generation showed that normal P1-rr and the P1-rr' epiallele could be recovered from the heterozygote. These results indicate that (1) failure of P1-rr' to paramutate P1-rr was not due to reversion of P1-rr', and (2) the P1-rr' allele did not revert upon exposure to a P1-rr allele.

The P1.2b::GUS Transgene Suppresses P1-rr but Not P1-wr

The suppressing effects of the P1.2b::GUS transgene were observed only for P1-rr, whereas no effects were observed in similar numbers of crosses conducted with another p1 allele, P1-wr. This result was obtained consistently in crosses involving both homozygous P1-wr/P1-wr and heterozygous P1-rr/P1-wr testers (Table 4). In crosses using a heterozygous P1-rr/P1-wr tester, all three independent transformation events of the P1.2b::GUS transgene caused suppression of P1-rr (average frequency of 10%), whereas there was no effect on the P1-wr allele (Table 4). The P1-wr allele also exhibited no phenotypic changes in the presence of the P1.0b::GUS and P2.0b::GUS transgenes (data not shown). These results indicate that the P1.2b::GUS transgene construct carrying the 1.2-kb enhancer fragment from the P1-rr transcription regulatory region specifically suppressed the endogenous P1-rr allele but had no detectable effect on the P1-wr allele. It remains to be seen whether the apparent resistance of P1-wr to paramutation by the P1.2b::GUS transgene is associated with specific sequences or structural features of the P1-wr allele.

DISCUSSION

Sequences That Induce P1-rr Paramutation Colocalize with an Upstream Enhancer Element

The conspicuous red kernel pericarp phenotype controlled by the p1 gene serves as a sensitive indicator of alterations in expression resulting from interactions between the endogenous p1 gene and transgene loci. The P1.2b::GUS, P1.0b::GUS, and P2.0b::GUS transformation constructs differed only in the content of the P1-rr upstream genomic fragments they contained; hence, the different effects observed should be due to the specific properties of these sequences. Crosses between the P1.2b::GUS transgene and the endogenous P1-rr allele produced cosilencing of both P1-rr and GUS expression in the progeny. The suppressed P1-rr' state is heritable in the absence of the transgene and itself behaves as a paramutagenic agent that can silence a naive P1-rr allele. In contrast, transgenes containing the proximal 1-kb enhancer fragment (P1.0b::GUS) or the proximal enhancer plus an additional 1 kb of upstream flanking DNA (P2.0b::GUS) had no significant effects on P1-rr expression.

Delineation of the minimal sequences required for the
induction and transmission of paramutation may lead to an understanding of the underlying mechanism(s). In paramutation, alleles that cause suppression are termed paramutagenic, alleles that become silenced are paramutable, and alleles that do not participate in paramutation are neutral. In some cases, different chromosomal components may be involved in paramutagenicity versus paramutability. At the maize r1 locus, for example, the relative paramutagenic strength of recombinant alleles derived from R1-mb and R1-st is proportional to the number of R1 gene repeats that each contains (Kermicle et al., 1995; Panavas et al., 1999), whereas paramutability of the R1-r allele requires a small (300 bp) sequence named \( \sigma \) that is located between two divergently transcribed r1 gene copies (Walker et al., 1995; Kermicle, 1996). The \( \sigma \) region contains remnants of the transposon doppia, the subterminal repeats of which were proposed to serve as targets of paramutagenic modification(s) (Walker, 1998). In contrast, the sequences involved in b1 and pl1 paramutation are unknown. Element(s) involved in b1 paramutation are genetically closely linked to b1 (within 0.1 centimorgan upstream of the b1 coding sequences; Patterson et al., 1995) but are possibly located at a substantial physical distance of \( \geq 50 \) kb (V.L. Chandler and M. Stam, personal communication).

The P1-rr gene presents an alternative situation in which the elements implicated in silencing are located at sites \( \sim 5 \) kb upstream and 8 kb downstream of the P1-rr transcription start site (Figure 1). When included in a transgene construct, a relatively small (1.2 kb) fragment can induce a silenced, paramutant state of P1-rr; interestingly, the same fragment has been shown by transient and stable transformation assays to contain an enhancer of P1-rr expression (Sidorenko et al., 1999, 2000). This finding supports an earlier prediction that DNA elements involved in paramutation may include regulatory sequences (Patterson et al., 1995). Tests are in progress to determine whether the enhancing and silencing activities within the 1.2-kb fragment derive from the same or from different elements. The 1.2-kb SalI fragment has no obvious similarities with doppia or other known transposon sequences.

Our results indicate that a P1.2b::GUS transgene containing the 1.2-kb SalI fragment can induce silencing and a paramutant state of P1-rr, although these results do not prove that the same 1.2-kb SalI sequences in the endogenous P1-rr allele are required for subsequent paramutation effects. It should be possible to use structural variants of the p1 gene to determine whether the 1.2-kb enhancer fragment, at its normal genomic location in P1-rr, is required for transmission of paramutation and/or sensitivity to paramutation.

**p1 Paramutation Is Associated with DNA Methylation**

Maize paramutation systems differ in DNA methylation of the affected loci (reviewed in Chandler et al., 2000). Intensive analyses have failed to detect any changes in DNA methylation associated with paramutation of the b1 and pl1 genes (Patterson et al., 1993; reviewed in Chandler et al., 1996, 2000; Hollick et al., 2000). In contrast, paramutation at r1 is correlated with increased methylation within promoters of both paramutagenic (R1-st) and paramutable (R1-r) alleles (Kermicle, 1996; Walker, 1998; reviewed in Chandler et al., 2000).

**Figure 4.** The P1-rr’ Silenced Phenotype Is Correlated with Reduced P1-rr Transcript Levels.

Phenotypes of the tested plants are shown at the top. Blots of total RNA from developing pericarp (20 days after pollination) were hybridized with P1-rr cDNA probe (Grotewold et al., 1991). Total RNA loading was normalized by hybridization of the same blot with soybean 18S rRNA (Shirley and Meagher, 1990). The P1-rr and P1-ww control lanes show samples from homozygous plants, whereas the silenced P1-rr’ plants (lanes 1, 2, and 3) were heterozygous with P1-ww. Thus, the expression level of control P1-rr was assigned a value of 200% to account for the twofold dosage of the P1-rr allele; the transcript levels of the P1-rr’ plants are presented as dosage-compensated values relative to P1-rr and normalized to the rRNA signals. The arrow indicates the major P1-rr transcript.
Interestingly, the phenotype produced by \( P1-rr' \) is similar to that of \( P1-pr \), a spontaneous epiallele of \( P1-rr \) isolated previously (Das and Messing, 1994); both alleles commonly produce kernels with reduced pericarp color but with dark pigmentation at the point of silk attachment (Figure 3). Both \( P1-rr' \) and \( P1-pr \) are heritable and can be maintained for multiple generations without significant reversion to \( P1-rr \). The phenotypic suppression observed with both \( P1-rr' \) and \( P1-pr \) is tightly correlated with increased DNA methylation (Das and Messing, 1994). Moreover, the patterns of methylation of both \( P1-rr' \) and \( P1-pr \) are very similar: restriction sites near both the upstream and downstream copies of the

Table 2. Heritability of the Suppressed \( P1-rr' \) Phenotype in the Absence of the 1.2b::GUS Transgene

<table>
<thead>
<tr>
<th>Generation</th>
<th>Degree of Suppression of Parental Ears</th>
<th>No. of Independent Transgenic Events Tested</th>
<th>Total Ears</th>
<th>No. of Crosses with Suppressed Progeny</th>
<th>Ear Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>( P1-rr )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Suppressed</td>
</tr>
<tr>
<td>BC1</td>
<td>Strong</td>
<td>3</td>
<td>9</td>
<td>9</td>
<td>59p</td>
</tr>
<tr>
<td>BC1</td>
<td>Weak</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>BC2</td>
<td>Strong</td>
<td>2</td>
<td>10</td>
<td>10</td>
<td>67</td>
</tr>
<tr>
<td>BC3</td>
<td>Strong</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>15</td>
</tr>
</tbody>
</table>

\[ ^a \] BC1 families were derived from crosses of \( P1-rr'/P1-ww \); \( 1.2b::GUS'/ \) plants and homozygous \( P1-ww \) testers. BC2 and BC3 families were derived from crosses between suppressed \( P1-rr'/P1-ww \); \( --/-- \) and homozygous \( P1-ww \) testers.

\[ ^b \] Two ears had darker pigmentation than their parents but were still suppressed significantly.
P1.2 enhancer fragment are hypermethylated, whereas no methylation changes are detected within the p1 coding sequence (Figure 3) (Das and Messing, 1994). These data suggest that DNA methylation is involved in either the establishment or the maintenance of the silenced states of P1-pr and P1-rr. Whether DNA methylation plays a causative role in silencing is unclear; a recent study of P11-Blotched, an epiallele of the maize p1 gene, concluded that chromatin structure, not DNA methylation, plays a primary role in epigenetic regulation (Hoekenga et al., 2000). Using a DNase I sensitivity assay, Lund et al. (1995) showed that in the kernel pericarp, the P1-pr allele has a more closed chromatin conformation within the P1.2 enhancer fragment than does the P1-rr allele. Analyses of P1-rr’ chromatin structure have not been done.

**Comparison of p1 and Other Paramutation Systems**

Both P1-rr’ (this article) and P1-pr (O.P. Das and J. Messing, personal communication; W. Goettel and J. Messing, personal communication) are paramutagenic to a naive P1-rr allele, although it is unclear whether they are equivalent in their paramutagenic activity. P1-rr’ exhibited variable levels of paramutagenic activity, ranging from 0 to >90% among the progeny of different crosses (Table 3). Also, the affected ears formed a continuum in terms of degree of phenotypic suppression (Figure 6). In contrast, paramutagenic B1’ has a strongly suppressed phenotype and is always strongly paramutagenic to paramutable B1-I. Paramutagenic P11’ exhibits variable levels of expression that tightly correlate with paramutagenicity; stronger phenotypic suppression is associated with stronger paramutagenicity (Hollick et al., 1995). For r1 paramutation, the relationship between the degree of suppression and paramutagenicity is more difficult to assess. Paramutagenic R1-st and R1-md are complex alleles; each contains functional r1 gene copies that may obscure phenotypic suppression of the paramutagenic copies (Eggleston et al., 1995; Kemnicel et al., 1995; Panavas et al., 1999).

One of the distinguishing characteristics of different paramutation systems is the stability of the paramutable and paramutagenic alleles. The paramutable alleles for b1 and p11, B1-I and P11-Rh, are very unstable and spontaneously produce paramutagenic derivatives, B1’ and P11’, respectively, at frequencies ranging from 1 to 10% (Coe, 1966;…

---

Figure 6. The P1.2b::GUS Transgene Induces a Paramutant State of the Endogenous P1-rr Allele.

**(Top)** Cross of homozygous P1-rr with a plant heterozygous for P1-rr/wP1-ww and hemizygous for the P1.2b::GUS transgene (event 29-4) resulted in 15 plants; nine were herbicide sensitive, and three of these plants produced ears with the suppressed P1-rr’ phenotype. The genotypes of 10 plants were determined by DNA gel blot analysis and are presented here; the genotypes of the other five plants were not determined precisely and hence are not shown. Phenotypes of plants used in crosses are shown in the photographs.

**(Bottom)** One plant homozygous for P1-rr’ and lacking the P1.2b::GUS transgene was backcrossed with a plant homozygous for naive P1-rr. Most of the resulting progeny exhibit the silenced P1-rr’ phenotype (photograph). See text for details. BC1, backcross 1.
Hollick et al., 1995; Patterson and Chandler, 1995). For \( r1 \), paramutable \( R1-r \) can spontaneously change to lower expression states, but these states are neither heritable nor paramutagenic (Brink et al., 1968). In contrast, spontaneous changes of the paramutable \( P1-rr \) allele to \( P1-pr \) occur at a much lower frequency (\( \approx 10^6 \) kernels) (Das and Messing, 1994).

The stability of the paramutagenic states also may vary. The paramutagenic \( B1' \) state is very stable and not known to revert spontaneously to \( B1-l \) in standard maize stocks (Coe, 1966; Patterson and Chandler, 1995), whereas paramutagenic \( P1' \) is metastable and reverts to paramutable \( P1-Rh \) at a significant rate (10 to 20%) when hemizygous or when heterozygous with neutral alleles (Hollick and Chandler, 1998). For the \( p1 \) gene, we found that the suppressed \( P1-rr' \) state was quite stable. Partial reversion of \( P1-rr' \) was observed in two of 144 progeny ears derived from strongly suppressed parents (data not shown). Possibly, continued propagation of these partial revertants would result in the gradual full recovery of red pigmentation, similar to that described for \( r1 \) paramutation (reviewed in Chandler et al., 2000).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Paramutagenic Activity of \( P1-rr' \) Is Linked with the \( p1 \) Locus.}
\end{figure}

\textbf{(Top)} Diagram of sequential crosses used to test whether the silencing effect of \( P1-rr' \) segregates with \( P1-rr \). In the first cross, \( P1-rr' \) segregates from the \( P1-ww \) allele. In the second cross, sibling ears carrying \( P1-rr' \), or \( P1-ww \) previously exposed to \( P1-rr' \), are crossed with homozygous naive \( P1-rr \). Expression of the naive \( P1-rr \) allele is scored in the following generation. See text for details.

\textbf{(Bottom)} Tables show the numbers of suppressed ears (\( P1-rr' \)) observed among the total ears in each family. The results show that ability to suppress \( P1-rr \) is linked with \( P1-rr' \) and is not transmitted through \( P1-ww \) nor through randomly segregating factor(s).

\begin{table}[h]
\centering
\begin{tabular}{ccc}
\hline
Family & \( P1-rr' \) & Total \\hline
1 & 5 & 15 & 33 \\
2 & 5 & 13 & 38 \\
3 & 0 & 13 & 0 \\
4 & 7 & 17 & 41 \\
5 & 6 & 12 & 50 \\
6 & 9 & 18 & 50 \\
7 & 8 & 18 & 44 \\
8 & 5 & 13 & 38 \\
9 & 1 & 17 & 6 \\
10 & 5 & 16 & 31 \\
11 & 4 & 12 & 33 \\
12 & 0 & 15 & 0 \\
13 & 3 & 8 & 38 \\
\hline
\end{tabular}
\caption{Number of Suppressed Ears (\( P1-rr' \)) in Each Family}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{ccc}
\hline
Family & \( P1-rr' \) & Total \\hline
1 & 0 & 13 & 0 \\
2 & 0 & 10 & 0 \\
3 & 0 & 14 & 0 \\
4 & 0 & 14 & 0 \\
5 & 0 & 10 & 0 \\
6 & 0 & 13 & 0 \\
\hline
Total & 0 & 74 & 0 \\
\hline
\end{tabular}
\caption{Number of Suppressed Ears (\( P1-rr' \)) in Each Family}
\end{table}

\textbf{Allele Specificity of Interaction between \( p1 \) Alleles and the \( P1.2b::GUS \) Transgene}

In this study we compared the response of the \( P1-rr \) and \( P1-rr' \) alleles to exposure to the \( P1.2b::GUS \) transgene. The standard \( P1-rr \) allele is not only susceptible to suppression
by P1.2b::GUS, it also becomes paramutagenic to a naive P1-rr allele. In contrast, the P1-wr allele exhibited no changes in its phenotype when exposed to P1.2b::GUS or P1-rr’. These results were obtained even when using a common heterozygous parent (P1-rr/P1-wr) and hence cannot be ascribed to background effects. The P1-rr and P1-wr alleles share a very high level (99%) of identity in the proximal promoter region and within most of the coding region. However, a significant structural polymorphism is located in the distal regulatory region, >5 kb upstream of the transcription start site. This region corresponds to the distal P1.2 enhancer fragment, wherein P1-rr contains a 690-bp sequence that P1-wr lacks (Chopra et al., 1996). Additionally, P1-wr contains approximately six copies of the p1 gene in a tandem array (Chopra et al., 1998). Further experiments will be required to determine whether differences in sequence composition or copy number are involved in the different sensitivity of P1-rr and P1-wr to paramutation.

Repeated structures and Paramutation

Repeated sequences have been implicated in several epigenetic phenomena, including paramutation (Assaad et al., 1993; Kermicle et al., 1995; Meyer, 1996; Ye and Signer, 1996; Dorer and Henikoff, 1997; Panavas et al., 1999). In r1 paramutation, both paramutagenic alleles, R1-st (Eggleston et al., 1995; Kermicle et al., 1995) and R1-mb (Panavas et al., 1999), have repetitive structures that are crucial for their paramutagenicity. A decreased number of tandem copies of r1 genes in recombinant R1-st or R1-mb alleles leads to diminished paramutagenicity, with single-copy alleles being nonparamutagenic (Kermicle et al., 1995; Panavas et al., 1999). Similar observations indicate the importance of repetitive structures for the paramutagenicity of a transgene locus (H2) in tobacco (Jakowitsch et al., 1999). In contrast, paramutation at the b1 and pl1 loci is not known to involve repeated sequences (reviewed in Hollick et al., 1997; Chandler et al., 2000).

The P1-rr allele has an unusual structure in which the coding region is flanked by two 5.2-kb directly repeated sequences. These flanking repeats and a related upstream gene, termed p2, are derived from a gene duplication event that was estimated to have occurred 2.7 million years ago (Zhang et al., 2000). Moreover, the 5.2-kb repeats flanking P1-rr contain multiple copies of a 1.2-kb direct repeat sequence: two complete copies are found downstream of P1-rr, whereas one complete and one partial copy are found upstream of P1-rr. The P1.2 enhancer-containing fragment is derived by digestion at SalI sites present in each of the 1.2-kb repeat sequences. Thus, sequences within the P1.2 enhancer fragment are repeated three to four times at sites flanking the P1-rr allele (Figure 1). The transgenic plants studied here have an additional increase in copy number of the P1.2 fragment that may somehow trigger epigenetic modifications. For example, one or more of these repetitive structures could serve as a preferential target for inactivation by methylation, as shown previously for other loci (Assaad et al., 1993; Vaucheret, 1993; Matzke et al., 1994). However, increased copy number of a P1-rr promoter fragment is not sufficient to induce P1-rr paramutation; this is demonstrated by comparisons of the P1.0b::GUS, P2.0b::GUS, and P1.2b::GUS transgene events containing similar copy numbers (P1.0b::GUS, one to 18 copies; P2.0b::GUS, five to nine copies; P1.2b::GUS, five to 15 copies) (Table 1). In the F1 generation, all three events of P1.2b::GUS silenced P1-rr, whereas four events each of P1.0b::GUS and P2.0b::GUS had no silencing effect (Table 1). Possibly, P1-rr paramutation may involve the combined effect of specific DNA sequences in a multicopy state.

Possible Mechanisms of p1 Silencing and Paramutation

The molecular mechanisms of establishment and maintenance of homology-dependent gene silencing, and in particular paramutation, remain obscure. Recently proposed models for paramutation in maize postulate the formation of

### Table 3. Paramutagenic Activity of P1-rr’ in Crosses with the Naive P1-rr Allele

<table>
<thead>
<tr>
<th>Genotype of Paramutagenic Source</th>
<th>Suppressed Ears/Total Ears</th>
<th>Average Frequency of Suppressed Ears (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-rr’</td>
<td>Cross 1</td>
<td>Cross 2</td>
</tr>
<tr>
<td>P1-rr’</td>
<td>7/12</td>
<td>14/15</td>
</tr>
<tr>
<td>P1-rr’</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>P1-rr’ P1.2b::GUS</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>P1-rr’ P1-ww</td>
<td>21</td>
<td>13</td>
</tr>
</tbody>
</table>

a This cross involved transgenic event 29-3. The rest of the crosses involved P1-rr’ plants suppressed by event 29-4.

b In this cross, naive P1-rr was used as a female parent and transgenic plant P1-rr’/P1-ww; P1.2b::GUS/— was used as a male parent. All other crosses used naive P1-rr as a male parent.
a local suppressive heterochromatin structure at the affected locus (Hollick et al., 1997; Chandler et al., 2000). A model for \( p1 \) silencing and paramutation needs to account for the following features: (1) the involvement of a specific DNA sequence, the upstream 1.2-kb enhancer fragment; (2) the heritability of the paramutant \( P1-rr' \) state in the absence of the inducing \( P1.2b::GUS \) transgene or the \( P1-rr' \) allele; and (3) the correlation of the silenced \( P1-rr' \) state with increased DNA methylation and reduced mRNA accumulation. Additionally, the silenced \( P1-rr' \) state is characterized by local chromatin compaction near the distal enhancer fragment (Lund et al., 1995). Together, these features are consistent with a model proposing repeat-induced formation of heterochromatin (Dorer and Henikoff, 1997; Chandler et al., 2000). This model proposes that interactions between the P1.2 enhancer fragments lead to transfer of chromatin-associated proteins from one locus to another, resulting in local chromatin compaction and heritable gene inactivation.

The postulated interaction of P1.2 enhancer fragments could be initiated by specific DNA binding proteins or by pairing of homologous chromosomal sequences. Although pairing of homologous chromosomes has not been reported in plants except in the later stages of flower development (Aragon-Alcaide et al., 1996), it is possible that pairing interactions may occur transiently in developing embryos and meristems, that is, at stages in which paramutation is likely to occur (Chandler et al., 2000).

An alternative mechanism for transgene-induced silencing of \( P1-rr' \) can be envisioned based on the finding that the P1.2 enhancer fragment contains sequences homologous with other genomic loci that are transcribed. One part of the P1.2 enhancer shares partial homology with expressed sequence tags isolated from maize root (accession number AI891238; 98% similarity over 225 bp; Gai et al., 2000) and kernel pericarp (Q cDNA; 97% similarity over 322 bp; E. Grotewold, personal communication; Grotewold and Peterson, 1990). Both transcripts probably originate from independent genomic loci, because each contains additional unique sequences. Possibly, these transcripts may trigger DNA methylation of homologous sequences within the P1.2 enhancer, resulting in promoter inactivation, as described by Mette et al. (1999, 2000). However, the RNA-dependent transcriptional gene silencing described by Mette et al. (1999, 2000) was reversible upon reduction of the levels of the promoter transcripts that induced silencing. In contrast, the silenced \( P1-rr' \) state is heritable for multiple generations, suggesting that \( p1 \) paramutation involves a more stable transcription inactivation mechanism.

A third possible silencing mechanism postulates the targeted degradation of \( P1-rr' \) transcripts by double-stranded RNAs according to the model of Wang and Waterhouse (2000). According to this model, during the transformation process the transgenes might integrate into the maize genome with rearranged copies of the P1.2 fragment present as inverted repeats. If this inverted repeat structure integrated near an endogenous promoter, then readthrough transcription could result in the production of double-stranded RNAs. These double-stranded RNAs might then trigger the degradation of \( P1-rr' \) transcripts, because the P1.2 enhancer fragment partially overlaps with the 3' end of \( P1-rr' \) transcripts (Grotewold et al., 1991). Although possible, this mechanism seems unlikely for \( P1-rr' \) silencing for two reasons. First, this model predicts that silencing would occur with particular transformation events that produce double-stranded RNAs; in contrast, we observed silencing by the P1.2b::GUS transgene in the progeny of three independent transformation events. Second, this model predicts that suppression of \( P1-rr' \) would be dependent on the continued presence of the P1.2b::GUS transgene, whereas we have shown that the suppressed state persists stably for several generations after segregational loss of the P1.2b::GUS transgene.

In summary, we show that a transgene containing a specific enhancer fragment of the \( P1-rr \) locus can trigger silencing and subsequent paramutation behavior of the endogenous \( P1-rr' \) gene. Additional transformation experiments in progress may further delimit the elements within the P1.2 fragment that mediate the silencing effects. The identification of these sequences will enable further experiments, including the isolation of sequence-specific DNA binding proteins and the analysis of chromatin structure, that may help to elucidate the mechanism(s) of \( P1-rr' \) paramutation.

### Table 4. \( P1-rr' \) but Not \( P1-wr' \), is Suppressed by the \( P1.2b::GUS \) Transgene

<table>
<thead>
<tr>
<th>( p1 ) Testers (^a)</th>
<th>Events Causing Suppression/Total Events</th>
<th>Families with Suppressed Plants/Total Families</th>
<th>Suppressed Plants/Total Plants</th>
<th>Average Frequency of Suppressed Plants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P1-wr )</td>
<td>0/3</td>
<td>0/4</td>
<td>0/41</td>
<td>0</td>
</tr>
<tr>
<td>( P1-rr )</td>
<td>3/3</td>
<td>7/8</td>
<td>25/126</td>
<td>19</td>
</tr>
<tr>
<td>( P1-rr/P1-wr )</td>
<td>3/3</td>
<td>4/7</td>
<td>4/40</td>
<td>10</td>
</tr>
</tbody>
</table>

\(^a\)Tester plants of the indicated \( p1 \) genotype were crossed with transgenic plants of genotype \( P1-ww/P1-ww; P1.2b::GUS/--; \) and ears produced by progeny plants were scored for phenotypic suppression of the \( P1-rr \) and/or \( P1-wr \) alleles.
METHODS

Plasmid Construction, Plant Transformation, and Genetic Stocks

The transgene plasmids used in this study were constructed as described (Sidorenko et al., 1999). Plasmid Pb::GUS (β-glucuronidase) contains a basal fragment of the P1-rr promoter (Pb) (Sidorenko et al., 1999), maize (Zea mays) Adh1 gene intron I (Dennis et al., 1984), Escherichia coli GUS reporter gene (Jefferson et al., 1987), and potato PinI gene terminator sequence (An et al., 1989). Plasmid P1.0b::GUS contains the P1.0 fragment (~1252 to ~234) fused 5’ of the Pb fragment of Pb::GUS, whereas plasmid P2.0b::GUS contains, in addition, the upstream adjacent 1-kbp HindIII fragment (~2249 to ~1252). Plasmid P1.2b::GUS contains a 1.2-kb Sall fragment (~6110 to ~4842) fused to the basal promoter fragment of Pb::GUS; the 1.2-kb Sall fragment was unintentionally reversed upon insertion into Pb::GUS. Transgenic plants were generated via biolistic bombardment of immature embryos or embryogenic callus, as described by Sidorenko et al. (2000). The P::GUS constructs were co-bombarded with plasmid Dp3528, which contains a 35S::BAR gene conferring resistance to the herbicide bialaphos. Transgene constructs were introduced into maize Hill germplasm (Armstrong, 1994), which carries a nonfunctional p1 allele (P1-ww). In some cases, the primary transgenic plants (T0) were crossed to a stock 1994), which carries a nonfunctional construct were introduced into maize (Zea mays) Adh1 gene intron I (Dennis et al., 1984), Escherichia coli GUS reporter gene (Jefferson et al., 1987), and potato PinI gene terminator sequence (An et al., 1989).

DNA and RNA Gel Blotting

Extraction of leaf genomic DNA was as described by Saghai-Maroodi et al. (1984) and Wise and Schnable (1994). Genomic DNA samples were digested with restriction enzymes, electrophoresed on 0.8% agarose gels, blotted to nylon membranes, and cross-linked under UV light. Blots were dried, rinsed in 0.15 M NaCl and 0.015 M sodium citrate, prehybridized for 4 hr, and hybridized for 14 to 16 hr at 65°C. Prehybridization and hybridization buffers were of the same composition (Angenent et al., 1992). For reprobing, blots were stripped in 0.1 M NaOH and 0.2% SDS, as described by Sidorenko et al. (2000). Transgene copy number was estimated from the numbers and intensities of bands on genomic DNA gel blots, as described by Sidorenko et al. (2000). In some cases, the presence of the P::GUS transgene was detected by DNA gel blot hybridization using a 788-bp Ncol-AlwNI fragment of the GUS gene as probe.

For RNA gel blot analysis, total RNA was extracted from 1 g of frozen immature (20 days after pollination) pericarp, as described by Coccioleone and Cone (1993). Total RNA was denatured and electrophoresed on glyoxal gels, as described by Angenent et al. (1992). RNA was blotted to nylon membranes in 0.25 M sodium phosphate buffer, pH 6.5, cross-linked under UV light, dried, and stored at room temperature. Conditions for prehybridization, hybridization, and stripping of blots were the same as those used for DNA gel blots. RNA loading was monitored by ethidium bromide staining. To normalize for differences in RNA loading, blots were rehybridized with a DNA probe from a soybean 18S rRNA (Shirley and Meagher, 1990); hybridization signals were quantitated by densitometry, and relative levels of P1-rr mRNA were determined after normalization with signals from the 18S rRNA probe.

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