Insertional Mutagenesis of the Maize *P* Gene by Intragenic Transposition of *Ac*

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ABSTRACT

The *P-rr* allele of the maize *P* gene regulates the synthesis of pigments derived from flavan-4-ol in the pericarp, cob glumes and other floral organs. We characterized 21 *P* alleles derived by intragenic transposition of *Ac* from three known positions. *Ac* transpositions can occur in either direction in the *P* gene, and with no apparent minimum distance: in one case *Ac* transposed just 6 bp from its original insertion site. However, the distribution of transposed *Ac* elements was markedly nonrandom: of 19 transposed *Ac* elements derived from a single *Ac* donor, 15 were inserted in a 1.1-kb region at the 5′ end of *P*, while none had inserted in an adjacent 3.2-kb intronic region. All of the *Ac* insertions affect both pericarp and cob glume pigmentation, providing further evidence that the *P-rr* allele contains a single gene required for both pericarp and cob glume pigmentation. The distribution of the inserted *Ac* elements and the phenotype conditioned by each allele suggests a structure of *P-rr* which is similar to that previously determined molecularly. Possible explanations for the nonrandom distribution of transposed *Ac* elements are discussed.

THE discovery of transposable elements by McCLINTOCK (1950) led to their use for gene isolation via transposon tagging. A number of genes have been cloned by tagging with the maize autonomous transposable element *Ac* (*Activator*) and its nonautonomous counterpart *Ds* (*Dissociation*). The finding that *Ac* transposes in other plants, including tobacco (BAKER *et al.* 1986), Arabidopsis, carrot (VAN SLUYS, TEMPE and FEDOROFF 1987), potato (KNAPP *et al.* 1988), tomato (YODER *et al.* 1988) and rice (MURAI *et al.* 1991), indicates that the use of *Ac* for gene isolation will likely be extended to additional species.

In addition to gene tagging, *Ac* is potentially useful as an insertional mutagen, similar to the use of *Tn5* to define complementation groups in prokaryotes (SCOTT *et al.* 1982). The *Ac* element should be favorable for this purpose since it tends to transpose to linked sites (GREENBLATT and BRINK 1962; GREENBLATT 1984; DOONER and BELACHEW 1989). The feasibility of this approach has been demonstrated by *Ac* insertional mutagenesis of the maize *P* (ORTON and BRINK 1966) and *R-navajo* (BRINK and WILLIAMS 1973) genes, and by *Ds* insertional mutagenesis of the maize *R-Sc* gene (KERMICLE 1980; KERMICLE, ALLEMAN and DELLAPORTA 1989). However, some aspects of *Ac* and *Ds* transposition could not be determined in these previous studies because the location of the donor transposon was unknown. More recently, WEIL *et al.* (1992) studied a series of alleles of the maize *waxy* gene resulting from intragenic transposition of a *Ds* element in the *Wx-m5* allele.

To learn more about short range *Ac* transposition, we studied the movement of *Ac* from defined donor sites to new locations within the maize *P* gene. The *P* gene regulates the biosynthetic pathway leading to the formation of a flavonoid-derived red pigment in certain floral organs (STYLES and CESKA 1977). The *P-rr* allele specifies red pericarp and cob glumes; *P-rr* has a complex structure (LECHELT *et al.* 1989) and produces at least two alternatively spliced transcripts. One of the *P-rr* transcripts encodes a protein with features resembling a transcriptional activator, including a region with significant homology to the DNA binding domain of several members of the *myb* family of protooncogene proteins. Further evidence that *P* is a transcriptional activator comes from the finding that *P* is required for the accumulation in the pericarp of RNA from *A1* and *C2*, two genes encoding enzymes for flavonoid biosynthesis (GROTEWOLD, ATHMA and PETERSON 1991a). The *P-vv* allele specifies variegated pericarp and cob glumes, and carries *Ac* inserted within a large intron of *P-rr* (CHEN *et al.* 1988; LECHELT *et al.* 1989; GROTEWOLD, ATHMA and PETERSON 1991a). From *P-vv*, we derived an allele termed *P-ovov-1114* which specifies *o*range *v*ariegated pericarp and cob glumes. In *P-ovov-1114*, *Ac* has transposed 153 bp toward the 5′ end of the *P* gene and inserted in the opposite orientation (PETERSON 1990). Because *P-ovov-1114* specifies a moderate level of pigmentation, subsequent transpositions resulting in higher or lower levels of *P* expression are easily recognized.

The sequence data presented in this article have been submitted to the EMBL/GenBank Data Libraries under the accession number Z11879.

Here we report the nucleotide sequence of the *P* gene and the positions of *Ac* insertions in 21 *P* alleles derived by intragenic transposition of *Ac*. The insertion sites are distributed both 5′ and 3′ of the donor *Ac* insertion site, indicating that *Ac* can undergo short-range transpositions in either direction. Each *Ac* insertion inhibits both pericarp and cob pigmentation, suggesting that *P-rr* contains a single gene required for pericarp and cob glume pigmentation. The number of red revertant pericarp sectors specified by each insertion allele depends upon whether the *Ac* element is inserted in translated or untranslated *P-rr* sequences. The structure of *P-rr* inferred from this mutational analysis is similar to that previously determined by molecular techniques. Thus, insertional mutagenesis with *Ac* can be used as a complement to molecular transcript mapping methods.

MATERIALS AND METHODS

Terminology, maize stocks and generation of mutants: The pericarp is derived from the ovary wall and is the outer covering of the mature kernel. Alleles of the *P* gene are conventionally identified by a suffix which indicates their expression in pericarp and cob glumes. *P-rr* specifies red pericarp and red cob, *P-wr* specifies white (colorless) pericarp and red cob, and *P-ww* gives white (colorless) pericarp and cob. Inbred line W23 (genotype *P-wr*) was obtained from the Maize Genetics Cooperation Stock Center, Urbana, Illinois. Inbred line 4Co63 (genotype *P-ww*) was obtained from the National Seed Storage Laboratory, Fort Collins, Colorado.

New *P-vv* alleles were derived from *P-ovov-1114* in a manner similar to that used to obtain *P-ww* mutants from *P-ovov-1114* (ATHMA and PETERSON 1991). Briefly, plants of genotype *P-ovov-1114/P-wr* or *P-ovov-1114/P-ww* were crossed by plants homozygous for *P-wr* or *P-ww*, and the resulting ears were screened for kernels with colorless or light variegated pericarp. Because the pericarp and egg cell are related by cell lineage, the mutant alleles could be recovered by growing kernels within mutant sectors (ANDERSON and BRINK 1952). One kernel with altered pericarp pigmentation from each ear was grown and the resulting plants were self-pollinated to make the new mutant alleles homozygous. New *P-vv* alleles were distinguished from the parental *P-ww* or *P-wr* alleles by Southern blot analysis using *P* locus hybridization probes, since the Southern blot patterns of each allele are distinctive. Plants carrying new *P-vv* alleles were tested for the presence of *Ac* by crossing to one or both of the *Ac* tester stocks *R-sc:124* variant 4 and *r-sc:m3*. *R-sc:124* variant 4 contains a chromosome-breaking *Ds* element located in chromosome 10 between *R-sc* and the centromere. In the absence of *Ac*, *R-sc:124* variant 4 specifies purple aleurone, while in the presence of *Ac*, colorless aleurone sectors result from breakage of chromosome 10 at the site of *Ds* insertion and loss of *R-sc* on an acentric fragment (J. KERMICLE, personal communication). The *r-sc:m3* allele carries a *Ds* insertion within the *R-sc* locus; in the absence of *Ac*, *r-sc:m3* specifies colorless aleurone, while in the presence of *Ac*, it produces a variegated colored aleurone due to the excision of *Ds* from *R-sc* (KERMICLE 1980).

Genomic cloning and sequencing: In *P-ovov-1114*, the *Ac* insertion in intron 2 contains an *Eco*RI site which divides the *P* gene into two *Eco*RI fragments of 13.0 and 14.5 kb. Genomic DNA from homozygous *P-ovov-1114* plants was digested with *Eco*RI, and size-selected fractions containing the 13.0- and 14.5-kb fragments were obtained by electrophoresis through SeaKem agarose and by centrifugation through glycerol gradients. The size-selected DNA was cloned in the *Eco*RI sites of lambda EMBL4, and the phage libraries were screened using *P* locus fragment 15 as probe (Figure 1). Because Fragment 15 is part of a 1.2-kb repeated sequence present on both the 13.0- and 14.5-kb fragments (stippled boxes in Figure 1), it was used as a probe to isolate clones carrying both these fragments. One positive clone was obtained from *ca.* 70,000 recombinant plaques in the library prepared from electrophoretically size-selected DNA; this clone carried the 14.5-kb *Eco*RI fragment containing the *P* 3′ region. Two positive clones were obtained from *ca.* 35,000 recombinant plaques in the library prepared from gradient centrifugation size-selected DNA; one of these carried the 13.0-kb *Eco*RI fragment containing the *P* 5′ region. Sequence from −100 to 4034 was obtained from subclones with unidirectional deletions generated by exonuclease III and mung bean nuclease (Stratagene). Sequence of the opposite strand was obtained using synthetic oligonucleotides to prime double strand plasmid templates. Sequence from 4035 to 7327 was obtained from the 3.3-kb *Sal*I fragment previously cloned from *P-vv* (LECHELT *et al.* 1989), by sequencing restriction fragments of this region subcloned in plasmid and M13 vectors. Additional sequence data was obtained from previously cloned *Bgl*II fragments carrying the *Ac* insertion and flanking DNA from *P-vv* and *P-ovov-1114* (PETERSON 1990). Sequence from 7328 to 7653 was obtained from a 1.2-kb *Sal*I fragment derived from *P-ovov-1114* and sequenced using synthetic oligonucleotide primers. Finally, synthetic oligonucleotide primers were used to sequence any remaining gaps.

Mapping of transposed *Ac* elements and sequencing of insertion sites: The positions and orientations of transposed *Ac* elements were determined by Southern blot analysis of genomic leaf DNA as described previously (ATHMA and PETERSON 1991). First, genomic DNA was digested with *Sal*I and Southern blots were hybridized with *P* locus probes 15 and 16 + 17 (Figure 1). Due to repetitive sequences at the *P* locus, probe 15 detects 3.3-, 3.0- and 1.2-kb *Sal*I fragments (see Figure 1; the 3.0-kb *Sal*I fragment lies immediately to the left of the left-most *Sal*I site in Figure 1). Probe fragments 16 + 17 detect 9- and 7-kb *Sal*I fragments (Figure 1). This combination of probes allowed detection of any *Ac* insertions in a 25-kb region of the *P* locus. Insertion of *Ac*, which contains no *Sal*I sites, results in a 4.5-kb increase in fragment size. The approximate location of *Ac* elements within *Sal*I fragments was determined by Southern analysis of genomic DNA digested with *Eco*RI and probed with *P* locus fragment 15. *Eco*RI cuts once near the center of *Ac*, and at sites outside the 5.2-kb direct repeats of the *P* locus (Figure 1). The *Ac* insertion sites were more accurately mapped by Southern analysis of DNA digested with *Bam*HI and probed with *P* locus fragments 8B and 15 (Figure 1); because *Bam*HI cuts at a site near the 5′ end of *Ac*, this also indicated *Ac* orientation. Polymerase chain reaction (PCR) amplification used an oligonucleotide primer homologous to *Ac* sequences together with a primer homologous to the flanking *P* genomic sequence. The oligonucleotide primers used are given in Table 1 (see also Table 2).

PCR conditions were as described by Perkin Elmer-Cetus. Reactions were heated at 94° for 5 min; then cycled 40 times for 1 min at 94°, 1 min at 50°, and 1 min at 72°; then extended for 20 min at 72°. Reaction products were analyzed by agarose gel electrophoresis and Southern hybridization using *P* gene specific probes. Sequences were

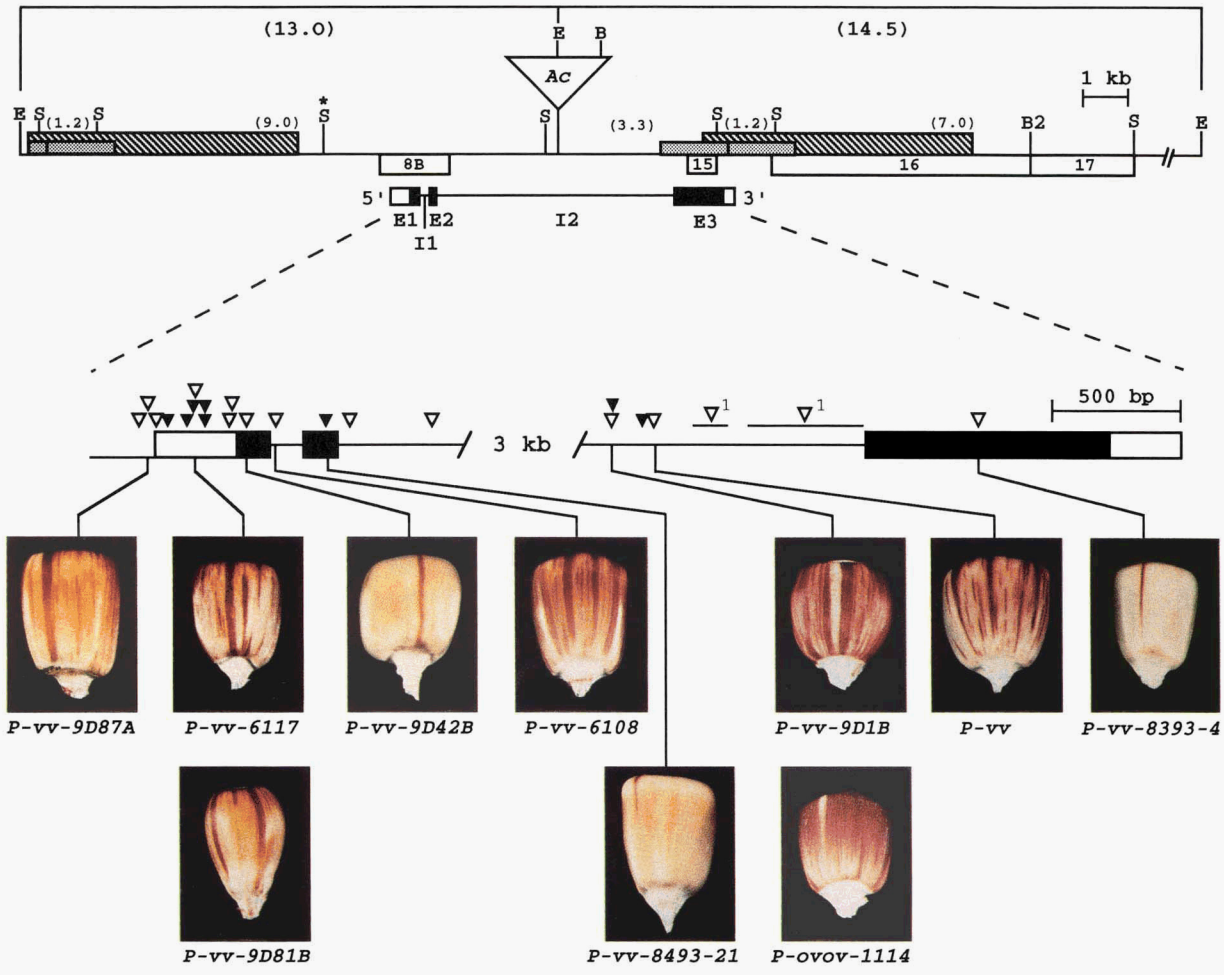

FIGURE 1.—(Upper) Restriction map of the *P* locus. The 5.2-kb direct repeats (hatched boxes) flanking the *P* gene, and 1.2-kb direct repeat sequences (stippled boxes) are indicated. The triangle indicates the insertion site of the transposable element *Ac* in the *P-ovov-1114* allele. Open boxes indicate the positions of restriction fragment probes 8B, 15, 16 and 17. Numbers in parentheses indicate the fragment sizes obtained upon digestion of genomic DNA with *Eco*RI (upper) and *Sal*I (lower). Restriction sites for *Sal*I (S), *Eco*RI (E) and *Bgl*II (B2) are indicated; not all *Bgl*II sites are shown. The asterisk above the third *Sal*I site from the left indicates that this site is not digested by *Sal*I in genomic DNA, possibly because of methylation. The structure of the 1.8 kb *P-rr* transcript is shown below the restriction map. Open boxes correspond to 5′ and 3′ untranslated regions, black boxes correspond to protein coding sequences, and the lines between them correspond to intron sequences. Exons are indicated by E1, E2 and E3; introns by I1 and I2. (Lower) Location of *Ac* insertions. An enlarged map of the 1.8-kb *P-rr* mRNA is shown. Open triangles represent *Ac* elements in the same orientation with respect to *P* as in *P-vv* (*i.e.*, with the 5′ end of *Ac* nearer the 5′ end of the *P* gene); filled triangles represent insertions in the opposite orientation (*i.e.*, same as *P-ovov-1114*). Photographs at bottom show the phenotypes resulting from insertion of *Ac* at representative sites. The upper and lower panels of photos are of kernels from plants with *Ac* insertions in the *P-vv* and *P-ovov-1114* orientations, respectively. Plants producing these kernels were heterozygous with an allele specifying colorless pericarp (either *P-ww* or *P-wr*). [1]*Ac* insertion sites mapped by Southern blotting but not sequenced. Horizontal lines indicate approximate locations.

determined using a Sequenase kit (USB) either after asymmetric PCR amplification (SAMBROOK *et al.* 1989) or after cloning into pBluescript plasmid vectors (Stratagene). For most alleles, the *Ac* insertion site was determined by amplifying and sequencing one of the *Ac/P* gene junction fragments; the 8 nucleotides immediately adjacent to *Ac* were assumed to represent the 8-bp duplication typically resulting from *Ac* insertion (DORING and STARLINGER 1984).

RESULTS

Transposition of *Ac* within *P*: Insertions of *Ac* in the *P* locus were mapped by Southern blotting using *P* specific probes (MATERIALS AND METHODS). Figure 2 shows the locations of transposed *Ac* elements in new *P* alleles derived from three progenitor alleles: *P-vv*, *P-ovov-1114* and *P-vv-9D32A*. The *P-vv* allele gave rise to *P-ovov-Valentine* (VALENTINE 1957), and *P-ovov-1114* (PETERSON 1990) (Figure 2, upper). From *P-ovov-1114*, we derived 19 *P* alleles with single *Ac* insertions (Figure 2, middle). Fifteen of the new *P* alleles carried *Ac* inserted in the 5′ region of the *P* gene, while in four alleles *Ac* had inserted near the 3′ end of intron 2, or in exon 3 (Figure 2). Several additional alleles derived from *P-ovov-1114* carried *Ac* insertions outside the *P-rr* transcribed region, at sites up to 10 kb from the *P-ovov-1114* donor site. However, in none of these latter alleles with single *Ac* insertions was the background pericarp pigmentation

TABLE 1

Oligonucleotide primers used in this study

Primer	Primer sequence	Position in *P* sequence
EP1PE	5′-GGATACACGCTGGCAGTCG-3′	69–51
PA-B7	5′-CACACCGGAGTCGATGTGG-3′	240–221
EP5-9	5′-AGCCAGCACAGCACACACACTG-3′	1–22
PA-A2	5′-TGTACGTACGTACGTACTCCG-3′	(−61)–(−41)
EP3-13	5′-AGGAATTCCGCCCGAAGGTAGTTGATCC-3′	637–616
EP5-18	5′-TATAAAAGTATACATGGTACCTCAC-3′	1028–1004
PA-A8	5′-CCAAGGAGGAAGAAGACATC-3′	658–677
EP4026-1	5′-CTGGCGAGCTATCAAACAGGACAC-3′	4216–4239
EP4026-2	5′-GCGCTAGTAGCTTGAATGAAGAG-3′	4558–4535
TPAc3	5′-GGAATTCGTTTTCGTTACCGGTATATC-3′	NA
TPAc5	5′-GGAATTCGTTTTTTACCTCGGGTTC-3′	NA

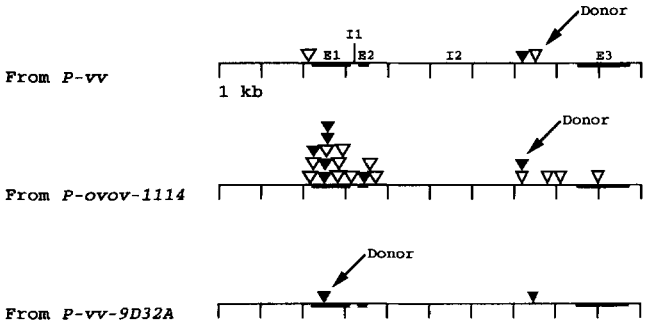

FIGURE 2.—Transposition of *Ac* from the progenitor alleles *P-vv* (upper), *P-ovov-1114* (middle) and *P-vv-9D32A* (lower). Exons 1, 2 and 3 of the 1.8-kb *P-rr* RNA are indicated by thick lines marked E1, E2 and E3; introns are indicated by I1 and I2. The donor and transposed *Ac* elements are indicated by triangles. Open triangles represent *Ac* elements in the same orientation with respect to *P* as in *P-vv*; filled triangles represent insertions in the opposite orientation (*i.e.*, same as *P-ovov-1114*). For the phenotypes of the alleles refer to Table 2.

reduced to colorless as in the standard *P-vv*. Finally, *P-ovov-1114* gave rise to *P-vv-9D32A*, which in turn gave rise to *P-ovov-12:1–1* (Figure 2, lower). In addition to the single *Ac* insertions described here, we obtained a number of alleles carrying two *Ac* elements; these are under investigation and will not be discussed further here. For phenotypes of the alleles, refer to Table 2.

***P* gene sequence:** To determine the effects of *Ac* inserted at specific sites within *P*, the positions of *Ac* insertions were determined and placed within the nucleotide sequence of the *P* gene. Figure 3 presents 7753 nucleotides of sequence of the *P* gene, beginning at 100 nucleotides 5′ of the transcription start site. The exons comprising two alternatively spliced *P* transcripts of approximately 1.8 and 0.95 kb are indicated in capital letters. The 1.8-kb transcript contains two small 5′ exons and a larger 3′ exon. The 0.95-kb transcript is produced by an alternative splicing reaction which joins the first two exons, common to both the 1.8- and 0.95-kb transcripts, to an exon contained within a 1.2-kb direct repeat sequence located immediately downstream of the *Sal*I site at position 7322. The 1.8-kb transcript is thought to be necessary for *P* function, since a 7-bp frameshift mutation in exon 3 results in a colorless phenotype (GROTEWOLD, ATHMA and PETERSON 1991b). The function, if any, of the 0.95-kb transcript is not known.

A striking feature of the *P* sequence is the length of introns 2 and 3, which at 4.6 and 6.7 kb are the longest plant introns reported to date. Contributing to the length of these introns is a 723 nucleotide sequence (Figure 3, positions 3074 to 3816) which may be a transposon insertion. This sequence is flanked by 10 nucleotide direct repeats (Figure 3, bold italics), and is absent from a *P*-homologous clone derived from the inbred line W22 (P. ATHMA, unpublished). This sequence does not have terminal inverted repeats characteristic of many plant transposons, nor have we observed excision of this sequence. However, a transposon with similar structural features (10 nucleotide flanking direct repeats, and no terminal inverted repeats) termed *Tz86* was identified in the maize *Shrunken* locus (DELLAPORTA *et al.* 1985). There is no obvious sequence similarity between the insertion in *P* and *Tz86*, nor in the 10 nucleotide direct repeats flanking each insertion.

Location of transposed *Ac* elements: The sites of *Ac* insertions were determined by PCR amplification and sequencing of *Ac/P* junction fragments (MATERIALS AND METHODS). The *Ac* elements in 21 *P* alleles are indicated by triangles in Figure 3. Open and filled triangles represent *Ac* insertions in the *P-vv* and *P-ovov-1114* orientations, respectively. The most 5′ *Ac* insertion (−41 relative to the transcription start) is in the *P-ovov* allele derived from *P-vv* by VALENTINE (1957). Fifteen additional insertions in the 5′ region were derived from *P-ovov-1114*: one at −12, nine within the 326 nucleotide 5′ untranslated leader, one in translater sequence of exon 1, one in intron 1, one in exon 2, and two in the 5′ region of intron 2. Altogether, 16 *Ac* insertions fall within a 1.2-kb region

TABLE 2

Alleles used in this study

Allele	Progenitor allele	*Ac* insertion site[a]	*Ac* orientation[b]	PCR primers[c]
P-ovov-Val[d]	*P-vv*	−41	VV	EP1PE + TPAc3
P-vv-9D87A	*P-ovov-1114*	−12	VV	EP1PE + TPAc3
P-vv-9D11B	*P-ovov-1114*	6	VV	EP1PE + TPAc3
P-vv-9D20A	*P-ovov-1114*	43	OVOV	PA-B7 + TPAc5
P-vv-9D32A	*P-ovov-1114*	133	OVOV	PA-B7 + TPAc5
P-vv-9D81B	*P-ovov-1114*	156	OVOV	EP5-9 + TPAc3
P-vv-6117	*P-ovov-1114*	166	VV	PA-A2 + TPAc5
P-vv-9D98A	*P-ovov-1114*	202	OVOV	PA-A2 + TPAc3
P-vv-9D79A	*P-ovov-1114*	205	OVOV	PA-A2 + TPAc3
P-vv-4189	*P-ovov-1114*	303	VV	EP3-13 + TPAc3
P-vv-6113	*P-ovov-1114*	312	VV	PA-A2 + TPAc5
P-vv-9D42B	*P-ovov-1114*	369	VV	EP3-13 + TPAc3
P-vv-6108	*P-ovov-1114*	472	VV	EP3-13 + TPAc3
P-vv-8493-21	*P-ovov-1114*	685	OVOV	EP5-18 + TPAc5
P-vv-9D14B	*P-ovov-1114*	781	VV	EP5-18 + TPAc3
P-vv-9D86A	*P-ovov-1114*	1086	VV	PA-A8 + TPAc5
P-vv-9D1B	*P-ovov-1114*	4335	VV	EP4026-1 + TPAc5
P-ovov-1114[e]	*P-vv*	4341	OVOV	NA[e]
P-ovov-12:1-1	*P-vv-9D32A*	4456	OVOV	EP4026-2 + TPAc5
P-vv	Unknown[f,g]	4494	VV	NA[e]
P-vv-8393-4	*P-ovov-1114*	5768	VV	NA[g]

[a] The *Ac* insertion site is given as the number of nucleotides from the *P* transcription start site to the most 3′ nucleotide of the 8-bp duplication predicted to be formed upon *Ac* insertion.

[b] The orientation of *Ac* in each allele is indicated by either VV (same orientation as in the *P-vv* allele; *i.e.*, with the 5′ end of *Ac* nearer the 5′ end of the *P* gene) or OVOV (opposite orientation).

[c] See Table 1 for primer sequences.

[d] VALENTINE (1957).

[e] PETERSON (1990).

[f] EMERSON (1914).

[g] GROTEWOLD, ATHMA and PETERSON (1991b).

including the first two exons of the *P* gene. There is only one allele with *Ac* inserted in the 3.2-kb region 5′ of the *P-ovov-1114* site. In this case, *Ac* transposed from the progenitor *P-ovov-1114* site to a position only 6 bp toward the 5′ end of the *P* gene, and inserted in the opposite orientation. Six alleles carry *Ac* inserted in the 3′ region of intron 2, but the exact insertion sites in two cases are not known. Finally, one allele carries *Ac* inserted in the translated region of exon 3.

Effects of *Ac* insertions on *P* gene expression: Figure 1 illustrates the pericarp pigmentation phenotypes specified by ten representative *P* alleles. There is a significant correlation between the grade of variegation specified by each insertion allele and the position of the insertion site in translated or untranslated sequences. That is, most alleles give a medium variegated or orange variegated phenotype with frequent red revertant sectors (Figure 1); these alleles carry *Ac* insertions in the *P* gene introns or untranslated regions of exons. In contrast, three alleles give very light variegated pericarp and cob glumes. Crosses to *Ds* testers (MATERIALS AND METHODS) show that the light variegated phenotype specified by these three alleles is not due to a high dose of *Ac*, which is known to delay the timing of *Ac* excision (MCCLINTOCK 1950). These three alleles–*P-vv-9D42B*, *P-vv-8493–21* and *P-vv-8393–4*–carry *Ac* insertions in the long open reading frame in exons 1, 2 and 3, respectively (Figure 1). Presumably, *Ac* excisions in these light variegated alleles occur at a similar frequency as in the more variegated alleles, but most of the resultant sectors are colorless due to remnants of the 8-bp duplication within the *P* reading frame.

There is a marked difference in the frequency of red sectors given by alleles carrying *Ac* in translated sequences. A single dose of either *P-vv-9D42B* or *P-vv-8393–4* gives approximately 2–3 visible red sectors on each kernel; in contrast, one dose of *P-vv-8493–21* gives only 1 visible red sector per 50–100 kernels. For *P-vv-9D42B*, which has *Ac* inserted in the open reading frame in exon 1, revertant sectors must, at a minimum, restore the *P* reading frame. In *P-vv-8493–21*, *Ac* is inserted in exon 2 midway between conserved residues of the *myb*-homologous domain thought to be critical for DNA binding (SAIKUMAR, MURALI and REDDY 1990). For *P-vv-8493–21*, revertant sectors may occur only through rare precise excisions which restore the original protein sequence.

DISCUSSION

Effects of *Ac* insertions on *P* expression: A notable feature of the *P* locus is the organ-specific expression

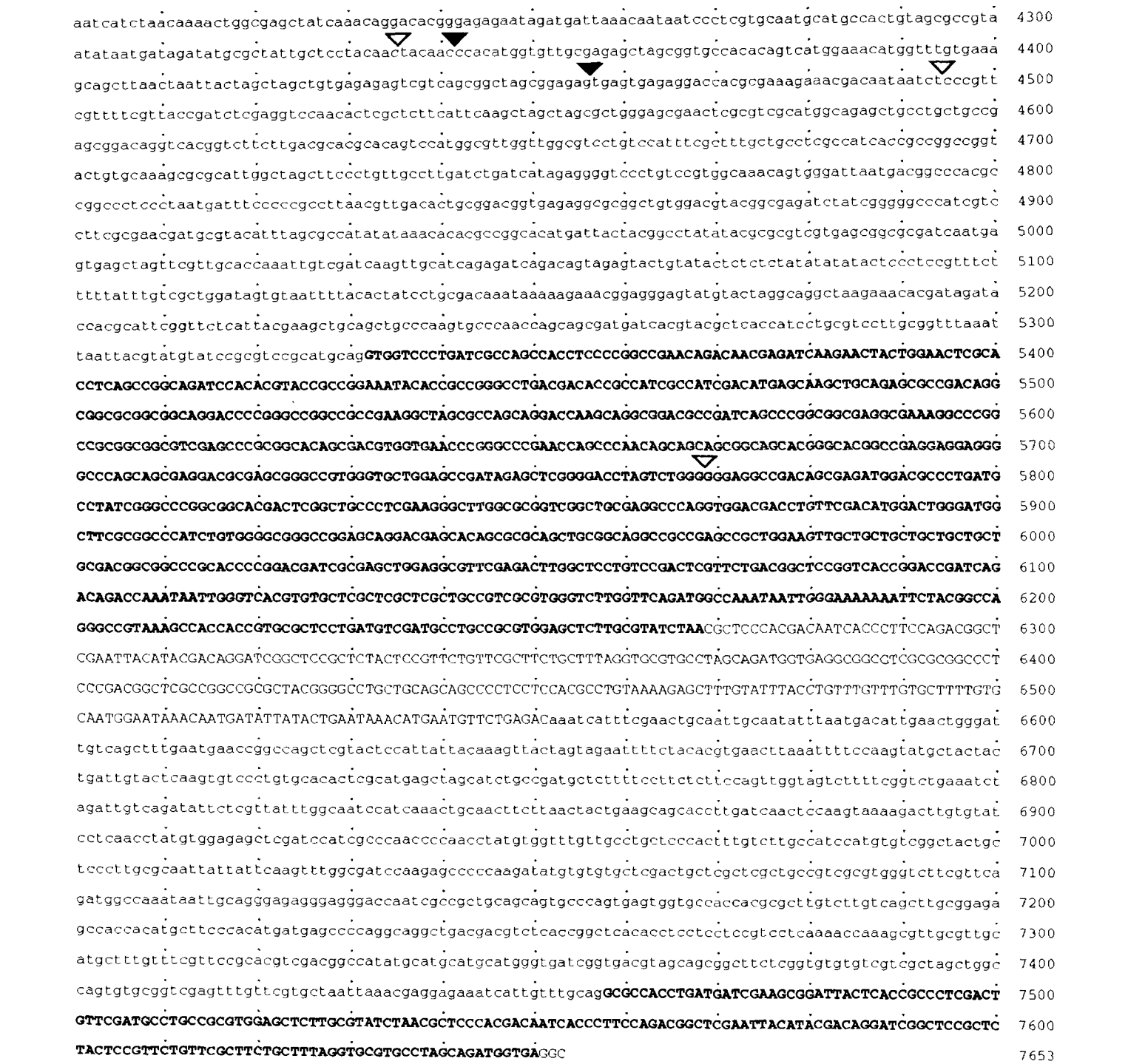

FIGURE 3.—Nucleotide sequence of the *P* gene. Exons and introns are indicated by upper- and lowercase letters, respectively; open reading frame is in bold. The 1.8-kb *P* transcript comprises exons 1, 2 and 3; the alternatively spliced 0.95-kb *P* transcript comprises exons 1, 2 and 4. *Ac* insertion sites are indicated by triangles; open triangles indicate *Ac* elements in the same orientation with respect to *P* as in the *P-vv* allele; filled triangles indicate *Ac* elements in the opposite orientation. Triangles indicating the insertion sites are placed to the right of the 8-bp sequence predicted to be duplicated upon *Ac* insertion. A 723-bp putative transposon in intron 2 (nucleotides 3084 to 3806) is indicated in italics; flanking 10-bp direct repeats are in bold italics. See text for details.

specified by the *P-wr* and *P-rw* alleles, which condition colorless pericarp/red cob and red pericarp/colorless cob, respectively. A long-standing question is whether *P-rr*, which pigments both pericarp and cob glumes, comprises two closely linked organ-specific genes (ANDERSON 1924). An example of complex gene organization is found at the maize *R* locus, in which certain *R* alleles comprise two components that confer plant or seed pigmentation (STADLER and NUFFER 1953; ROBBINS *et al.* 1991). For the *P* locus, genetic crosses did not give any clear evidence for recombination between pericarp and cob-specific components; how-

ever, the populations may not have been sufficiently large to detect recombinants between two tightly linked genes (ANDERSON 1924). We reasoned that insertional mutagenesis with *Ac*, which is known to have a preference for localized transposition, could disclose the presence of multiple functional units at *P-rr* if they in fact exist. Although our screening method (MATERIALS AND METHODS) may not have detected mutants which affect cob glume pigmentation only, we should have detected alleles specifying variegated pericarp and red cob glumes; such a phenotype would be expected from *Ac* insertion into a pericarp-specific *P* component. However, all the alleles analyzed here have similar effects on pericarp and cob glume pigmentation. The fact that no alleles with single *Ac* insertions give organ-specific pigmentation suggests that, in *P-rr*, the same gene component(s) are required for pericarp and cob glume pigmentation. Furthermore, since all of the mutant alleles carry *Ac* insertions within or very near the transcriptional unit previously mapped by molecular methods (GROTEWOLD, ATHMA and PETERSON 1991a), we conclude that *P-rr* contains a single gene required for both pericarp and cob glume pigmentation.

Effect of *Ac* orientation: As mentioned above, in the *P-vv* allele *Ac* is inserted in the second intron of the *P* gene. Analysis of RNA from homozygous *P-vv* pericarps indicated that the *P* message terminates within the *Ac* element, resulting in a nonfunctional chimeric *P/Ac* transcript (LECHELT *et al.* 1989). In *P-ovov-1114*, *Ac* excised from the *P-vv* site, transposed to a new site in the second intron 153 bp toward the 5′ end of the *P* gene, and reinserted in the opposite orientation. This transposition resulted in increased pericarp pigmentation and significant amounts of normal-sized *P* transcripts due to splicing of *Ac* sequences from the *P-ovov-1114* transcript. We proposed that the difference in expression between the *P-vv* and *P-ovov-1114* alleles is most likely due to the different orientations of *Ac* (PETERSON 1990). In the *P-vv* allele, in which *Ac* is transcribed in the same direction as the *P* gene, transcription terminates within *Ac*. In the *P-ovov-1114* allele, *Ac* is transcribed in the opposite direction as the *P* gene; *P-ovov-1114* transcripts can proceed through *Ac*, and the *Ac* sequences can be spliced from the message. This model is supported by the phenotypes specified by six additional *Ac* insertions in the *P* gene second intron: five insertions in the same orientation as *P-vv* (Figure 1, open triangles) give standard variegated pericarp, while one insertion in the same orientation as *P-ovov-1114* (Figure 1, closed triangles) gives orange variegated pericarp. It is noteworthy that the opposite orientations of *Ac* in the *P-vv-9D1B* and *P-ovov-1114* alleles correlate with the variegated and orange variegated phenotypes, respectively, even though the *Ac* insertion sites in these alleles are just six bp apart.

Although *Ac* orientation in intron 2 is correlated with the variegated or orange variegated phenotypes, this correlation does not hold throughout the *P* gene. The *Ac* element in the *P-ovov-Valentine* allele is located 41 bp upstream of the transcription start site in the *P-vv* orientation; this insertion may interfere with transcription regulation. In the 5′ untranslated leader region, four alleles carry *Ac* insertions in the *P-vv* orientation and five have *Ac* in the *P-ovov* orientation; yet all condition a standard variegated pericarp phenotype. Analysis of transcripts from each of these alleles is required to determine the molecular impact of *Ac* in each case. Nevertheless, it is clear from these results that changes in the position and orientation of *Ac* insertions within *P* can produce marked phenotypic differences. ORTON and BRINK (1966) reported similar phenotypic differences among *P* alleles derived by transposition of *Ac* from flanking sites back into the *P* locus, and they proposed that these differences resulted from *Ac* insertion at different sites in the *P* gene. Our results provide further confirmation of the hypothesis that "changes in state" of genes carrying transposable elements can occur by shifts in position and/or orientation of the element within the affected gene (PETERSON 1976).

Insertion site specificity: The *Ac* insertion sites of 19 *P* alleles are compared in Figure 4. Interestingly, the sequence **GCxAG** occurs immediately adjacent to the *Ac* insertion in four out of 19 sequences, while five of the 19 alleles match at 3 of 4 bases of the **GCxAG** motif. The complementary sequence **CTxGC** is located at the opposite end of the duplicated 8 nucleotides in two alleles, and two alleles match the **CTxGC** sequence at 3 of 4 bases at this position. The site of *Ds1* insertion in *Bz-wm* is adjacent to the sequence **CTxGC** (SCHIEFELBEIN *et al.* 1988), while the site of double *Ds* insertion in *sh-m6233* matches the **CTxGC** motif at three of four positions (WECK *et al.* 1984). However, several insertion sites in the *P* gene, as well as most other *Ac* and *Ds* insertion sites published to date, bear little resemblance to the consensus. No sequence specificity for *Ac/Ds* insertions has been previously reported, although it has been noted that *Ac* and *Ds* insertions tend to be located near short direct duplications (DORING and STARLINGER 1984). Similar duplications are found near several insertion sites in *P* (Figure 4).

The shortest transposition distance we observed is in the case of *P-vv-9D1B*, in which *Ac* transposed only 6 bp to the 5′ side of the *P-ovov-1114* site and inserted immediately 3′ of the sequence TACAAC (Figure 4). This is the same sequence at which *Ac* inserted in *P-ovov-1114* after transposition from *P-vv*. Recently we presented evidence of even earlier transposon inser-

Allele	Insertion Site Sequence
P-vv-9D87A	ATTATGGCCG GCCGTGGC GTGCCCTCTC
P-vv-9D11B	CGTGCCCTCT CTAGCCAG CACAGCACAC
P-vv-9D20A	AGTGCAAGCT GTAGTGAG ACCTGCGCGA
P-vv-9D32A	AACTCCCTTG GACGCACG CGCGCGCGCG
P-vv-9D81B	GCGCGACCAG CTGCTAAC CGTGCGCAAG
P-vv-6117	CTGCTAACCG TGCGCAAG TAGTAGTGCG
P-vv-9D98A	GCCGGCCGGG ATCGCTAG CTCGATCGAT
P-vv-9D79A	GGCCGGGATC GCTAGCTC GATCGATCGG
P-vv-4189	AGGGCAGACG CTAGCTGT TGCCGGGAGC
P-vv-6113	GCTAGCTGTT GCCGGGAG CTAGCCGGCG
P-vv-9D42B	AGGTGGGGCT CAAGCGAG GGAGGTGGAC
P-vv-6108	TGCAGGTAAA CCAAAGCC GGCCGCGCGC
P-vv-8493-21	AGAAGACATC ATCATCAA GCTCCACGCC
P-vv-9D14B	AGTAGTAGTA CTACTACT TCCTTCCTTT
P-vv-9D86A	AAGGATCTCT GGCACAAG ACTAGAACAG
P-vv-9D1B	CGCTATTGCT CCTACAAC TACAACCCAC
P-ovov-1114	TGCTCCTACA ACTACAAC CCACATGGTG
P-ovov-12:1-1	TCAGCGGCTA GCGGAGAG TGAGTGAGAG
P-vv-8393-4	TCGGGGACCT AGTCTGGG GGGAGGCCGA
Consensus:	AG--G---C- ---GC-AG --C-G--C/G-G 8 10--9---10- --- 10 9-12 10 --8-8--8/8-8

FIGURE 4.—Sequences at *Ac* insertion sites, and derived consensus sequence. The central 8 nucleotides represent the 8 nucleotides predicted to be duplicated upon *Ac* insertion. Nucleotides matching the consensus sequence are indicated in bold. Sequences are aligned as they are oriented in *P*; alignment such that all sequences are in the same orientation with respect to *Ac* does not improve the consensus, although the **GCxAG** consensus is retained in the central 8 nucleotides. Direct repeat sequences are underlined. *P-vv* and *P-ovov-Valentine* are omitted because the *Ac* insertion in the former has no flanking direct repeat, and the sequence of the *Ac* insertion in the latter is slightly different from the same region in *P-ovov-1114* (P. ATHMA, unpublished).

tion at this site (GROTEWOLD, ATHMA and PETERSON 1991b); the *Ac* insertion in *P-vv-9D1B* is another indication that this is a preferred site for transposon insertion.

Nonrandom distribution of *Ac* insertion sites: A striking feature of our data is the pronounced clustering of transposed *Ac* elements. The 1.1-kb region located 3.2 kb to the 5′ side of the *P-ovov-1114* donor site carries 16 of the 21 transposed *Ac* elements derived from *P-ovov-1114* and *P-vv*. We detected three *Ac* insertions in the 2-kb region on the 3′ side of the *P-ovov-1114* donor site; this region covers the third exon of the 1.8-kb *P* mRNA. In contrast, only one *Ac* insertion was found in the 3.2 kb region immediately to the 5′ side of the *P-ovov-1114* donor site, and this insertion (*P-vv-9D1B*) was located just 6 bp to the 5′ side of the *P-ovov-1114* site. The 3.2-kb region with one *Ac* insertion is contained within the 4.6-kb *P* intron 2, but it is unclear why there should be so few *Ac* insertions in this region. One possibility is that *Ac* insertion in this region does not give a phenotype different from that specified by the progenitor *P-ovov-1114* allele. However, the available evidence (see above) suggests that insertion in the *P-vv* orientation should produce a variegated phenotype similar to that specified by *P-vv*. Also, we note that *Ac* "jumped over" this region in two cases in which alleles specifying orange variegated pericarp were derived from alleles giving standard variegated pericarp: the transposition from *P-vv* to the *P-ovov-Valentine* site, and the transposition from *P-vv-9D32A* to the *P-ovov-12:1-1* site (Figure 2). It is unlikely that *Ac* insertion in this region disrupts an essential gene, because deletion of this entire region by recombination between the flanking 5.2-kb direct repeats has no obvious effect on plant vigor (ATHMA and PETERSON 1991). Neither does there seem to be a minimum distance of *Ac* transposition, as evidenced by the transpositions of *Ac* from the *P-vv* to *P-ovov-1114* sites (153 bp) and the *P-ovov-1114* to *P-vv-9D1B* sites (6 bp).

Might the clustering of transposed *Ac* elements result from the association of *Ac* transposition and DNA replication identified by GREENBLATT and BRINK (1962)? GREENBLATT (1984) reported a striking asymmetry in distribution of *Ac* insertions after transposition from *P-vv*: the 4 map unit region proximal to *P* was devoid of *Ac* insertions, whereas the 4-map unit interval distal to *P* contained the highest number of *Ac* insertions. GREENBLATT proposed that *Ac* transposes from a replicated donor site to an unreplicated target site, and that the asymmetrical distribution of transposed *Ac* elements in the vicinity of *P* reflects replication of the *P* locus and surrounding DNA in a proximal to distal direction. One might attempt a similar explanation of our data by postulating a replication origin situated within the region devoid of *Ac* insertions. However, this model is incompatible with three observations. First, the transpositions of *Ac* from the *P-vv* site to the *P-ovov-1114* site (153 bp toward the 5′ end of *P*), and from the *P-ovov-1114* site to the *P-vv-9D1B* (6 bp toward the 5′ end of *P*) would require transposition of *Ac* toward the hypothetical replication origin, which is inconsistent with the stipulation that *Ac* transposes into unreplicated DNA. Second, the replication model predicts that each transposition generates a potential twin sector (GREENBLATT 1968). However, twinned sectors derived from *P-ovov-1114* are rare (P. ATHMA and T. PETERSON, in preparation), and none of the alleles described here was derived from twinned sectors. Third, a prediction of the replication model is that transposition of *Ac* from its site in *P-vv-9D32A* (inserted in the *P* 5′ leader) in the 3′ direction should have resulted in insertion at a site at least 4.5 kb (the length of *Ac*) further 3′ than the site of insertion in *P-ovov-1114* (assuming that the rate of DNA replication is uniform). Instead, the *P-ovov-12:1-1* insertion site is located approximately 100 bp to the 3′ side of the *P-ovov-1114* insertion site, in the same region at the 3′ end of intron 2 containing five other *Ac* insertions (Figure 2).

The absence of *Ac* insertions in the 3.2-kb region of intron 2 may arise from some other aspect of the

transposition mechanism. Alternatively, the region lacking *Ac* insertions may have a particular chromatin structure refractory to *Ac* insertion. We are currently seeking additional intragenic transpositions of *Ac* from the *P-vv* and *P-ovov-Valentine* insertion sites to test whether *Ac* insertions in the 3.2-kb region can occur.

There is a disproportionately high number of *Ac* insertions in the 5′ region (15 insertions in 1.1 kb) compared to the exon 3 translated region (one insertion in 1.0 kb). This uneven distribution is due, at least in part, to deliberate selection for insertions which give a significant amount of variegation (medium variegated phenotype). In a related study, we found that *P-vv* and *P-ovov-1114* can mutate to *P-ww* by *Ac*-induced recombination between the 5.2-kb direct repeats flanking the *P* gene. The resulting *P-ww* alleles have a common 17-kb deletion of the *P* gene and are designated *P-ww-d*. We estimated that the frequency of intragenic transpositions is 10–20% the frequency of *P-ww-d* mutants (ATHMA and PETERSON 1991). However, kernels in mutant sectors arising from either deletions or intragenic transpositions commonly have a pigmented silk attachment region ("dark crown") with occasional rays of pigment extending down the kernel (EMERSON 1917; ATHMA and PETERSON 1991). Thus, it is difficult to distinguish kernels carrying deletions from those carrying intragenic transpositions into *P* translated sequences which would confer a very light variegated phenotype. To avoid a high background of *P-ww* deletion mutants, we preferentially selected kernels from sectors with a medium variegated phenotype; this selection undoubtedly contributed to the paucity of insertions in *P* gene translated sequences. However, this selection cannot account for the region devoid of insertions in intron 2.

Recently, WEIL *et al.* (1992) characterized 15 alleles of the maize *Waxy* gene derived by intragenic transposition of the *Ds* element in the *Wx-m5* allele. The donor *Ds* was located near the 5′ end of the *Waxy* gene, and although *Ds* insertions were detected throughout *Waxy*, a greater frequency of insertion occurred at sites near the 3′ end of the gene. It was postulated that, upon short-range transpositions, *Ds* preferentially inserts at sites at least 3 kb from the donor site (WEIL *et al.* 1992). A similar tendency during short-range transposition of *Ac* might contribute to the high number of insertions found near the 5′ end of the *P* gene (Figure 1). However, this model is not consistent with our finding of two very short transpositions in the *P* gene (6 bp and 153 bp; see above).

Bidirectional *Ac* transposition within *P*: At the maize *Bronze* gene, both intragenic transpositions of *Ds* (DOWE, ROMAN and KLEIN 1990) and extragenic transpositions of *Ac* (DOONER and BELACHEW 1989) are bidirectional. Similarly, extragenic transpositions of *Ac* from the *Waxy* gene are bidirectional (SCHWARTZ 1989). In contrast, GREENBLATT (1984) reported that extragenic transpositions of *Ac* from *P-vv* to sites within 4 map units of *P* were in one direction only. However, our results do not show the strict polarity of transposition predicted from GREENBLATT's data and the replicon hypothesis discussed above (GREENBLATT 1984). Rather, intragenic transpositions of *Ac* from *P-ovov-1114* can occur in both directions (Figure 2). It may be significant that, unlike the intragenic transpositions reported here, the *Ac* elements mapped by GREENBLATT (1984) had transposed out of the *P* gene to sites sufficiently distant to be separable from *P* by meiotic recombination; perhaps the difference in distribution of insertion sites reflects a difference between the mechanism of short- and long-range transpositions. In any case, our results establish that *Ac* can transpose in either direction in the *P* gene.

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