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Generating Novel Allelic Variation Through *Activator* Insertional Mutagenesis in Maize

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ABSTRACT

The maize transposable element *Activator* (*Ac*) has been exploited as an insertional mutagen to disrupt, clone, and characterize genes in a number of plant species. To develop an *Ac*-based mutagenesis platform for maize, a large-scale mutagenesis was conducted targeting the *pink scutellum1* locus. We selected 1092 *Ac* transposition events from a closely linked donor *Ac*, resulting in the recovery of 17 novel *ps1* alleles. Multiple phenotypic classes were identified corresponding to *Ac* insertions in the 5′-UTR and coding region of the predicted *Ps1* gene. To generate a stable allelic series, we employed genetic screens and identified 83 germinally heritable *ps1* excision alleles. Molecular characterization of these excision alleles revealed a position-dependent bias in excision allele frequencies and the predominance of 7- and 8-bp footprint products. In total, 19 unique *ps1* excision alleles were generated in this study, including several that resulted in weak mutant phenotypes. The analysis of footprint alleles suggests a model of *Ac* excision in maize that is consistent with recent *in vitro* studies of *hAT* element excision. Importantly, the genetic and molecular methods developed in this study can be extended to generate novel allelic variation at any *Ac*-tagged gene in the genome.

THE maize transposable elements *Activator* (*Ac*) and *Dissociation* (*Ds*) are powerful insertional mutagens in plants and have been used to isolate genes (Kunze and Weil 2002), to define promoter and enhancer elements (Athma *et al.* 1992; Moreno *et al.* 1992; Sundaresan *et al.* 1995; Chin *et al.* 1999; Greco *et al.* 2003; Wu *et al.* 2003; Jin *et al.* 2004), and to create genetic mosaics for clonal analysis (Emerson 1917; Dawe and Freeling 1990; Peng and Harberd 1997; Jenik and Irish 2001). An important feature of both *Ac* and the nonautonomous derivative *Ds* is the tendency for short-range transposition. In studies of *Ac* transposition from the *p1* and *bz1* loci, nearly 60% of all transpositions were to sites within 5 cM of the donor *Ac* (*dAc*) (Greenblatt 1984; Dooner and Belachew 1989). This general feature of *Ac/Ds* has been exploited in maize to mutagenize genes that lie in close proximity to the donor element (Orton and Brink 1966; Brink and Williams 1973; Kermicle *et al.* 1989; Athma *et al.* 1992; Moreno *et al.* 1992; Weil *et al.* 1992; Alleman and Kermicle 1993).

The power of *Ac* as an insertional mutagen was perhaps most fully exploited in the dissection of the *p1* locus. Utilizing *Ac* donor elements closely linked to *p1*, >400 *Ac*-induced *p1* alleles have been generated (Orton and Brink 1966; Athma *et al.* 1992; Moreno *et al.* 1992). By carefully examining the variegation patterns of these alleles, promoter elements, enhancer elements, intron/exon boundaries, and critical amino acids were defined throughout coding and noncoding regions of the locus (Moreno *et al.* 1992). The elegance of such studies is perhaps most striking for maize, where time and cost constraints associated with constructing an allelic series of near-isogenic, stably expressed transgenic lines are prohibitive.

Ac and *Ds* can also be utilized to create genetically stable excision or "footprint" alleles. When *Ac* or *Ds* inserts into the genome, it generates an 8-bp target-site duplication (Muller-Neumann *et al.* 1984; Pohlman *et al.* 1984). *Ac* and *Ds* excision events are often imprecise (Baran *et al.* 1992; Moreno *et al.* 1992), resulting in the creation of novel footprint alleles (Muller-Neumann *et al.* 1984; Pohlman *et al.* 1984). At the *waxy1* locus, two *Ds* excision alleles resulting in 9- and 6-bp insertions altered the enzymatic activity of the WAXY1 protein (Wessler *et al.* 1986). Similarly, *Ds* excision alleles at the *r1* locus resulted in subtle perturbations of *R* function and defined critical domains in the R protein necessary for nuclear localization and protein–protein interactions (Liu *et al.* 1996, 1998). Interestingly, a *Ds* excision allele at *shrunken2* resulted in an 11–18% increase in seed weight through the alteration of allosteric properties of the protein (Giroux *et al.*

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1996). These studies have shown that the activity, localization, and function of a protein can be dramatically or subtly altered through *Ac* or *Ds* excision.

Despite the many useful features of *Ac/Ds*, relatively few genes have been characterized using these elements in maize. The primary limitation has been the lack of precisely mapped and evenly distributed *Ac* elements throughout the maize genome (Brutnell and Conrad 2003). However, in recent years several large-scale genomics programs have been developed to position *Ac* elements on genetic and physical maps of the maize genome (Cowperthwaite *et al.* 2002; Kolkman *et al.* 2005). The genetic materials created through these programs now provide the opportunity to exploit *Ac* to characterize thousands of genes throughout the maize genome.

To explore the potential of *Ac* as a tool in large-scale insertional mutagenesis programs, we conducted a regional mutagenesis of the *pink scutellum1* (*ps1*) locus. The *Ps1* gene encodes the enzyme lycopene β-cyclase that catalyzes the first committed step in xanthophyll biosynthesis (Singh *et al.* 2003). We previously utilized an *Ac* element located 4 cM from *ps1* to clone the gene and generated seven unstable *ps1* alleles (Singh *et al.* 2003).

In this study, we have selected 1092 independent *Ac* transposition events from the same donor *Ac* and recovered 17 novel *ps1* alleles. Of the 17 alleles, 14 carried *Ac* insertions that were precisely positioned at the *ps1* locus, whereas 3 of the *ps1* alleles retained a signature of *Ac* excision. Thus, all of the recovered *ps1* alleles were likely induced by *Ac*, but 18% did not carry a molecular tag for cloning. We have also developed a genetic strategy to enrich for the recovery of *Ac* footprint alleles. Sequence analysis of 57 footprint alleles was consistent with the "endonuclease model" of excision repair (Coen *et al.* 1989) whereby double-strand breaks are generated on each side of *Ac* following excision, resulting in the formation of hairpins at the donor locus. We propose that these hairpins are resolved by a selective endonucleolytic attack at a site immediately adjacent to the ligation site and discuss the implications of these findings for developing *Ac/Ds*-based mutagenesis programs in maize.

MATERIALS AND METHODS

Genetic stocks and selection schemes: All alleles were maintained in the common genetic background of a color-converted W22 inbred line (Dooner and Kermicle 1971). *Ac* regional mutagenesis at *ps1* from donor site *bti97156::Ac* was previously described (Singh *et al.* 2003).

To identify rare *Ac* excision events, two genetic schemes were developed that exploit a *Ds* reporter to monitor *Ac* copy number. *Ac* activity was monitored with the *Ds* reporter, *r-sc:m3*, as previously described (Brutnell and Dellaporta 1994). In the absence of *Ac*, the *Ds* insertion at *r-sc:m3* is stable, resulting in a colorless aleurone. In the presence of *Ac*, *Ds* excises at a time and frequency that is dependent on *Ac* copy number, resulting in purple sectored aleurone. An increase in *Ac* copy number delays *Ds* excision, resulting in kernels with smaller spots, and is generally referred to as the "negative dosage effect" (McClintock 1951).

In the first genetic scheme, kernels were selected from self-pollinated ears of lines carrying the donor *Ac* (*bti97156::Ac*, *dAc*) and the *ps1::Ac* allele (see supplemental Figure S1 at http://www.genetics.org/supplemental/). To enrich for *Ac* excision events, coarsely spotted kernels were selected, indicating that a single active *Ac* element was inherited through the male gametophyte. Because the donor and *ps1 Ac* insertions are closely linked in *cis*, recombination events are rare. To identify potential excision alleles, single-copy *Ac* kernels (*dAc* +/+ + or + *ps1*::*Ac*/+ +) from self-pollinated *dAc ps1::Ac*/+ + ears were planted in the field. DNA was extracted from seedling tissues and DNA blot analysis was performed as previously described (Singh *et al.* 2003) to identify plants that no longer carried *Ac* at the *ps1* locus. Plants carrying these putative *ps1* footprint alleles were self-pollinated, and ears were screened to identify families that segregated a single active *Ac* element and a *ps1* mutant phenotype. Colorless kernels were selected from these ears (+ *ps1*/+ + or + +/+ +), and the plants self-pollinated to generate a stock that did not carry *Ac*.

A second genetic scheme was developed to identify novel *ps1* alleles that condition a subtle mutant phenotype as described in the results. In this strategy, kernel selections were based solely on *Ac* copy number and not on *ps1* mutant phenotypes. To select *Ac* excision alleles, lines doubly hemizygous for the *dAc* and the *ps1::Ac* allele (*dAc ps1::Ac*/+ +) were testcrossed to the *r-sc:m3* tester line. Lines carrying a single *Ac* element were selected on the basis of kernel variegation pattern, and DNA was isolated from seedling tissues. To screen for the presence of the *dAc*, primers 53035.7 and TBP35 were used to amplify the DNA flanking the *dAc* insertion. Primer pairs used to amplify the *Ac* junction at the *ps1* locus are listed in supplemental Table S1 and primer sequences are listed in supplemental Table S2 at http://www.genetics.org/supplemental/. Lines carrying putative *ps1* excision alleles were self-pollinated to generate segregating families.

Fine mapping of *ps1* alleles: To precisely map *Ac* insertion sites at *ps1*, phenotypically wild-type kernels were selected from families segregating *ps1* mutant kernels. DNA was extracted from seedling tissues and DNA blot analysis was performed to identify heterozygous individuals carrying an *Ac* insertion at *ps1* using a *ps1*-specific DNA fragment (Singh *et al.* 2003). DNA flanking each of the *Ac* insertions and excision sites from *ps1-d22*, *ps1-d23*, and *ps1-d24* were amplified using primers shown in Table S1 at http://www.genetics.org/supplemental/. To define *ps1* footprint alleles, kernels carrying a single *Ac* were grown in the field and DNA was extracted from seedling tissue. PCR was performed to determine the presence of *Ac* at the donor site and *ps1*. Putative excision alleles were then amplified using *Ps1*-specific primers listed in Table S1 at http://www.genetics.org/supplemental/. As these primers amplified both footprint and wild-type *Ps1* alleles, multiple PCR products were cloned and sequenced. The PCR reactions consisted of ~30 ng DNA, 0.25 μM of each primer, 0.2 mM dNTP's (Promega, Madison, WI), 1× Taq polymerase buffer with $MgCl_2$ (Promega), 0.5 M betaine, 4% DMSO, and 2 units of Taq polymerase (Promega). The reaction was denatured at 94° for 2 min and followed by 30 cycles of 94° for 30 sec, 57° for 45 sec, and 72° for 2 min. All cycles terminated with a final extension at 72° for 7 min. Amplification reactions were fractionated on 1.2% agarose gels and PCR products were purified using the QIAquick gel extraction kit (QIAGEN, Valencia, CA) following the manufacturer's recommendations. Products were

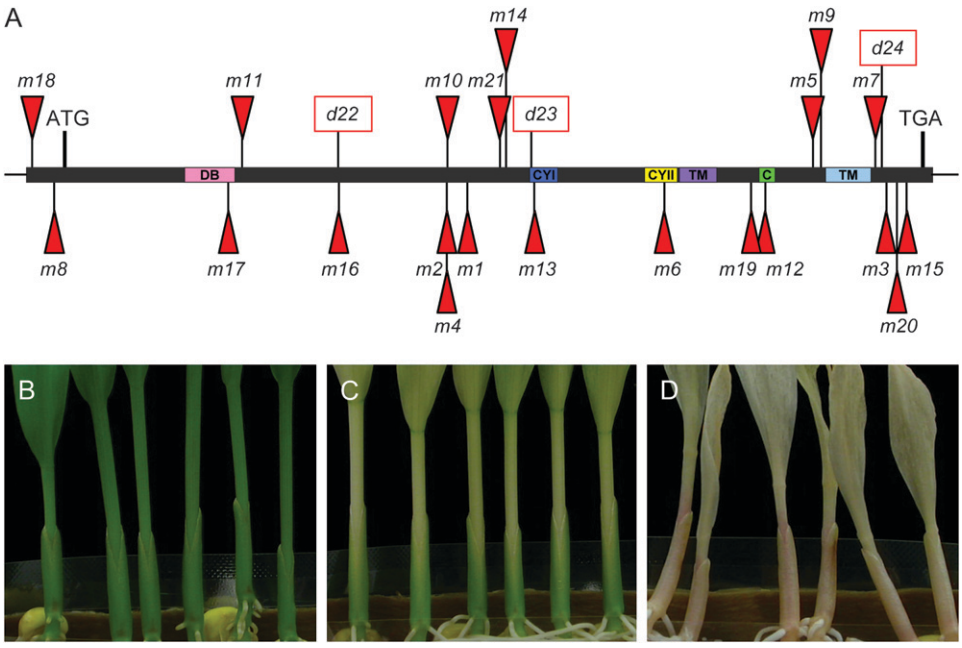

FIGURE 1.—*Ac*-induced *ps1* allelic variation. (A) *Ac* insertions in *ps1*. The long horizontal black bar represents the coding and noncoding regions of the intronless *Ps1* gene. Red triangles above the gene represent *Ac* insertions in 5′–3′ orientation in each *ps1-m::Ac* allele; red triangles below the gene are *Ac* insertions in 3′–5′ orientation. Orientation is related to *Ac* transcriptional start sites. Rectangular open boxes represent *Ac*-induced *ps1-d* alleles with the signature of *Ac* excision. Previously identified conserved regions (CUNNINGHAM *et al.* 1996) are shown by colored boxes: DB, dinucleotide-binding motif; CYI, cyclase motif I; CYII, cyclase motif II; TM, predicted transmembrane helix; C, charged region. Representative seedling phenotypes that are homozygous for (B) the W22 *Ps1* allele, (C) *ps1-m18::Ac*, and (D) *ps1-m7::Ac* are shown.

ligated into pGEM T-easy vector (Promega) and sequenced as previously described (SINGH *et al.* 2003).

Germination screen: Maize kernels were sterilized in a 20% bleach solution by gentle rocking for 20 min followed by five rinses with sterile water and then was left to soak in water overnight at room temperature with gentle rocking. Ten kernels for each genotype were placed in a seed germination pouch (Mega International, St. Paul) and watered daily. Seed pouches were placed vertically in the growth chamber and grown under 40 μmol m^2 s^{-1} white light. Germination rates were scored and plants were photographed 7 days after planting.

RESULTS

Insertional mutagenesis at *Ps1* gene: The *Ps1* gene of maize was cloned utilizing the transposable element *Ac* as an insertional mutagen (SINGH *et al.* 2003). In this previous study, seven independent *Ac* insertion alleles were recovered throughout the coding region of *Ps1* from a population of 386 transposition events. To further characterize the genetic fine structure of the *ps1* locus, we expanded the scope of the mutagenesis program. An additional 1092 transposition events were recovered as single kernel selections from an *Ac* donor (*bti97156::Ac*) resident 4 cM from the *ps1* locus. The resulting self-pollinated ears were screened and 17 families were identified that segregated pink/viviparous kernel phenotypes.

To characterize these putative *ps1* alleles, DNA blot analysis was first performed to map potential *Ac* insertion sites (see MATERIALS AND METHODS). This survey indicated that in 14 of 17 alleles, an *Ac* element was present at the *ps1* locus. However, we did not detect an *Ac* element or any large-scale rearrangements of *ps1* sequence associated with the remaining 3 *ps1* alleles. Thus, we sequenced the 5′-UTR complete coding region and the 3′-UTR of the *Ps1* gene from each of the 3 alleles. Comparison of the progenitor W22 *Ps1* sequence with the sequence from the mutant alleles identified polymorphic sites in each of the non-*Ac*-tagged alleles. In each case, there was a small duplication of the *ps1* sequence. Derivative *ps1-d22* carried a 4-bp insertion, *ps1-d23* carried an 8-bp insertion, and *ps1-d24* carried an 8-bp insertion at positions 925, 1253, and 1857, respectively, of the *Ps1* gene (GenBank AY206862). These insertions corresponded to tandem duplications of *Ps1* sequence typical of *Ac* excision events (SCOTT *et al.* 1996). In summary, of the 1092 kernels selected as carrying a *tr-Ac*, 14 families were recovered with *Ac* insertions at *ps1*, and 3 *ps1* mutant alleles that did not carry an *Ac* insertion were recovered. Thus, *Ac*-induced alleles of *ps1* were recovered in 1.6% of kernel selections (17/1092), and 18% of the *ps1* alleles recovered were not tagged with an *Ac* element.

DNA flanking each *Ac* insertion was amplified and sequenced to precisely position the *Ac* insertion within the *ps1* locus (see MATERIALS AND METHODS). Insertions that resulted in a mutant phenotype were identified in 5′-UTR and throughout coding regions as summarized in Figure 1A. *Ac* elements were distributed in both orientations and at roughly equal distribution throughout the *ps1* locus ($\chi^2 = 1.19$, d.f. $= 1$, $P > 0.2$). A uniform distribution of *Ac* is consistent with studies of *Ac* insertion at *p1* (ATHMA *et al.* 1992; MORENO *et al.* 1992) and transpositions of *Ac* from *bz1* and *waxy1* (COWPERTHWAITE *et al.* 2002) where no insertion site preference for the proximal or distal half of genes was detected. This is in marked contrast to the distribution of *Mutator* elements in maize or *Ds* insertions in

TABLE 1

Seed germination ratio of *ps1::Ac* alleles

ps1::Ac allele[a]	*Ac* orientation	% germination[b]	Seedling phenotype	Seedling lethal
Ps1-W22	NA	100 (100/100)	Green	No
ps1-m18::Ac	5–3′	98 (59/60)	Virescent	Yes
ps1-m8::Ac	3′–5′	98 (59/60)	Pale green mesocotyl	No
ps1-m17::Ac	3′–5′	0 (0/20)	NA	Yes
ps1-m11::Ac	5′–3′	0 (0/20)	NA	Yes
ps1-m16::Ac	3′–5′	0 (0/20)	NA	Yes
ps1-m2::Ac	3′–5′	0 (0/20)	NA	Yes
ps1-m4::Ac	3′–5′	0 (0/20)	NA	Yes
ps1-m10::Ac	5′–3′	0 (0/20)	NA	Yes
ps1-m1::Ac	3′–5′	0 (0/20)	NA	Yes
ps1-m21::Ac	5′–3′	0 (0/20)	NA	Yes
ps1-m14::Ac	5′–3′	0 (0/20)	NA	Yes
ps1-m13::Ac	3′–5′	0 (0/20)	NA	Yes
ps1-m6::Ac	3′–5′	0 (0/20)	NA	Yes
ps1-m19::Ac	3′–5′	0 (0/20)	NA	Yes
ps1-m12::Ac	3′–5′	0 (0/20)	NA	Yes
ps1-m5::Ac	5′–3′	0 (0/20)	NA	Yes
ps1-m9::Ac	5′–3′	0 (0/20)	NA	Yes
ps1-m7::Ac	5′–3′	88 (35/40)	White with green tip	Yes
ps1-m3::Ac	3′–5′	59 (53/90)	White with green tip	Yes
ps1-m20::Ac	3′–5′	5 (2/40)	White with green tip	Yes
ps1-m15::Ac	3′–5′	34 (20/59)	Virescent	Yes

[a] *ps1::Ac* alleles are listed according to the *Ac* insertion site in the *Ps1* gene.
[b] Germination ratio was scored as the average of three replicates.

Arabidopsis that preferentially target the 5′ half of genes (Parinov *et al.* 1999; May *et al.* 2003).

Although the *Ps1* gene contains no introns, *Ac* insertions at *ps1* conditioned a range of phenotypic variation that was position dependent. To quantitate this variation, we exploited the fact that the *Ps1* gene is required for both abscisic acid (ABA) production and normal carotenoid content of leaf tissues (Singh *et al.* 2003). A loss of ABA production leads to precocious germination due to a failure to suppress germination late in the grain-filling process. These kernels do not survive the desiccation process following harvest. Thus, the percentage of germination is an indirect measurement of ABA activity and PS1 function.

Seed germination assays were performed as described in materials and methods (data are in Table 1). *Ac* insertions in the middle of the *Ps1* gene resulted in a severe mutant phenotype, characterized by vivipary and a failure to germinate after the ear was dried. These phenotypes are consistent with the absence of functional PS1 protein, thus preventing the accumulation of ABA. Insertions in the 5′-UTR and near the carboxy terminus of the predicted protein were less severe. For example, germination rates for seedlings homozygous for both the *ps1-m8::Ac* and the *ps1-m18::Ac* allele were 98%. In contrast, *Ac* insertions at the 3′-end of the *Ps1* gene conditioned much lower germination rates. Individuals homozygous for *ps1-m7::Ac*, *ps1-m3::Ac*, *ps1-m20::Ac*, and *ps1-m15::Ac* conditioned germination rates of 88, 59, 5, and 34%, respectively. Thus, the site of *Ac* insertion in the *Ps1* gene has a dramatic effect on germination rate.

Insertions in the 5′-UTR permit the accumulation of ABA pools that are sufficient to protect the embryo during desiccation, leading to very high rates of germination. Insertions in the central and 3′ region of the gene are likely to produce truncated protein products that disrupt PS1 function, thereby limiting ABA production. The reduced levels of ABA may result in delayed developmental arrest and reduce the desiccation tolerance of the embryo, resulting in a greatly reduced germination rate.

In addition to ABA production, PS1 function is also required for the synthesis of photoprotective carotenoids. As observed for germination rates, the position of the *Ac* insertion at *Ps1* also had a dramatic effect on the seedling phenotype (Figure 1, C and D). Insertions in the 5′-UTR, including *ps1-m18::Ac* and *ps1-m8::Ac*, resulted in seedlings with slightly pale green leaf sheath tissue and virescent leaf blade tissue (Figure 1C). In contrast, insertions near the 3′ region of the gene, including *ps1-m7::Ac*, *ps1-m3::Ac*, *ps1-m15::Ac*, and *ps1-m20::Ac*, resulted in albescent or slightly pink leaf tissues, due to the accumulation of lycopene in seedling tissues (Figure 1D; Singh *et al.* 2003). Thus, the leaf phenotypes correlated well with the germination assays, suggesting that *Ps1* functions similarly in seed and seedling tissues.

Creating novel alleles of *ps1* by *Ac* excision: To fully exploit *Ac* as tool to create novel and genetically stable allelic variation, we developed two genetic schemes to rapidly identify germinally inherited *Ac* excision alleles on the basis of canonical "negative dosage effect" of *Ac* (McClintock 1951). The first scheme (see materials and methods) was dependent on identifying a *ps1* mutant phenotype from self-pollinated ears of *ps1::Ac* heterozygous plants (see supplemental Figure S1 at http://www.genetics.org/supplemental/). Thirty-seven putative *ps1* alleles were generated and 11 *ps1* excision alleles were further characterized using this selection method (Table 3, scheme I). One of the limitations of this scheme is the potential to miss subtle mutant phenotypes that are the most informative for structure–function studies as discussed below. Thus, we developed a second strategy that was used to recover the majority of the *Ac* excision alleles. This second strategy was based on a two-step screen that was solely dependent on detecting germinal *Ac* excision events and not *ps1* phenotypic variation. Importantly, this scheme can be readily applied to any *Ac* insertion that is closely linked to a donor insertion and is illustrated in Figure 2A.

To generate transpositions using the two-step strategy, plants hemizygous for both the *Ac* donor (*bti97156::Ac*) and the *ps1::Ac* allele were testcrossed by pollen carrying the *Ds* reporter gene *r-sc:m3* (see materials and methods). The majority of the testcross progeny were expected to carry both *Ac* alleles or no *Ac* as the two *Ac* insertions are tightly linked in *cis* (<4 cM). However, *Ac* excision events that result in the loss of *Ac* from the genome will generate chromosomes carrying a single *Ac* element. These kernels were identified as coarsely spotted due to the decrease in *Ac* copy number (see materials and methods). The second step was to discriminate between excision alleles from *ps1* and the donor *Ac* loci using a PCR-based strategy. Two pairs of primers were designed to amplify the junction of the *Ac* and flanking DNA. If *Ac* excision occurred at the donor site, a PCR product was expected from the *ps1* insertion site and not from the donor allele. Conversely, when excision occurs from the *ps1* locus, only the *Ac* donor site is expected to amplify in the PCR assay. Once the putative *ps1* footprint alleles were identified, PCR products spanning the excision sites were cloned and sequenced as described in materials and methods. Plants carrying putative *ps1* excision alleles were then self-pollinated and progeny were screened for *ps1* phenotypic variation.

Utilizing the second genetic scheme, we identified 342 coarsely spotted kernels from testcross ears as carrying putative *Ac* excision alleles. Seedlings were genotyped for the presence or absence of *bti97156::Ac* and *ps1::Ac* as described above. A representative gel image is shown in Figure 2B. Two PCR reactions were performed for each of the 342 DNA samples examined. As shown in Figure 2B (lanes 3, 4 and 7, 8), PCR

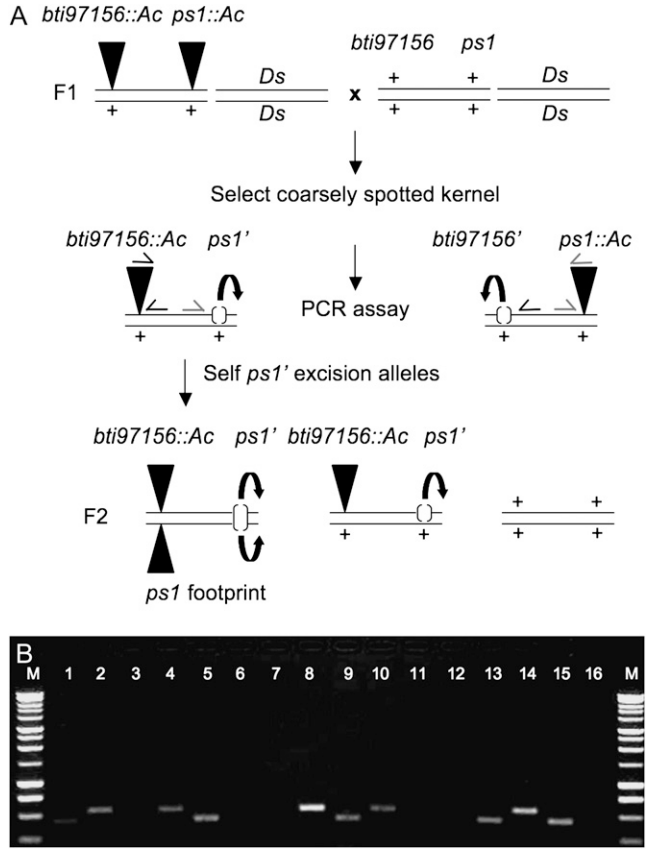

Figure 2.—Genetic scheme II used to identify novel *Ac* excision alleles. (A) Genetic strategy developed to recover *ps1* footprint alleles. Triangles represent *Ac* insertions. "+" represents the wild-type allele. Large parentheses represent putative excision alleles. *Ac* excision events are indicated by curved arrows. Solid arrows denote primers designed to amplify the donor *Ac* (*dAc*, *bti97156::Ac*). Shaded angled arrows represent primers designed to amplify the *Ac* insertion site at *ps1* locus. (B) A representative PCR gel image. When *Ac* is present, *Ac*-specific and locus-specific primers result in amplification products of a predicted size. If *Ac* is absent from the locus, no products are detected. The 1-kb DNA ladder (Promega) is labeled as M. Eight individual plants were genotyped using two PCR reactions. Odd-numbered lanes show products that result from amplification of the *dAc* (lanes 1, 5, 9, 13, and 15), whereas even-numbered lanes show expected products when *Ac* is inserted at the *ps1* locus (lanes 2, 4, 8, 10, and 14). Lanes without amplification products indicate a potential *Ac* excision allele.

products were detected when using primers specific for the *Ac* insertion at *ps1*, and no product was amplified using primers specific to the donor allele. These results indicated that the individuals retained the *Ac* insertion at *ps1* and were not characterized further. Amplification products from putative *ps1* excision alleles are also shown in Figure 2B (lanes 5, 6 and 15, 16). Here, the expected band sizes were detected using the donor-*Ac* primer pair, but not with the *ps1*-specific primers, suggesting that the closely linked *dAc* was present and that the *Ac* at *ps1* had excised. These hemizygous individuals

TABLE 2

Ac* excision frequency at different loci in *ps1

ps1 allele	*Ac* insertion site	*ps1::Ac* excised	Donor *Ac* excised	Both *Ac*'s present	Neither *Ac* present	Total
ps1-m18::Ac	5′-UTR	0	1	0	1	2
ps1-m8::Ac	5′-UTR	0	17	7	8	32
ps1-m17::Ac	Dinucleotide-binding motif	3	3	4	9	19
ps1-m11::Ac		0	4	1	3	8
ps1-m10::Ac		0	1	1	6	8
ps1-m21::Ac		1	0	0	2	3
ps1-m14::Ac		2	2	1	7	12
ps1-m13::Ac	Cyclase motif 1	4	1	2	17	24
ps1-m6::Ac	Cyclase motif 2	7	3	10	6	26
ps1-m12::Ac	Charged region	24	41	9	20	94
ps1-m3::Ac	3′-end	1	13	0	40	54
ps1-m20::Ac	3′-end	0	1	0	1	2
ps1-m15::Ac	3′-end	4	10	1	43	58
Total		46	97	36	163	342

were grown to maturity and self-pollinated to generate families segregating a putative *Ac* excision allele.

Two exceptional classes were also detected. In 36 individuals, amplification products were detected with both sets of primers, indicating that both *Ac* insertions were still present. As discussed below, these events were likely recovered due to nonconcordance of embryo and endosperm genotypes. A second exceptional class of 163 seedlings did not contain either parental *Ac* allele. Our inability to detect the parental *Ac* insertions was not likely due to failed PCR reactions as positive controls were performed for both PCR reactions and DNA samples. Instead, it is likely that selections for a single copy of *Ac* in the genome led to the recovery of unlinked transpositions that were inherited with the non-*Ac*-containing parental chromosome. These results are summarized in Table 2 and show that, of the 342 kernel selections, 46 putative *ps1* excision alleles were identified (13% of total) from eight independent *ps1::Ac* alleles.

Novel stable *ps1* footprint alleles: In total, 83 stable *ps1* alleles were generated from both selection schemes (37 from the first scheme and 46 from the second). However, only 19 unique *ps1* excision alleles were generated (Table 3). The relatively small number of unique alleles is due to the predominance of one or two excision products associated with each *Ac* insertion. For example, of the 24 independent *ps1* footprint alleles recovered from *ps1-m12::Ac*, 17 carried an identical 7-bp insertion, 6 carried an identical 8-bp insertion, and 1 had a unique 7-bp insertion. Thus, among the 24 excision products generated from *ps1-m12::Ac*, a 7-bp insertion is the predominant footprint sequence and only 3 unique footprint alleles were created. The majority of the excision events showed imperfect 7- or 8-bp duplications of *ps1* sequence at the site of *Ac* insertion. In most cases, the nucleotides immediately flanking the *Ac* insertion suffered transversion mutations (*e.g.*, C to G).

Phenotypic diversity at *ps1* locus through stable allelic series: Although most *ps1* excision alleles retained a 7- or 8-bp imperfect target-site duplication, we identified four *ps1* mutants that carried 6- or 9-bp insertions. As shown in Table 3, footprint allele *ps1′-m9.1* carries a 9-bp insertion derived from *ps1′-m9::Ac*. This allele is predicted to encode a novel PS1 protein carrying an in-frame three-amino-acid insertion at the *Ac* excision site. Footprint alleles *ps1′-m6.1*, *ps1′-m21.1*, and *ps1′-m15.1* carry 6-bp insertions and are derived from *ps1-m6::Ac*, *ps1-m21::Ac* and *ps1-m15::Ac*, respectively. Each insertion allele is predicted to encode a protein with an in-frame two-amino-acid insertion at the *Ac* excision site.

As shown in Figure 3B, individuals homozygous for these four footprint alleles display a range of phenotypic variation from near wild type to embryo lethality. Excision allele *ps1′-m21.1* carries an insertion in a nonconserved region of the PS1 protein (Figure 3A). Seed and seedling tissues were phenotypcially indistinguishable from wild type, and germination was 100% in the seed germination assay. Excision allele *ps1′-m15.1* also conditioned a 100% germination rate but seedlings were slightly pale green with pink mesocotyl tissue. The two-amino-acid insertion in *ps1′-m15.1* is close to the 3′-end of the *Ps1* gene but at a site that is conserved among *Ps1* orthologs and suggests that even a slight perturbation of protein sequence at this site negatively affects protein stability or activity. Kernels homozygous for the *ps1′-m9.1* allele were slightly pink and seeds germinated at a rate of 90%. Geminated seedlings were virescent and died at the third-to-fourth leaf stage. Although this mutant phenotype is more severe than either the *ps1′-m21.1* or the *ps1′-m15.1* allele, it is less severe than the parental *ps1-m9::Ac* allele, suggesting that the excision allele encodes a PS1 protein with limited activity. The three-amino-acid insertion in *ps1′-m9.1* is at a relative conserved region but closer to the central portion of the

TABLE 3

Novel *ps1* footprint alleles

ps1 allele	Footprint sequence		Size (bp)	No. of alleles	Scheme[a]	Allele severity[b]
ps1-m17::Ac	GCGCCATC	GCGCCATC	*Ac*			Strong
	GCGCCATg	cCGCCATC	8	3	II	Strong
ps1-m2::Ac	GGTACCAG	GGTACCAG	*Ac*			Strong
	GGTACCAc	GTACCAG	7	1	I	Strong
ps1-m21::Ac	CCCCGAAG	CCCCGAAG	*Ac*			Strong
	CCCCGAA	CCCGAAG	6	1	II	Wild type
ps1-m14::Ac	CCGAAGGG	CCGAAGGG	*Ac*			Strong
	CCGAAGG	gCGAAGGG	7	2	II	Strong
ps1-m13::Ac	GCCATGCC	GCCATGCC	*Ac*			Strong
	GCCATGCg	cCCATGCC	8	2	II	Strong
	GCCATGCgg	CCATGCC	8	2	II	Strong
ps1-m6::Ac	GGTGCACC	GGTGCACC	***Ac***			Strong
	GGTGCAC	cGTGCACC	7	3	II	Strong
	GGTGCACg	GTGCACC	7	3	II	Strong
	GGTGCAC	GTGCACC	6	1	II	Strong
ps1-m12::Ac	GCGGCAGA	GCGGCAGA	*Ac*			Strong
	GCGGCAG	cCGGCAGA	7	17	II	Strong
	GCGGCAGt	cCGGCAGA	8	6	II	Strong
	GCGGCAGt	CGGCAGA	7	1	II	Strong
ps1-m9::Ac	GCCACACT	GCCACACT	*Ac*			Strong
	GCCACAC	ggcCCCACACT	9	1	I	Weak
ps1-m7::Ac	GTCGAGGC	GTCGAGGC	*Ac*			Strong
	GTCGAGGg	cTCGAGGC	8	6	I	Strong
	GTCGAGG	cTCGAGGC	7	1	I	Strong
ps1-m3::Ac	AAGGGCAC	AAGGGCAC	*Ac*			Strong
	AAGGGCAg	tAGGGCAC	8	2	I, II	Strong
	AAGGGCA	GGCAC	4	1	I	Strong
ps1-m15::Ac	TTGGCAAC	TTGGCAAC	*Ac*			Strong
	TTGGCAA	aTGGCAAC	7	2	II	Strong
	TTGGCAA	TGGCAAC	6	2	II	Weak

Underlined sequences represent 8-bp duplications flanking the original *Ac* insertion.
[a] Scheme (I or II) refers to the method used to identify the excision allele as detailed in MATERIALS AND METHODS.
[b] Allelic strength is based on germination assay: ≥90%, weak; <90%, strong.

PS1 protein. Interestingly, the two-amino-acid insertion present in *ps1′-m6.1* is located in the highly conserved cyclase motif II (SINGH *et al.* 2003). This conserved motif has been hypothesized to play a role in generating β-ionone rings from lycopene that are necessary for the production of the ABA precursors γ-carotene and β-carotene (CUNNINGHAM *et al.* 1996). Seedlings homozygous for *ps1′-m6.1* display a very strong *ps1* mutant phenotype characterized by a pink endosperm, viviparous kernels, and a 0% germination rate. Given that this in-frame insertion is most severe, it is likely that this insertion disrupts the β-ring cyclization function of the enzyme, thus preventing production of γ- and β-carotenes and inducing the accumulation of lycopene. In the absence of xanthophylls, ABA also fails to accumulate, resulting in a viviparous phenotype.

DISCUSSION

***Ac* regional mutagenesis:** In this study, we conducted a large-scale mutagenesis of the *ps1* locus to examine the efficacy of *Ac* mutagenesis in maize. Using a closely linked *Ac* element as a donor locus, we identified 17 independent *Ac*-induced *ps1* alleles. DNA blot and sequence analysis indicated that all but 3 *ps1* alleles recovered were due to a single *Ac* insertion. The other 3 alleles carried a signature of *Ac* excision, indicating that all mutants recovered were induced by *Ac*. Nevertheless, 18% of recovered mutants did not carry an *Ac* insertion. In the context of *Ac* mutagenesis programs, these findings suggest that 15–20% of mutants recovered from such programs might not carry *Ac* insertions. Indeed, in a comprehensive survey of 1225 *Ac* transposition events generated from the *waxy1* and *bz1* loci, mutant phenotypes cosegregated with *Ac* in only 10% of the families (COWPERTHWAITE *et al.* 2002). Thus, it is likely that at least some of the mutants recovered were the result of *Ac* or *Ds* insertion and subsequent excision that generated nonfunctional alleles. However, it is likely that *Ds* and *Ac* excision alleles represent a minor proportion of mutants recovered in nondirected *Ac*-tagging programs. Assuming a similar rate of *Ac*

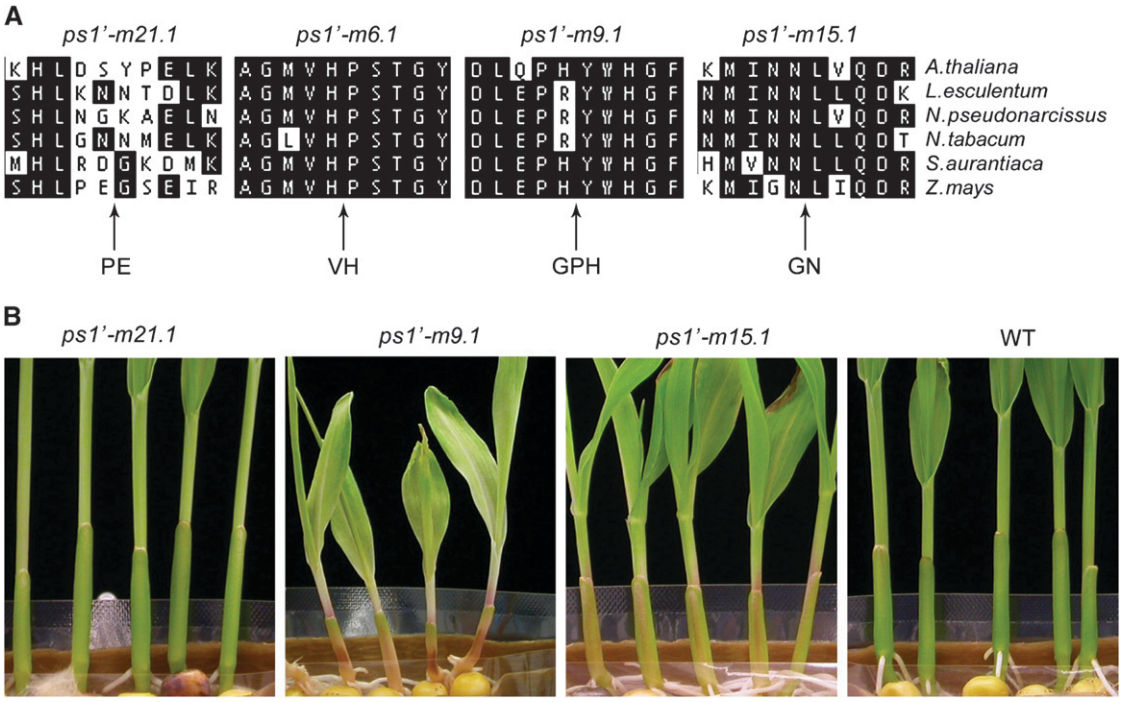

FIGURE 3.—*ps1* footprint alleles carrying in-frame insertions. (A) Plant lycopene-β-cyclase partial sequence alignments (SINGH *et al.* 2003). The sequence of in-frame amino acid insertions in each *ps1* footprint allele is denoted by arrows. (B) Phenotypes of seedlings homozygous for *ps1* alleles induced by in-frame amino acid insertions. Seedlings homozygous for the *ps1'-m6.1* allele failed to germinate.

footprint allele formation as observed here, at most 2% of the mutant families recovered in the Cowperthwaite study would be the result of *Ac* excision alleles (~20% of the *Ac*-induced mutations). As previously discussed (COWPERTHWAITE *et al.* 2002), the increased rate of mutation associated with the presence of an active *Ac* element in the genome, may be the result of an increased rate of *Ds* element transposition. However, it is also possible that the presence of an active transposon in the genome increases the rate of spontaneous mutation or reduces the efficiency of DNA damage repair mechanisms.

A genetic scheme for generating footprint alleles: Although *Ac* and *Ds* are clearly useful tools for site-directed mutagenesis, one serious drawback is that very large populations must be screened to identify rare excision events that lead to subtle alterations in protein structure and visible mutant phenotypes. Thus, it is perhaps not surprising that previous studies have been restricted to the analysis of genes that condition obvious seed phenotypes (*e.g.*, *waxy1*, *r1*, *bz1*, and *sh2*) (WESSLER *et al.* 1986; SCHIEFELBEIN *et al.* 1988; GIROUX *et al.* 1996; LIU *et al.* 1996, 1998). To expand the repertoire of tools for the global analysis of gene function in maize, we have developed a high-throughput genetic screen to enrich for independent and germinally heritable *Ac* excision alleles. This screen is based on *Ac/Ds*-mediated variegation and thus is independent of phenotypic variation conditioned by the gene of interest.

Two genetic schemes were developed to identify novel stable *ps1* footprint alleles that exploit the negative dosage of *Ac* to select for excision events. An essential feature of these approaches is that the two *Ac* elements are closely linked in *cis*. Otherwise, recombination between two loosely linked *Ac* insertions would result in a number of false positives. However, as *Ac* tends to insert at closely linked sites (GREENBLATT 1984; DOONER and BELACHEW 1989), most schemes that have been developed to utilize *Ac* in directed tagging strategies exploit this tendency for closely linked transposition (DELLAPORTA and MORENO 1994; BRUTNELL and CONRAD 2003). Thus, the majority of *Ac*-tagged mutants should carry a *dAc* closely linked in *cis* and be amenable to the strategies outlined here.

As discussed above, we favor the scheme in which *Ac* excision events were identified solely through changes in *Ac* copy number, thus enabling the recovery of weak *ps1* alleles that may escape phenotypic screens. Indeed, one of the alleles generated in this study (*ps1'-m21.1*) was phenotypically indistinguishable from wild type yet carried a predicted two-amino-acid insertion in the coding region. Using this scheme, we attempted to generate germinal *Ac* excision alleles from *ps1::Ac* insertions that were distributed throughout coding and noncoding regions of *ps1* and recovered germinal excision alleles from eight alleles. Surprisingly, we did not detect *Ac* excision alleles from all of the *ps1::Ac* alleles (Table 2). Furthermore, we observed an asymmetry in excision

frequency between the donor *Ac* and several *ps1* insertion alleles. Approximately two times as many alleles were recovered as a result of *Ac* excision from the donor *Ac* relative to the *ps1* insertion (97:46, respectively, in Table 2). In particular, all *Ac* excision events recovered from lines carrying *ps1-m8::Ac* were generated from the donor locus (17/17). It is unclear what may have generated this bias in excision allele frequency. It is possible that differences in methylation status at the *Ac* element itself may influence excision frequencies. *Ac* elements with increased methylation show greatly reduced rates of germinal excision (Brutnell and Dellaporta 1994; Brutnell *et al.* 1997). However, all *Ac* insertions examined at *ps1* contributed to *Ac* dosage and thus were likely to be largely hypomethylated (Chomet 1988). A similar bias in excision frequency has been reported for *Ds* insertions at the *waxy1* locus (Eisses *et al.* 1997). In this instance, a dominant suppressor of gametophytic *Ds* excision that was closely linked to the *Ds* insertion at *waxy1* was identified. A closer examination of *Ac* methylation in *ps1-m8::Ac* may help to clarify the likely mechanism underlying the low frequency of germinal excision events.

In addition to identifying *Ac* excision alleles, two exceptional kernel classes were also recovered. Of the 342 seedlings that were genotyped, 163 that did not carry either the *dAc* or the *ps1::Ac* insertion were identified (Table 2). The majority of these seedlings likely carried *Ac* insertions unlinked to the *ps1* locus that cosegregated with the non-*Ac*-containing parental chromosome. The other seedling class carried both parental *Ac* alleles. These progeny were likely the result of *Ac* excision events that occurred during a mitotic division of megagametogenesis. In this scenario, the megaspore mother cell inherits both parental *Ac*'s. During a subsequent mitotic division, *Ac* excises from either the donor locus or *ps1*, reducing the copy number of *Ac* in the lineage fated to give rise to a polar cell. This excision event is not inherited by the lineage contributing to the egg nucleus, leading to a coarsely spotted endosperm and nonconcordance of endosperm and embryo genotypes. As 36 seedlings were found to carry both *Ac* insertions, we calculate a minimal frequency of nonconcordance at 11%. For comparison, ~40% of all *Ds* excisions from the *r1* locus mediated by *Ac-im* occur during gametophytic development (Conrad and Brutnell 2005) whereas at least 34% of *Ac* transpositions from the *bz-m2(Ac)* likely occur at a cell division during megagametogenesis (Dooner and Belachew 1989). Thus, our calculated frequency of nonconcordance is likely to be an underestimate of the true frequency of gametophytic transposition. Indeed, additional scenarios can be envisioned where excision of *Ac* during gametophytic development would lead to the recovery of kernels that lack both parental *Ac* elements in the embryo, but that had inherited a single copy of *Ac* in the endosperm lineage. Thus, some of the events scored as unlinked transposition events (163) are likely *ps1* or *dAc* excision alleles. In summary, 342 kernel selections resulted in the recovery of 46 *ps1* excision alleles from 8 *ps1::Ac* insertions, and 13% (46/342) of the total kernel selections resulted in stable *ps1* footprint alleles. Given that the rate of germinal *Ac* excision is ~1–2% (Brutnell and Dellaporta 1994), our selection scheme results in an ~10-fold enrichment for germinally heritable *Ac* excision alleles.

Mechanism of footprint formation: One of the most comprehensive studies of *Ds* excision was conducted by Weil and colleagues at the *waxy1* locus (Scott *et al.* 1996). In their study, 621 somatic transposition events were examined from six *Ds* insertions distributed throughout the *waxy1* locus. They observed that (1) multiple excision products are generated from each *Ds* allele, (2) the predominant excision product varies with *Ds* insertion site, and (3) the frequency of each class of excision product varies for each insertion. Here, we have examined only *Ac* excision events that are germinally inherited and have likely recovered many that occurred during gametophytic development. Nevertheless, our findings are largely congruent with the findings of Weil and colleagues, suggesting that the mechanism of *Ac* excision and repair does not differ significantly from *Ds* excision across tissue types and developmental stages in maize.

Studies of *Ds* excision in yeast (Weil and Kunze 2000) and *Hermes* excision *in vitro* (Zhou *et al.* 2004) strongly suggest a model of *hAT* element excision in which a hairpin intermediate joins DNA flanking the transposon insertion (Coen *et al.* 1986). On the basis of our *ps1* footprint sequences, we propose a modified "endonuclease model" for *Ac* excision (see Figure 4). In this model, *Ac*-mediated cleavage initiates 5′ to the nucleotide immediately flanking the *Ac* insertion (arrows in Figure 4). Nucleophyllic attack by the free 3′-hydroxyl group joins the top and bottom strands of the flanking DNA, resulting in hairpin formation as recently proposed for *Hermes* excision (Zhou *et al.* 2004). The predominant endonucleolytic attack occurs at the nucleotide immediately 3′ to the ligation site and never at the site of ligation (triangles in Figure 4). Repair of the single-stranded gaps followed by ligation of the donor DNA will result in an imperfect 8-bp target-site duplication with transversion mutations at each nucleotide immediately flanking the site of *Ac* insertion (Figure 4A). Limited exonucleolytic digestion at the single-stranded donor sites followed by ligation of the donor ends will result in 7- or 6-bp insertions (Figure 4, B and C). This model can account for 54 of the 57 *ps1* footprint alleles sequenced in this study. The exceptional alleles contain 8- and 9-bp insertions and may have resulted from endonucleolytic cleavage 2 and 3 nucleotides 3′ to the ligation site, respectively. Thus, we propose that endonucleolytic attack of the donor hairpins is never initiated at the site of ligation and occurs

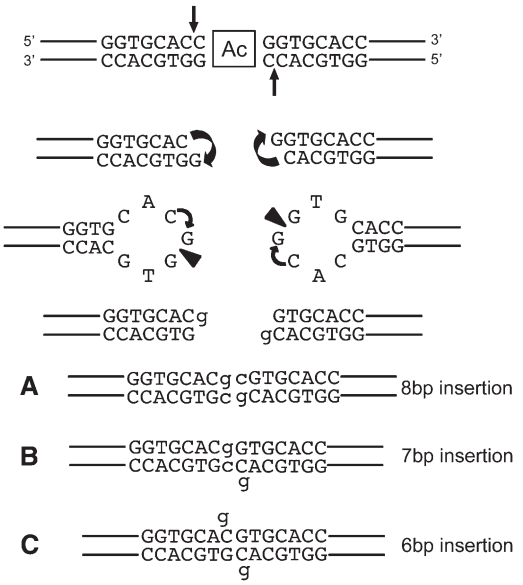

FIGURE 4.—Modified endonuclease model for *ps1* footprint formation. *Ac*-mediated cleavage is indicated by arrows. Curved arrows represent the nucleophylic attack forming DNA hairpins at donor site. Endonuclease cleavage sites are indicated by triangles. Nonhomologous end-joining and repair synthesis results in the formation of an (A) 8-bp insertion with transversions at the junction. Limited exonucleolytic digestion results in (B) 7-bp or (C) 6-bp insertions.

rarely at sites more than one nucleotide from the site of ligation. The failure to cleave at the ligation site may reflect a steric hindrance of the transposase molecule or host protein that likely mediates the *trans*-esterification reaction. A model in which endonucleolytic attack of donor hairpins occurs at sites 3′ to the ligation site is also consistent with the majority of *Ds* excision products detected at the *waxy1* locus by Weil and colleagues (SCOTT *et al.* 1996) and with a recent study of *Ds* transposition in rice (PARK *et al.* 2006). The exceptional cases that are inconsistent with our model may reflect subtle differences in *Ac vs. Ds* excision or the repair process in somatic *vs.* gametophytic tissues. Nevertheless, this model predicts that the vast majority of germinal excision alleles recovered from *Ac* mutagenesis programs will carry imperfect 7- or 8-bp insertions. We are now exploiting this finding to develop "*Ac* casting" strategies (SINGH *et al.* 2003) that enrich for closely linked *Ac* transpositions by designing PCR primers that will selectively anneal to these 7- or 8-bp imperfect excision events (L. CONRAD, unpublished results).

Allelic engineering: *Ps1* encodes lycopene-β-cyclase and is essential for the synthesis of photoprotective carotenoids such as zeaxanthin and for abscisic acid biosynthesis (SINGH *et al.* 2003). Disruption of the *Ps1* gene leads to the accumulation of lycopene, photobleaching of seedling tissues, and precocious germination. The maize *Ps1* gene is intronless and encodes a predicted 491-amino-acid protein (SINGH *et al.* 2003). An examination of *Ac* insertion alleles defined domains within the PS1 protein that were critical for activity. Insertions in either orientation within the central coding region of the *ps1* gene resulted in a 0% germination rate in our germination assay, presumably due to a failure to splice *Ac* sequences from the *Ps1* transcript or due to transcription termination within *Ac*, resulting in a truncated PS1 protein. Our finding that all *Ac* insertions at the 3′-end of *Ps1* condition very low germination rates and seedling lethality indicates that the 3′-end of the *Ps1* gene is less critical but still indispensable for proper PS1 protein function. The *Ac* insertions in the 5′-UTR of *Ps1* conditioned a very weak mutant phenotype, indicating that a functional PS1 protein was generated in these lines. Thus, despite the absence of introns and the relatively small size of the *Ps1* gene (~2.0 kb), extensive phenotypic variation that was correlated to the position of *Ac* insertion within the gene was observed. As previously shown at the *p1* locus (ATHMA *et al.* 1992; MORENO *et al.* 1992), our findings indicate that *Ac* will serve as a useful tool in genetic fine-mapping studies to define the boundaries of genes throughout the maize genome.

One powerful application of *Ac* mutagenesis is to create stable excision alleles for structure–function studies and to introduce novel allelic variation into breeding programs where genetic stability is essential. The predominant *Ac* excision alleles detected in this study were 7- or 8-bp duplications, resulting in frameshift mutations. This finding is similar to the predominant footprint following *Ds* excision in somatic tissues of maize (SCOTT *et al.* 1996). However, we did recover 6- and 9-bp excision alleles from four independent *Ac* insertion sites within *Ps1*. Furthermore, in previous studies of *Ds* excision, insertions of three, six, and nine nucleotides were recovered at the *waxy1*, *bz1*, *sh2*, and *r1* loci (WESSLER *et al.* 1986; SCHIEFELBEIN *et al.* 1988; GIROUX *et al.* 1996; LIU *et al.* 1996, 1998). As excision events that generate insertions in multiples of three nucleotides will yield in-frame amino acid insertions within the protein-coding region, this class is the most useful in site-directed mutagenesis programs with a goal of creating proteins with slightly altered function. Importantly, all footprint alleles generated through *Ac* or *Ds* excision maintain the native context of the altered gene, ensuring similar transcription rates and processing events that are not feasible through the use of transgenics.

In summary, through the use of the genetic scheme outlined in Figure 2, it should be possible to identify germinal *Ac* excision alleles from any locus where two closely linked *Ac* insertions are present. As this screen is independent of the phenotype induced by *Ac* excision, and utilizes a reporter gene that is expressed in the aleurone, a large number of testcross progeny can be easily screened. The ability to screen large numbers of kernels will increase the likelihood of recovery of rare

3-, 6-, and 9-bp duplications and should facilitate site-directed mutagenesis programs utilizing *Ac*.

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