The *Activator/Dissociation* Transposable Elements Comprise a Two-Component Gene Regulatory Switch That Controls Endogenous Gene Expression in Maize

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ABSTRACT

The maize *Activator/Dissociation* (*Ac/Ds*) elements are able to replicate and transpose throughout the maize genome. Both elements preferentially insert into gene-rich regions altering the maize genome by creating unstable insertion alleles, stable derivative or excision alleles, or by altering the spatial or temporal regulation of gene expression. Here, we characterize an *Ac* insertion in the 5'-UTR of the *Pink Scutellum1* (*Ps1*) gene and five *Ds* derivatives generated through abortive transposition events. Characterization of *Ps1* transcription initiation sites in this allelic series revealed several that began within the terminus of the *Ac* and *Ds* elements. Transcripts originating within *Ds* or *Ac* accumulated to lower levels than the wild-type *Ps1* allele, but were often sufficient to rescue the seedling lethal phenotype associated with severe loss-of-function alleles. Transcription initiation sites were similar in *Ac* and *Ds* derivatives, suggesting that *Ac* transposase does not influence transcript initiation site selection. However, we show that *Ac* transposase can negatively regulate *Ps1* transcript accumulation in a subset of *Ds*-insertion alleles resulting in a severe mutant phenotype. The role of maize transposons in gene evolution is discussed.

THE maize *hAT* family members *Activator/Dissociation* (*Ac/Ds*) are composed of the autonomous *Ac* and nonautonomous *Ds* transposable elements (KUNZE and WEIL 2002). *Ac* is 4565 bp and encodes a 3.5-kb open reading frame (ORF_a) that directs the synthesis of an 807-amino-acid-transposase (TPase) protein (FEDOROFF *et al.* 1983; KUNZE *et al.* 1987). The TPase transcript initiates at several sites within a 100-bp interval at the 5' end of the element and spans most of *Ac* (KUNZE *et al.* 1987). The lack of canonical CAAT and TATA boxes in the promoter region may account for the multiple transcriptional initiation sites within *Ac* and the low levels of *Ac* transcript (DYNAN 1986; FUSSWINKEL *et al.* 1991; FRIDLINDER *et al.* 1998). *Ac* contains ~240 bp subterminal repeats and 11 bp terminal inverted repeats at each end. The subterminal repeat regions at both *Ac* ends contain TPase binding sites that are essential for TPase recognition and subsequent transposition of both *Ac* and *Ds* (MULLER-NEUMANN *et al.* 1984; POHLMAN *et al.* 1984a,b; COUPLAND *et al.* 1989; KUNZE and STARLINGER 1989).

Ds elements are structurally diverse but share the feature that they are capable of nonautonomous transposition (reviewed in DORING and STARLINGER 1986;

KUNZE 1996). Most *Ds* elements arise from *Ac* internal deletions that ablate TPase activity. Some *Ds* elements, such as *Ds9*, are derived from simple internal deletions of *Ac* sequence (POHLMAN *et al.* 1984b). More complex structures can also be generated when one *Ds* element inserts into or near another *Ds* element (COURAGE-TEBBE *et al.* 1983; KLEIN *et al.* 1988; DORING *et al.* 1989; RALSTON *et al.* 1989; DOONER and BELACHEW 1991). These complex elements are capable of inducing chromosome breakage (ENGLISH *et al.* 1995), a phenomenon first discovered by McClintock in 1946 (McCLINTOCK 1946; DORING *et al.* 1989). *Ac* and *Ds* are also capable of inducing large-scale structural rearrangements of the genome such as chromosome translocations, inversions, and deletions (DOONER and BELACHEW 1991; ZHANG *et al.* 2009).

Ac and *Ds* element insertions can regulate and modify flanking gene expression in a variety of ways (reviewed in WESSLER 1988; FESCHOTTE *et al.* 2002). *Ac/Ds* elements can alter the RNA splicing patterns (SIMON and STARLINGER 1987; WESSLER *et al.* 1987; GROTEWOLD *et al.* 1991) or affect the timing and tissue-specific accumulation of adjacent gene transcripts (KLEIN *et al.* 1988; SCHIEFELBEIN *et al.* 1988a; SULLIVAN *et al.* 1989; DOWE *et al.* 1990; MORENO *et al.* 1992). VOLLBRECHT *et al.* (2000) have also described a *Ds*-insertion allele of *knotted1* that shows altered expressivity in the presence of *Ac*. The mutant phenotype is most enhanced (*i.e.*, more knots on leaves) in the presence of *Ac-st1*, whereas in the absence of *Ac*, the mutant phenotype is relatively

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.110.124149/DC1>.

Sequences for the *ps1(Ds)* alleles have been deposited in GenBank under identifiers HQ641289–HQ641293.

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weak. *Ac-st1* is an allele of *Ac* that accumulates higher levels of ORFa transcript (BRUTNELL 1995) but is identical in sequence to *Ac* (J. MESSING, unpublished data). Although the precise mechanism by which *Ac-st1* mediates the effect on *knotted::Ds* is unknown, the interaction between *Ds* and *Ac* results in ectopic accumulation of *Kn1* transcripts in leaves (VOLLBRECHT *et al.* 2000).

To explore the mechanisms by which *Ac/Ds* may control adjacent gene expression, we characterized an *Ac* insertion in the *ps1* gene, *ps1-m8::Ac* [hereafter referred to as *ps1(Ac)*]. The *Ps1* gene encodes lycopene β -cyclase (LCYB), an essential enzyme in β -carotene biosynthesis (SINGH *et al.* 2003). In previous studies, a series of *Ac*-induced alleles of *ps1* were generated through a large-scale *Ac* regional mutagenesis (SINGH *et al.* 2003; BAI *et al.* 2007). Among a collection of 24 independent *ps1* alleles, all but 1 allele conditioned a seedling lethal phenotype when homozygous due to insufficient accumulation of xanthophylls in seedling tissues (BAI *et al.* 2007). The one viable insertion allele, *ps1(Ac)*, carries a single *Ac* insertion in the 5'-untranslated region (UTR) 16 bp upstream of the predicted *Ps1* start codon in a 3' to 5' orientation relative to *Ps1* transcription (BAI *et al.* 2007). This single insertion allele and the nonautonomous *Ds* deletion derivatives generated from this allele (CONRAD *et al.* 2007) are the focus of this report.

Quantitative real-time PCR and 5' rapid amplification of cDNA ends (RACE) techniques were utilized to examine *Ps1* transcript pools in mutant and wild-type individuals and to map *Ps1* transcription start sites (TSS), respectively. A series of *Ds* derivatives was generated from *ps1(Ac)* to examine the contribution of *Ac* transposase on *ps1* expression. The *Ps1* TSS in these *Ds* derivatives were also mapped by 5' RACE analysis and the *ps1* transcript pools among the *Ds* derivatives quantified. We found that in some *Ds* alleles, the presence of *Ac* results in a significantly reduced accumulation of *Ps1* product leading to seedling lethality. We discuss the implications of these results in elucidating the molecular mechanisms of transposon-mediated control of endogenous gene expression.

MATERIALS AND METHODS

Plant stocks: All materials were maintained in the color-converted W22 inbred genetic background (DOONER and KERMICLE 1971). Line *ps1(Ac)*, originally defined as *ps1-m8::Ac*, was discovered through an *Ac* regional mutagenesis of the *ps1* locus from donor site *bti97156::Ac* as previously described (BAI *et al.* 2007). *Ds* insertional alleles *ps1(Ds1)*, *ps1(Ds2)*, *ps1(Ds3)*, *ps1(Ds4)*, and *ps1(Ds5)* were derived from *ps1(Ac)* through a two-step genetic selection scheme as previously described (CONRAD *et al.* 2007).

To monitor *Ac* copy number in the maize genome, we used a *Ds* tester line, *r-sc:m3* as previously reported (BRUTNELL and DELLAPORTA 1994). This line contains a *Ds* insertion at the *r1* locus that is responsible for anthocyanin production in the aleurone and scutellum tissues. In the absence of *Ac*, *Ds*

insertions at *r1* are stable, which results in a colorless aleurone. In the presence of *Ac*, *Ds* excises from *r1* and restores gene function producing purple sectors in the aleurone. The timing of *Ds* excision is delayed with increasing *Ac* copy number in the genome, resulting in a "negative dosage effect" (MCCLINTOCK 1951). The *Ac-immobilized* (*Ac-im*) element encodes the *Ac* transposase protein but is incapable of autonomous transposition (CONRAD and BRUTNELL 2005). The *Ac-stabilized1* (*Ac-st1*) allele is a derivative *Ac* line that does not display a negative dosage effect (CHOMET 1988).

Phenotypic screens: Seeds were surface sterilized and placed in a seed germination pouch as described in BAI *et al.* (2007) or sown directly in pots without treatments. Seedlings were grown in growth chambers under the following conditions: 31° day/22° night, 50% humidity, 12 hr night and 12 hr light of intensity at 500 $\mu\text{mol}/\text{m}^2/\text{sec}$. Photos were taken 7 days after planting.

Nucleotide sequences: Sequences for the *ps1(Ds)* alleles have been deposited in GenBank under identifiers HQ641289–HQ641293.

RACE: Total RNA was extracted from seedlings harvested at the three-leaf stage using TRIzol reagent following the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Approximately 200 μg total RNA was used to isolate mRNA with the Oligotex mRNA mini kit (Qiagen, Valencia, CA). Approximately 200 ng mRNA was treated using the GeneRacer kit according to the manufacturer's recommendations (Invitrogen). Only capped full-length mRNA pools were captured and used to construct RACE libraries. The first-strand cDNA synthesis was performed using SuperScript III (Invitrogen) with GeneRacer Oligo dT primer (Invitrogen). An RNase H treatment was performed (Invitrogen) followed by two rounds of nested PCR to amplify the cDNA product. The first-round PCR reaction was performed using 0.6 μM GeneRacer 5' Primer (Invitrogen) and 0.2 μM *Ps1* gene-specific primer PS1-38 (5'-GACCTTGGCCTGGTGAAGACGA-3') from 1 μl first-strand cDNA template. The nested PCR reaction was performed using 0.2 μM GeneRacer 5' Nested Primer (Invitrogen) and 0.2 μM *Ps1* gene-specific primer PS1-36 (5'-AACTCGTCCACCCACACGCCGTA-3') with 1 μl first-round PCR product. The PCR reactions consisted of 2.5 units Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen), 1 $\times$ Platinum *Taq* buffer, 0.2 mM dNTP's, 0.5 M betaine, 4% DMSO and 2 mM magnesium sulfate. A touchdown PCR program was used under the following conditions: denaturing at 94° for 2 min, 5 cycles at 94° for 30 sec and 72° for 2 min, 5 cycles of 94° for 30 sec and 70° for 2 min, 25 cycles of 94° for 30 sec, 65° for 30 sec, and 68° for 2 min, with a final extension at 68° for 10 min. RACE products were visualized by gel electrophoresis, subcloned into pCR4-TOPO cloning vector (Invitrogen) and sequenced.

Real-time PCR: Total RNA was extracted as described above, purified using an RNeasy mini kit, and treated with RNase-free DNase (Qiagen). cDNA libraries were reverse transcribed from purified total RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). *Taq-Man* gene primers and a 3' minor groove binder (MGB) probe specific to *ps1* were used in real-time PCR as previously described (BAI *et al.* 2009). For each allele, three biological replicates and for each replicate three technical replicates were performed. The relative *ps1* gene expression was calculated as previously described (BAI *et al.* 2009).

RESULTS

***Ac* insertion in the 5'-UTR directs transcription at the *ps1* locus:** To explore the mechanisms of *Ac*-regulated

gene expression, we performed a detailed characterization of the *psI(Ac)* allele. Unlike all other *Ac*-induced *psI* alleles described (SINGH *et al.* 2003; BAI *et al.* 2007), seedlings homozygous for the *psI(Ac)* grew to maturity and set seed. When seeds are planted and grown in seed germination pouches as shown in Figure 1, homozygous *psI(Ac)* seedlings display a slightly pale green and pink leaf sheath, due to the accumulation of lycopene in seedling tissues (our unpublished data). When grown in a field nursery, plants homozygous for *psI(Ac)* display a weak mutant phenotype including thinner stalks, slower growth, and delayed flowering time, indicating that the *Ac* insertion at the 5'-UTR disrupts but does not completely abolish PS1 function.

To characterize *PsI* transcript profiles in *psI(Ac)* homozygous mutant plants, *psI* mRNA pools were quantified using real-time PCR (see MATERIALS AND METHODS). Relative gene expression levels of *psI* alleles were calculated and are shown in Figure 1C. Transcripts encoded by *psI(Ac)* were reduced to ~14% of wild-type levels, suggesting that the *Ac* insertion interfered with *PsI* transcription initiation or transcript stability. Despite the greatly reduced transcript pools, these results indicate that sufficient PS1 protein is able to accumulate to produce viable offspring in self-pollinated homozygous *psI(Ac)* mutants.

Rare transcripts initiate within terminus of *Ac* element: To further characterize the transcriptional regulation of the *psI(Ac)* allele, we performed 5' rapid amplification of cDNA ends (RACE) (see MATERIALS AND METHODS) to map the *psI* TSS. The 5' RACE products were amplified from both wild-type and homozygous *psI(Ac)* mutant tissues. A pool of PCR products was amplified, and 47 independent clones for both the wild-type and mutant allele were sequenced to define the TSS. It should be noted that the same primer sets were used to map the TSS for both *PsI* and *psI(Ac)* alleles and were designed to sequence 321 bp downstream of the ATG start codon. Furthermore, 5' RACE was performed on capped full-length mRNA templates as described in MATERIALS AND METHODS. Thus, it is likely that all the TSS mapped reflect genuine TSS and the relative abundance of the products is directly comparable between *PsI* and *psI(Ac)* alleles. Transcription initiation sites are shown in Figure 1, D and E.

The majority of wild-type *PsI* transcripts initiate within a window of -49 to -93 bp upstream of the start codon ATG (Figure 1D). The lack of a strong consensus TSS is consistent with the absence of a strong consensus TATA motif as seen in mammalian systems (CARNINCI *et al.* 2006) and suggests that other promoter elements are utilized to drive *PsI* transcription. In *psI(Ac)*, the predominant *psI* transcripts initiate within the *Ac* terminus 18 nt from the 5' end of *Ac* and read out into the flanking *psI* sequence (Figure 1E). These initiation sequences are ~300 bp from the predominant *Ac* transposase TSS (ORF α ; KUNZE *et al.* 1987), and *Ac* and *psI*

transcription is directed in opposite orientation. Such 5' *Ac* read-out promoter activity has not been previously reported in maize, though a similar activity has been described for a *Ds* insertion in transgenic tomato plants (RUDENKO *et al.* 1994). In addition to the TSS initiating within *Ac*, several TSS were mapped within the coding regions of *psI(Ac)*. However, these transcripts are unlikely to encode functional protein products, since they would lack 31 amino acids of the mature protein, including plastid targeting sequences (SINGH *et al.* 2003). In summary, multiple TSS are utilized in both the wild-type and *Ac* insertion allele, suggesting plasticity in the control of *PsI* expression.

***Ds* derivatives at *psI-m8*:** The *Ac*-encoded TPase binds to subterminal repeats within *Ac* and *Ds* elements to catalyze the transposition reaction (KUNZE and STARLINGER 1989). Thus, we examined the possibility that the *PsI* promoter activity associated with *psI(Ac)* is mediated through *Ac* binding at end sequences. To test this hypothesis, we examined promoter activity in the absence of TPase expression and in the presence of low and high levels of TPase expression.

To eliminate TPase expression from *psI(Ac)*, a genetic scheme was used to recover five *Ds* derivatives from the *Ac* insertion at *psI(Ac)* (CONRAD *et al.* 2007). Four of the five *psI(Ds)* alleles were identified as internal deletion derivatives that do not produce functional TPase protein but retain the element at the original genomic location as the donor *Ac*. A schematic of the *Ds* allelic structures at the *psI(Ac)* locus is shown in supporting information, Figure S1. The *psI(Ds1)*, *psI(Ds3)*, *psI(Ds4)*, and *psI(Ds5)* alleles each suffered an internal deletion of varying length: 1177 bp, 775 bp, 455 bp, and 1805 bp, respectively, from a 4565-bp intact *Ac*. In each of these four *psI(Ds)* alleles, the *Ds* element has intact terminal and subterminal repeats. The *psI(Ds1)* allele contains 39 bp, and *psI(Ds3)* contains 77 bp of "filler" DNA, respectively, inserted at the deletion junction as shown in Figure S1. The fifth *psI(Ds)* allele, *psI(Ds2)*, is unique in that sequences at the 5' end of the *Ac* element including the subterminal and terminal inverted repeats were deleted together with the internal sequence. One base pair of the target site duplication adjacent to this *Ds* insertion was also deleted. In addition to the 3563-bp deletion in *psI(Ds2)*, filler DNA was also present at the deletion junction, which originated from both *Ac* and *psI* 5'-UTR sequences that flanked the insertion site (CONRAD *et al.* 2007).

To dissect the function of *Ac* TPase in regulating *psI* expression, *psI* transcription initiation sites were mapped by 5' RACE in *psI(Ds)* derivatives. The *psI(Ds1)* allele was not selected for this assay because it has a similar structure to *psI(Ds3)*, *psI(Ds4)*, and *psI(Ds5)*. Homozygous *psI(Ds2)*, *psI(Ds3)*, *psI(Ds4)*, and *psI(Ds5)* seedlings were harvested and mRNA used in 5' RACE experiments (see MATERIALS AND METHODS). The 5' RACE products were amplified from each cDNA library

FIGURE 1.—Phenotypic and transcriptional analysis of wild-type and *psI(Ac)* alleles. (A) Wild-type W22 and (B) homozygous *psI(Ac)* seedling phenotypes of 7-day old plants grown in germination pouches. (C) Relative *psI* transcript levels in wild-type and homozygous *psI(Ac)* seedlings. Error bars (SE) represent the variation between three biological replicates. Mapped TSS of *psI* transcripts in seedling tissues are shown for (D) wild-type and (E) *psI(Ac)*. Each arrow denotes a TSS identified as an independent 5' RACE clone. The 8-bp *Ac* target site duplication is highlighted in boldface type and underlined. The *Ac* insertion in *psI(Ac)* is shown as a blue triangle with the insertion orientation indicated (5' or 3'). The 5' terminal sequences of *Ac* insertion are in blue. The PS1 start codon is represented in red. Nucleotide positions are relative to this ATG.

and a minimum of 12 PCR products were cloned and sequenced for each genotype to map TSS. As shown in Figure 2, multiple TSS were identified for each allele and the profile of each was similar among all *Ds* derivatives characterized. In particular, the predominant *psI* transcription initiation site in *psI(Ds3)*, *psI(Ds4)*, and *psI(Ds5)* was within the *Ac* terminus 18 nt from the 5' end. Additional TSS were also detected downstream of the ATG in plants carrying these *psI(Ds)* alleles as was observed with *psI(Ac)*. However, it is unlikely that any of these downstream TSS restore functionality as the predicted encoded protein would lack a plastid transit peptide. The finding that the predominant TSS are conserved between these *Ds* alleles and the *Ac* allele indicates that *Ac* transposase is not necessary for TSS selection.

The *psI(Ds2)* allele is unique in that it carries ~1 kb of 3' *Ac* end sequences and completely lacks the 5' *Ac* end sequences that immediately flank the *psI* gene in *psI(Ac)*. As the derivative is missing the end of the element it is most similar to *fAc* elements described previously by Dooner (RALSTON *et al.* 1989). However, we refer to it here as a *Ds* element as it was derived from *Ac* and is no longer capable of autonomous transposition. Interestingly, *psI* transcription still initiates within the *Ds* element in *psI(Ds2)* but no consensus sequences were identified and TSS initiated further upstream at sites ~47–115 bp from the *PsI* start codon. Thus, it appears that 5' terminal sequences of *Ac* do influence the TSS selection but are not absolutely required for transcription initiation.

A comparison of the mutant phenotypes conditioned by the various *Ds* alleles (Figure 3) is consistent with the

results of TSS site selection. The *psI(Ds1)*, *psI(Ds3)*, *psI(Ds4)*, and *psI(Ds5)* alleles condition similar mutant seedling phenotypes that are not appreciably different from the parental *psI(Ac)* allele. Seedlings are very slightly pale green, but grow to maturity in the field and set seed. However, *psI(Ds2)* shows a severe mutant phenotype; seedlings are albescent and die soon after germination. In summary, these results suggest that *Ac/Ds* sequences at the 5' end of the element promote *psI* gene expression independently of *Ac* TPase.

High levels of TPase expression in *psI(Ds)* alleles:

Although *Ac* TPase did not noticeably influence the mutant seedling phenotype, plants that carried the *Ds* insertion alleles [with the exception of *psI(Ds2)*] displayed weaker mutant phenotypes in the field than the original *psI(Ac)* allele (data not shown). This finding suggests that *Ac* transposase may negatively regulate gene expression from *PsI* through interactions with *Ac* or *Ds* sequences upstream of *PsI*. Thus, we reasoned that very high levels of *Ac* TPase could negatively influence the phenotype of the *Ds* insertion alleles. To dissect the role that TPase may play in regulating *PsI* gene expression, we examined the influence of *Ac* on *psI(Ds)* allelic expression using the *Ac* derivatives *Ac-immobilized* (*Ac-im*) (CONRAD and BRUTNELL 2005) and *Ac-stabilized1* (*Ac-st1*) (CHOMET 1988). The *Ac-im* element encodes a functional TPase but is incapable of autonomous transposition due to a 10-bp deletion of 5' end sequences (CONRAD and BRUTNELL 2005). *Ac-st1* shows a dosage insensitive *Ds*-mediated variegation pattern (B. MCCLINTOCK, unpublished data) that is correlated to a high steady-state level of *Ac* expression (VOLLBRECHT *et al.* 2000) and hypomethylation of the

FIGURE 2.—Results of 5' RACE analysis for *ps1(Ds)* alleles. The *Ds* insertions are shown as a blue triangle in a 3' to 5' orientation. Sequences originating from *Ac/Ds* are in blue, whereas sequences derived from *ps1* are in black. Parentheses represent deletions within *Ds* elements.

5' sequences of the element (BRUTNELL 1995). The *Ac-st1* allele likely produces high levels of TPase protein that delays the developmental timing of the transposition (HEINLEIN 1996). Thus, we compared the seedling phenotypes and expression of the *Ps1* gene in the absence of TPase [*ps1(Ds)*, +/+], with low levels of TPase [*ps1(Ds)*, *Ac-im*/+] and with high levels of TPase [*ps1(Ds)*, *Ac-st1*/+].

To introduce TPase into *ps1(Ds)* derivatives, a two-step genetic strategy was developed. Pollen collected from plants homozygous for *ps1(Ds1)*, *ps1(Ds3)*, *ps1(Ds4)*, and *ps1(Ds5)* was used to pollinate plants homozygous for *Ac-im* or *Ac-st1* in the field. To create stocks with the *ps1(Ds2)* allele, crosses could only be performed with heterozygous individuals because homozygous *ps1(Ds2)* are seedling lethal. Heterozygous progeny carrying the *ps1(Ds)* mutant alleles and *Ac* were then backcrossed to plants homozygous for their respective *ps1(Ds)* mutant alleles. Using this two-step genetic scheme, testcross ears were harvested and kernels homozygous for *ps1(Ds1)*, *ps1(Ds3)*, *ps1(Ds4)*, or *ps1(Ds5)* alleles were phenotypically identified in approximately one-half of the kernels as they conditioned slightly pink endosperm tissue. Of these kernels, half carried one copy of *Ac-im* or *Ac-st1* (spotted) and half lacked *Ac* (nonspotted). For the *ps1(Ds2)* allele, heterozygous plants were used to generate mutant progeny and similar kernel selections were made [e.g., *ps1(Ds2)*

mutant kernels were selected with or without *Ac-im* and *Ac-st1*].

To visualize the effects of TPase on *ps1(Ds)* alleles, homozygous *ps1(Ds)* mutant kernels exhibiting three different levels of *Ac* expression (+/+, *Ac-im*/+, or *Ac-st1*/+) were grown in growth chambers (see MATERIALS AND METHODS). Plants homozygous for *ps1(Ds2)* display a seedling lethal albescent phenotype (Figure 3A). The introduction of one copy of *Ac-im* (Figure 3B) or *Ac-st1* (Figure 3C) did not noticeably affect the expressivity of the mutant phenotype in the homozygous *ps1(Ds2)* individuals.

The *ps1(Ac)* derivative alleles *ps1(Ds1)*, *ps1(Ds3)*, *ps1(Ds4)*, and *ps1(Ds5)* are very weak mutants and condition similar phenotypes. Homozygous *ps1(Ds3)* and *ps1(Ds5)* seedlings were selected as representatives of these four derivative mutants (Figure 3, D and G, respectively). Introduction of a low level of TPase expression through the addition of a single copy of *Ac-im* did not noticeably influence the mutant seedling phenotypes in either *ps1(Ds3)* or *ps1(Ds5)* plants as shown in Figure 3, E and H, respectively. Interestingly, the introduction of high levels of TPase expression by adding a single copy of *Ac-st1* resulted in a virescent seedling lethal phenotype [Figure 3, F and I for *ps1(Ds3)* and *ps1(Ds5)*, respectively] that is similar to the seedling phenotype of lines homozygous for the *ps1(Ds2)* allele, with the exception that occasional revertant sectors are

FIGURE 3.—Seedling phenotypes of homozygous *psI(Ds)* alleles. (A) *psI(Ds2)*, (B) *psI(Ds2) + Ac-im*, (C) *psI(Ds2) + Ac-st1*, (D) *psI(Ds3)*, (E) *psI(Ds3) + Ac-im*, (F) *psI(Ds3) + Ac-st1*, (G) *psI(Ds5)*, (H) *psI(Ds5) + Ac-im*, and (I) *psI(Ds5) + Ac-st1*.

observed in the presence of *Ac-st1* (Figure 3, F and I), which was confirmed by DNA blot analysis (data not shown).

***psI* transcript levels in *psI(Ds)* derivatives:** To understand the phenotypic variation found in the *psI(Ds)* derivatives, real-time PCR was performed to examine the relative *psI* transcript levels in *psI(Ds2)* and *psI(Ds5)* genotypes containing different *Ac* doses (Figure 4). The *psI(Ds5)* allele was selected for this assay to represent the four weak *psI(Ds)* derivatives. TaqMan real-time PCR primers and probe were used to compare *psI* transcript abundance in seedling tissues (see MATERIALS AND METHODS). Relative to the transcript of the *psI(Ac)* allele, the *psI(Ds2) +/+*, *psI(Ds2) Ac-im/+*, and *psI(Ds2) Ac-st1/+* genotypes all showed *psI* transcript levels that were approximately fivefold lower in abundance. The *psI(Ds5) +/+* and *psI(Ds5) Ac-im/+* genotypes accumulated *psI* transcripts at a level similar to that of *psI(Ac)*. However, *psI* transcript accumulation was sub-

FIGURE 4.—Real-time PCR assay of *psI* relative expression in homozygous *psI(Ac)*, *psI(Ds2)*, and *psI(Ds5)* seedling tissues. Gene expression is plotted as fold change relative to the *psI(Ac)* allele. Error bars (SE) represent the variation between three biological replicates.

stantially lower in *psI(Ds5) Ac-st1/+* mutant tissues relative to *psI(Ds5) +/+*, *psI(Ds5) Ac-im/+*, or *psI(Ac)* seedlings. These results suggest that the presence of *Ac-st1* negatively affects the accumulation of the *psI* transcripts.

TSS in *psI(Ds)* alleles containing TPase: To understand whether or not additional TPase expression regulates *psI* transcription, *psI* TSS were examined by 5' RACE in *psI(Ds2)* and *psI(Ds5)* derivatives in the presence of TPase. Homozygous mutant seedlings of *psI(Ds2) +/+*, *psI(Ds2) Ac-im/+*, *psI(Ds2) Ac-st1/+*, *psI(Ds5) +/+*, *psI(Ds5) Ac-im/+*, and *psI(Ds5) Ac-st1/+* were harvested and mRNA used in 5' RACE experiments. TSS were mapped by cloning and sequencing a minimum of 30 PCR products from each genotype. As shown in Figure 5, multiple TSS were detected among all genotypes characterized. Importantly, transcription initiation sites were similar in the presence of TPase expressed from either *Ac-im* or *Ac-st1*. Interestingly, several TSS were mapped upstream of the *Ds* insertions in *psI(Ds5)* lines carrying *Ac-im* and *Ac-st1*. These minor products represent *Ds* excision alleles and show that the preferred TSS in revertant alleles are similar to those in the original *PsI* allele, indicating the *Ds* element displaces but does not eliminate endogenous promoter sequences. Taken together, these findings strongly suggest that *Ac-st1* inhibits the expression of the *psI(Ds5)* allele by reducing the rates of transcription initiation rather than affecting transcript site selection.

DISCUSSION

Plant transposable elements are capable of regulating flanking gene expression through numerous mechanisms including providing novel TSS (MASSON *et al.*

FIGURE 5.—Transcription initiation sites of *psI* in homozygous *psI*(*Ds*) derivatives. TSS from at least 30 independent clones were mapped for each allele and are represented as arrows. Other features are as described in Figure 2.

1987; SCHIEFELBEIN *et al.* 1988a; BARKAN and MARTIENSSEN 1991; RUDENKO *et al.* 1994; KIGER *et al.* 1999), introducing novel enhancer elements (NAITO *et al.* 2009), altering the splicing of the genes in which the transposon resides (WESSLER *et al.* 1987; MENSSEN *et al.* 1990; WEIL and WESSLER 1990), and subjecting flanking genes to epigenetic regulation (CHOMET *et al.* 1987; SLOTKIN and MARTIENSSEN 2007).

Here we describe a two-component gene regulatory switch whereby *Ds* insertions in the promoter of the *PsI* gene bring the gene under the control of *Ac*. In the presence of an *Ac*-overexpression allele (*i.e.*, *Ac-st1*) transcript pools from *PsI* are reduced significantly,

resulting in a seedling lethal phenotype. In the absence of *Ac* or in the presence of a single standard *Ac* element, expression of the *PsI* gene is sufficient to result in a green seedling that reaches maturity and sets viable seeds. A model describing this interaction is presented in Figure 6. In a wild-type seedling, transcription is initiated from several sites in a window ~50–90 bp upstream of the ATG (represented by a single arrow in Figure 6A). An *Ac* insertion in the 5'-UTR displaces the endogenous TSS 4.5 kb upstream of the translation start site. Nevertheless, *psI* transcripts accumulate to ~14% of wild-type levels and are initiated from sequences within the ends of *Ac* [relative levels represented by

poly(A) transcripts in Figure 6]. Transposase is not required to drive transcription from within the *Ds* element as insertion alleles can also provide TSS in the absence of *Ac* (Figure 6C). We propose that a transcription factor complex is recruited to the subterminal repeat region at the *Ac/Ds* terminus (Figure 6, B and C). The weak promoter activity results in a low abundance of *psl* transcript pools. However, these pools are sufficient for plant survival. In the presence of *Ac-st1*, excessive *Ac* transposase binds to the *Ds* subterminal repeat regions and inhibits recruitment of a transcription factor complex (Figure 6D). Thus, *psl* transcripts are greatly reduced resulting in a severe mutant phenotype. The absence of *Ac* binding sites at the 5' end of the element in *psl(Ds2)* renders this allele insensitive to this effect. Importantly, the *psl(Ac)* and *psl(Ds)* alleles represent new regulatory circuits that are controlled through both novel *cis* (i.e., *Ac/Ds* end sequence) and *trans* (i.e., transposase) regulatory components.

Our findings suggest that *Ac* transposase is capable of suppressing gene expression when *Ac* or *Ds* inserts in 5'-UTR sequences of target genes. Similar "suppressible" gene expression systems have been described for the maize *Mu* and *Spm* families. For instance, a *Mu* transposon inserted in 5'-UTR of the *hcf106* gene provides transcription initiation sites when *Mu* is inactive (BARKAN and MARTIENSSEN 1991). However, transcription from the *hcf106* gene is suppressed when an active source of *Mu* transposase is present elsewhere in the genome (BARKAN and MARTIENSSEN 1991). A similar phenomenon has been described for *Spm* (McCLINTOCK 1954, 1965). In *Spm*-suppressible alleles, *dSpm* insertions reduce but do not completely eliminate the expression of the target gene in the absence of *Spm*. In the presence of *Spm*, gene expression is inhibited but *dSpm* excisions restore gene function (McCLINTOCK 1954). The molecular characterization of *Spm*-suppressible alleles at *a2-m1* (MENSSEN *et al.* 1990), *a1-m1* (SCHWARZ-SOMMER *et al.* 1985), and *bz-m13* (SCHIEFELBEIN *et al.* 1988b) revealed that these insertions were in coding regions of the gene and likely involved the misprocessing of transcripts in the presence of *Spm* (GIERL *et al.* 1988). McCLINTOCK (1962) also characterized *Spm*-dependent alleles in which flanking gene expression was dependent on the presence of a functional *Spm* element. In *Spm*-dependent alleles of *a1*, an insertion of the *dSpm* element in the promoter region drives flanking gene expression in the presence but not in the absence of *Spm* (MASSON *et al.* 1987). The *Kn1-2F11* allele described by VOLLBRECHT *et al.* (2000) could be considered an *Ac*-dependent allele; only in this instance *Ac* enhances ectopic accumulation of *Kn1*, resulting in a dominant mutant phenotype.

The *Ac*-suppressible alleles of *psl* are most similar to the *Mu*-suppressible alleles at *hcf106*, in which insertions in the 5'-UTR result in decreased transcript accumulation in the presence of high levels of transposase. In addition, our observations that transcripts are initiated

FIGURE 6.—A model of *Ac*-mediated regulation of *psl* gene expression. (A) In wild-type tissues, a transcriptional complex binds to promoter sequences and initiates transcription of *Ps1*. *Ps1* transcript abundance is represented by lines with poly(A) tails. (B) In *psl(Ac)*, a transcriptional complex binds to *Ac* terminal sequences and initiates transcription from sequences within the *Ac* element. The *psl* transcript pool is reduced to ~14% of wild-type levels. *Ac* TPase is also present in the genome, and the resulting low levels of *Ac* TPase may interact with terminal *Ac* end sequences to slightly inhibit transcription. (C) In *psl(Ds)* alleles with 5' terminal *Ac/Ds* sequences, transcription initiates from the end sequences within the element. *psl* transcript pool abundance is reduced to ~20%. There is no *Ac* TPase in the genome. (D) The presence of *Ac-st1* in the genome produces high levels of *Ac* TPase that then compete with the transcriptional complex for binding at *Ds* terminal sequences. Transcription of the *psl* gene is inhibited. Transcription factor binding sites within *psl* are denoted in blue, whereas transcription factor binding sites from the *Ac/Ds* terminus are denoted in red. Triangles represent *Ac* or *Ds* insertions at the *psl* locus. TF, transcription factor.

from both within the 5' end of the *Ac/Ds* elements and in the flanking regions of the *psl(Ac/Ds)* alleles are consistent with the finding that TSS also initiate in the ends of *Mutator* in the *hcf106* allele (BARKAN and MARTIENSSEN 1991). In both instances flanking gene expression is inhibited in the presence, but not in the absence of the autonomous element. However, *Spm*-suppressible alleles characterized to date are associated with the insertion of elements in transcribed regions of the gene. Thus, the mechanism of suppression shared by *Ac/Ds/Mu* appears to differ from the mechanism used by the *Spm* system.

An interesting contrast between the *Mu* and *Ac/Ds* systems is that *Mu* active lines generally contain a high copy number of *Mu* insertions. Germinal insertions increase rapidly with each generation (MAY *et al.* 2003) and global silencing of all endogenous copies occurs rapidly and frequently (MARTIENSSEN and BARON 1994; MAY *et al.* 2003). In *Ac* active lines, copy numbers are generally maintained at low levels and silenced at a relatively low frequency (BRUTNELL and DELLAPORTA 1994). It is interesting to speculate that the stronger preference of *Ds* elements to insert into or near genes relative to *Mu* elements (VOLLBRECHT *et al.* 2010) may

provide a mechanism to negatively regulate both *Ds* copy number and *Ac* copy number. As *Ac* dosage increases (*i.e.*, copy number), expression of genes flanking *Ds* promoter insertions would be subject to negative regulation. In the case of *Ps1*, this results in a seedling lethal phenotype and the selection for lines that carry reduced copy numbers of *Ac* and *Ds* promoter insertions. To examine this potential for *Ac* suppression more globally, we mapped known *Ds* insertions relative to the 5'-UTR of annotated genes in the maize genome (VOLLBRECHT *et al.* 2010). As shown in Table S1, we identified 114 *Ds* insertions that are likely to act as *Ac*-suppressible alleles (3' to 5' orientation) and another 161 *Ds* insertion alleles that may serve as potential *Ac*-suppressible alleles (5' to 3' orientation). Thus, while *Mu* activity may be targeted directly through gene silencing mechanisms (SLOTKIN *et al.* 2005), more indirect regulation of *Ac/Ds* copy number may occur through the detrimental effect of insertion into or near essential maize genes.

The identification of additional *Ac*-suppressible alleles could be exploited for functional genomics studies. A program is currently under development to distribute thousands of *Ds* insertions throughout the maize genome as platforms for regional mutagenesis (AHERN *et al.* 2009; VOLLBRECHT *et al.* 2010). These insertions will be used to generate multiple *Ds*-insertion alleles at defined target genes. Those that insert in promoter regions could be potential targets for the "fine-tuning" of flanking gene expression by the modulation of *Ac* expression levels. We are currently testing this concept using the *ps1(Ds5)* allele to monitor expression of *Ps1* in the presence of several derivatives of *Ac-st1* that have altered transposase expression levels. The ability to influence flanking gene expression through *Ac/Ds* also has potential to regulate gene expression in transgenic systems. Several thousand *Ds* elements have been distributed throughout the rice and *Arabidopsis thaliana* genomes (SUNDARESAN *et al.* 1995; KOLESNIK *et al.* 2004) and those that insert into 5'-UTR sequences in a 3' to 5' orientation relative to target gene transcription are good candidates for *Ac*-suppressible insertion alleles.

Although transcription is initiated from within the end sequences of both *Ac/Ds* (this study) and *Mu* (BARKAN and MARTIENSSSEN 1991), it is unlikely that *Ac/Ds* or *Mu* end sequences are sufficient to drive high level expression of flanking gene transcription. As noted by Barkan and Martienssen, the ends of *Mu* do not harbor a canonical TATA motif, but do have sequences similar to Initiator (Inr) elements described in mammalian genes (SMALE and BALTIMORE 1989). TATA motifs are also absent from the ends of *Ac/Ds* and although a read-out promoter activity has been described for a *Ds* insertion in tomato (RUDENKO *et al.* 1994), the TSS was ~300 bp from the 5' end compared to ~18 bp from the 5' end of the *Ac/Ds* element in this study. This suggests

that sequences within the end of the elements function as transcription initiation sites if they are inserted in a suitable context (*e.g.*, near an endogenous gene promoter). A more detailed characterization of additional 5'-UTR insertions should help further delimit the promoter activity of *Ac/Ds* in driving flanking gene expression.

To examine additional regulatory motifs in the *ps1* gene, we scanned the regions surrounding the TSS for signatures of transcription initiation. The multiple transcriptional initiation sites associated with wild-type and mutant *ps1* alleles are consistent with the absence of consensus CAAT and TATA boxes in the promoter region (SMALE and KADONAGA 2003). In many promoters lacking a TATA element, the Inr functions at the site of transcription initiation (SMALE and BALTIMORE 1989). The Inr motif is contained within the transcription start site and is a strong core promoter element that is functionally analogous to the TATA box (O'SHEA-GREENFIELD and SMALE 1992). In yeast, the Inr extends from nucleotides -6 to +11 and has the consensus sequence of Py Py A(+1) N T/A Py Py (SMALE 1997). Within the consensus sequence, an A at +1, a T or A at +3, and a C or T at -1 are most critical for determining the strength of an Inr (SMALE 1997). In maize, an Inr element has been identified as directing transcription from the TATA-less gene *Abp1* (ELROUBY and BUREAU 2000). In our study, most of the transcripts identified initiated with an A at +1. The predominant transcripts have sequences similar to Inr element, but with mismatch(es).

The wild-type *Ps1* TSS lie in a window of -22 to -93 bp upstream of the start codon ATG. The predominant transcript initiation sites within *Ac* and most *Ds* insertions are located from -34 bp to +10 relative to the ATG. In *ps1(Ds2)*, the TSS fall in a window from 47 to 115 bp upstream of the ATG. These findings suggest that the proximity of TSS to the ATG is relatively conserved among the *ps1* transcripts detected, within a window of -115 to +10 bp relative to the ATG. This may reflect the ability of sequences surrounding the start codon to aid in the recruitment of RNA polymerase II.

Previous studies in *Drosophila* have suggested that sequences downstream of the Inr help direct transcription initiation sites. One such element, the downstream promoter element (DPE), is highly conserved (A/G G A/T C/T G/A/C) among *Drosophila* TATA-less promoters and is located at +28 to +32 relative to the start of transcription (BURKE and KADONAGA 1996; KUTACH and KADONAGA 2000). The DPE is also found in human core promoters and is conserved but not identical to the one described in *Drosophila* (BURKE and KADONAGA 1997). In our study, sequences at +28 to +32 relative to the predominant TSS in wild-type *Ps1* allele are identical to the *Drosophila* DPE motif with a single base pair mismatch. The predominant transcript found within the *Ac/Ds* end starting at 18 bp from the 5' end also

contains a sequence from +37 to +41 that matches the *Drosophila* DPE at all but 1 bp. The sequence at +33 to +37 of the transcript with TSS at 105 bp upstream of ATG in *ps1(Ds2)* is identical to the DPE and sequence at +38 to +42 has all but 1 bp, same as the DPE. Collectively, these results suggest that an Inr-DPE core promoter functions to direct transcription in maize *ps1* alleles. It appears that in *ps1(Ac/Ds)* alleles, the physical distance separating the Inr from the downstream DPE element is variable but similar to the spacing observed between the Inr and DPE elements in both *Drosophila* and human promoters. Thus, gene regulation in maize may involve similar Inr-DPE modules. Studies to investigate this possibility are now possible with the sequencing of the maize genome (SCHNABLE *et al.* 2009).

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The *Activator/Dissociation* Transposable Elements Comprise a Two-Component Gene Regulatory Switch That Controls Endogenous Gene Expression in Maize

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FIGURE S1.—Schematic structures of *ps1*(*Ac/Ds*) alleles. The light grey boxes represent *Ps1* coding regions. Solid black lines represent non-coding sequences of *ps1*. Dark grey box represents the *Ac* or *Ds* elements inserted in the *ps1* 5'UTR. Deletions within *Ds* are shown as gaps. Open box shows filler DNA at the *Ds* internal deletion junction. Dark triangles represent the terminal inverted repeats at the end of *Ac/Ds* termini.

TABLE S1**Putative *Ac*-suppressible *Ds* insertion alleles**

Table S1 is available for download as an Excel file at <http://www.genetics.org/cgi/content/full/genetics.110.124149/DC1>.

**Ds* insertion sites are based on B73 genome build 5a.59 and are positioned relative to the transcription start site.