





# Excision and reinsertion of *Ac* macrotransposons in maize

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## Abstract

Eukaryotic Macrotransposons (MTNs) can be formed by 2 nearby elements flanking a segment of host DNA. The maize *Ac* transposon can form *Ac::MTNs*, but little is known about *Ac::MTN* transposition activities. Here, we studied 3 *Ac::MTNs* at the maize *p1* locus, each of which is composed of a segment of maize *p1* genomic DNA (up to 15 kb) bounded by a *fractured Ac* element (*fAc*, 2039 bp), and a full-length *Ac* element in direct orientation. The resulting *Ac::MTNs* are of 16, 16.5, and 22 kb total length. From these 3 *Ac::MTNs*, we identified 10 independent cases of macrotransposition, and observed similar features of transposition between *Ac::MTN* and standard *Ac/Ds*, including characteristic excision footprints and insertion target site duplications. Nine out of the 10 *Ac::MTN* reinsertion targets were genetically linked to the donor sites, another similarity with *Ac/Ds* standard transposition. We also identified a MTN-like structure in the maize B73 reference genome and 5 NAM founder lines. The MTN in diverse lines is flanked by target site duplications, confirming the historic occurrence of MTN transposition during genome evolution. Our results show that *Ac::MTNs* are capable of mobilizing segments of DNA long enough to include a typical full-length plant gene and in theory could erode gene colinearity in syntenic regions during plant genome evolution.

**Keywords:** Macrotransposon; excision; reinsertion; transposition frequency; *p1*

## Introduction

*Ac* (Activator) and *Ds* (Dissociation) were the first transposable elements discovered and described by Barbara McClintock in the 1940s (McClintock 1948, 1950, 1951). As Class II transposons in the hAT superfamily, *Ac/Ds* elements utilize a “cut and paste” mechanism of transposition. The autonomous *Ac* element is 4,565 bp in length, contains 11-bp imperfect terminal inverted repeats (TIRs), and encodes a single transposase (TPase) gene. *Fractured Ac* (*fAc*) and nonautonomous *Ds* elements commonly lack a complete TPase coding sequence; however, they retain one or both functional transposon termini, respectively, and are capable of transposition in the presence of *Ac* (Kunze et al. 1987; Kunze and Starlinger 1989; Kunze and Weil 2002).

Standard *Ac* transposition only involves the termini of a single element. During transposition, *Ac/Ds* elements produce excision footprints at donor sequences, often characterized by local nucleotide changes; i.e. one to several basepairs deletion, transition or transversion (Rinehart et al. 1997). *Ac/Ds* insertion creates 8-bp target site duplications (TSDs) that flank the newly inserted element (Kunze and Weil 2002). *Ac/Ds* transposition also shows a high tendency for insertion into local, genetically linked sites (Greenblatt 1984; Dooner and Belachew 1989). Within the maize *p1* locus, reinsertion occurs at sites ranging from 6-bp to 15-kb from the donor element (Athma et al. 1992; Moreno et al. 1992;

Weil et al. 1992). In addition, *Ac/Ds* elements display strong preferential insertions into gene rich regions and exon/intron sequences (Chen et al. 1987; Bennetzen et al. 1994; Rabinowicz et al. 1999; Conrad and Brutnell 2005; Ahern et al. 2009; Vollbrecht et al. 2010). These features make the *Ac/Ds* system an effective tool for regional gene tagging in maize and other plants.

Unlike standard transposition, alternative transposition reactions (AT) recruit compatible termini from separate, nearby transposons. A variety of configurations of *Ac/Ds* termini, such as reverse- and directly oriented ends, were reported to undergo AT. While standard transposition results in a net movement of the transposon, AT can generate larger-scale changes in genome structure. For example, reversed ends transposition (RET) produces deletions, inversions, translocations, and duplications (Zhang and Peterson 2004; Zhang et al. 2006, 2009, 2013; Huang and Dooner 2008). One study identified 3 tandem duplications induced by RET of *dhAT-Zm* in the maize B73 genome, demonstrating the occurrence of RET during genome evolution (Zhang et al. 2013). The second type of AT, termed sister chromatid transposition (SCT), involves the directly oriented transposon termini located on sister chromatids (Weil and Wessler 1993); SCT can induce the formation of deletions and duplications of varying sizes (Zhang and Peterson 1999). The third configuration, macrotransposon (MTN), is delineated by the external 5' and 3' termini of nearby *Ac/Ds*

Received: February 24, 2022. Accepted: April 18, 2022

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elements, flanking variable sizes of intertransposon segment (ITS). Excision and reinsertion of an *Ac/Ds::MTn* was first described by Huang and Dooner (2008); this *Ac::MTn* included a 6.5-kb ITS containing maize *bz1* and *stc1* sequences. In contrast to RET or SCT, *MTn* cannot induce large chromosomal rearrangements or chromosomal breakage (Yu et al. 2010), but only mobilizes the included ITS from one position to another.

In this study, we investigated the transposition activity of 3 *Ac::MTn* elements in maize. We recovered 10 independent *Ac::MTn* excision and reinsertion events, and compared features of macrotransposition to that of standard *Ac/Ds* transposition. In general, *Ac::MTn* shares similar patterns of transposition activity compared to standard *Ac* transposition, such as sequence features of excision footprints and formation of 8-bp TSDs flanking the insertion targets. *Ac::MTn* also prefers insertion into genetically linked and genic sequences. We identified a case of a likely *Ac::MTn* in the endogenous maize B73 genome, suggesting a historic occurrence of *MTn* transposition event. We show that *Ac/Ds::MTn* is capable of mobilizing segments of DNA long enough to include a typical full-length plant gene, and may have contributed to the erosion of gene colinearity in syntenic regions during genome evolution. Our results help better elucidate the various roles of transposable elements in shaping genome evolution.

## Materials and methods

### Maize stocks and screen

In maize nomenclature, *p1* alleles can express color in the pericarp (the maternal tissue surrounding the seed) and/or the cob. The first letter designates pericarp color and the second designates cob color. Thus, the progenitor lines *P1-rr::MTn* (*P1-rr458*; *P1-rr460*; and *P1-rr908*) have color in both the cob and the pericarp due to the intact *p1*. The progeny lines *p1-ww::MTn* have no pigmentation in either cob nor the pericarp due to the loss of *p1* function. To screen for the macrotransposition candidates, we crossed the progenitor lines *P1-rr::MTn* (*P1-rr458*; *P1-rr460*; and *P1-rr908*) with *Ac* tester lines *p1-ww4Co63*; *rm-3::Ds* (Kermicle 1980). The *Ac* tester line *p1-ww4Co63*; *rm-3::Ds* is an *r1* mutant line with a *Ds* insertion. With an active *Ac* from the *P1-ww::MTn*, the *Ds* can be excised from *r1* gene to restore the aleurone pigmentation. We screened for seeds or sectors with colorless kernel pericarp indicating loss of *p1* function, and then confirmed the *Ac* activity by screening for purple spots in the aleurone. Seeds or sectors with loss of *p1* function but retained *Ac* activity were considered as candidates of *MTn* transposition.

### Molecular biological methods

Leaf tissue from young plants was collected and ground in liquid nitrogen. Total DNA was prepared by using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Porebski et al. 1997). HotMaster Taq polymerase from Eppendorf (Hamburg, Germany) was used in PCR experiments. PCR samples were heated at 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, 60°C for 30 s, and 65°C for 2 min. Another cycle at 65°C is extended for 8 min. Primers used are listed in Supplementary Table 1. For inverse PCR, we first digested genomic DNA with *HpyCHIV4*, then ligated with T4 DNA ligase (New England Biolabs). First-round PCR with primers 6 and *p1\_22643f* was performed on the ligation mixture, and second-round PCR with primers *Ac4508f* and *p1\_22927f* on the products of the first PCR. The PCR products were purified by Gel/PCR DNA fragment extraction kit (IBI Scientific, Peosta, IA, USA), and sequenced directly by the DNA Synthesis and Sequencing Facility, Iowa State University

using Sanger sequencing. Southern blot was performed according to published protocols (Sambrook et al. 1989); the washing stringency used is 0.5% SDS, 0.5×SSC at 60°C. Hybridization probe 15 from the *p1* gene has been described in Athma and Peterson (1991).

### Genome search for *Ac::MTn* structures B73 and NAM lines

We used the 150bp terminal sequences from *Ac* 5' and 3' termini as query sequences under default setting in a BLAST search against maize B73 reference genome (B73 RefGen\_v2) (Schnabbe et al. 2009). Two separate lists of hits were generated from 2 query termini. The list from *Ac* 5' terminus contains 63 hits, and the list from *Ac* 3' terminus contains 51 hits. We grouped the hits that are ≤100 kb apart from each other by their map locations among the total of 114 hits. Based on 2 possible termini compositions that are expected in *Ac::MTns*, we extracted groups that each contain 3–4 hits and include both *Ac* 5' and 3' termini. From the total of 2 hits groups that satisfy the initial search, we observed 1 group including 2 *Ac* 5' termini and 1 *Ac* 3' terminus in the orientations expected for *Ac::MTns*. This candidate was further evaluated in the most updated B73 assembly Zm-B73-REFERENCE-NAM-5.0. The sequence from the new genome assembly indicates a double *Ds* with one element insert into the other in the same orientation. We observed 8-bp TSD sequences flanking the internal *Ds*, confirming the *Ac* transposase-induced insertion. We also observed 8-bp TSD flanking the candidate *MTn*, indicating a historic transposition event of the *MTn*. We did a BLAST search against the NAM founder lines and identified highly conserved *MTn* from 5 diverse lines including B97, NC358, NC350, CML 247, and CML322. The TSD sequences are all intact in the NAM lines.

### Annotation analysis for *p1-ww::MTn* alleles

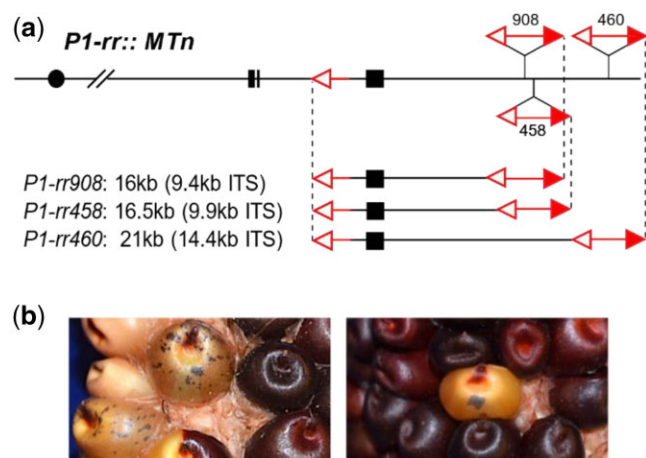
The annotation analyses of the confirmed sequences for 10 *p1-ww::MTn* alleles include mapping physical and genetic positions and identifying gene models. We blasted against Zm-B73-REFERENCE-NAM-5.0 assembly and IBM2 to obtain the physical positions and genetic coordinates for each *Ac::MTn* insertion, respectively. We extracted evidence-based gene models for the flanking sequences of each insertion from the official gene models for Zm-B73-REFERENCE-NAM-5.0 (Hufford et al. 2021).

## Results

### Excision and reinsertion events are identified in *p1-ww::MTn* alleles

In this study, we used kernel pericarp pigmentation as a phenotypic marker to detect structural changes at the maize *p1* locus. The *p1* gene encodes an R2R3 Myb transcription factor that regulates flavonoid pigment biosynthesis in pericarp, cob, tassel glumes, and silk (Lechelt et al. 1989; Grotewold et al. 1991, 1994). Pigmentation of pericarp and cob are easily scored, and thus were used to indicate different allele-specific expression patterns of *p1*: *P1-rr* specifies red pigmentation in both pericarp and cob, while *p1-ww* shows no pigmentation in either pericarp or cob (Grotewold et al. 1991, 1994; Athma et al. 1992).

Yu et al. (2010) identified 5 *Ac::MTn* alleles (*P1-ovov455*, *P1-rr905*, *P1-rr908*, *P1-rr458*, and *P1-rr460*) at the maize *p1* locus in a previous study. All of the *Ac::MTn* alleles are derived from the common progenitor *p1-uv9D9A* and thus their *fAc* elements are inserted in the same location as that in *p1-uv9D9A*. Three *Ac::MTn* alleles were further studied here including *P1-rr908*,

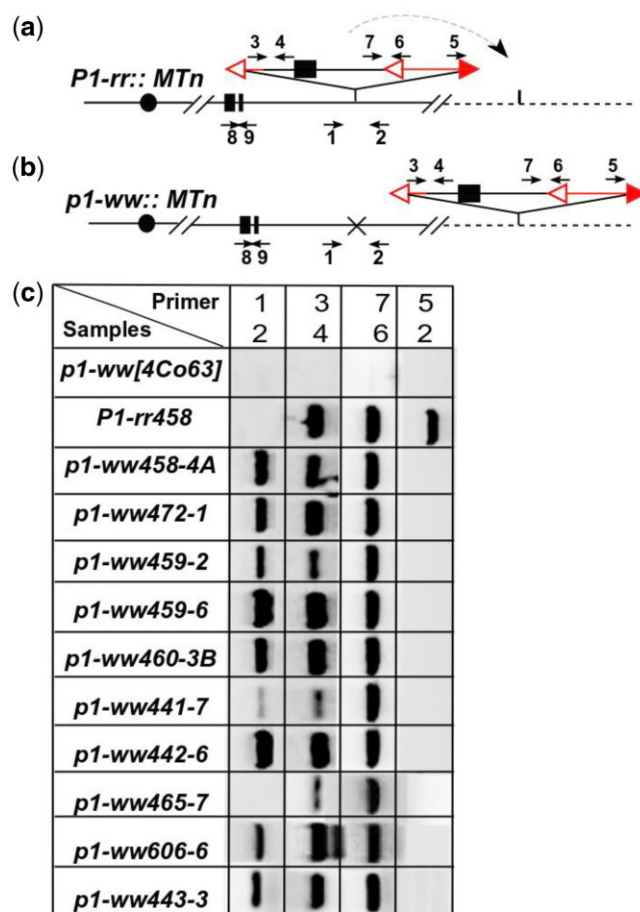


**Fig. 1.** *Ac::MTn* at *p1* locus. a) Schematic structure of *Ac::MTn* alleles at *p1* locus. Filled boxes are maize *p1* gene exons 1, 2, and 3 (left to right). open and filled red arrowheads indicate 3' and 5' *Ac* termini, respectively. Three *Ac::MTn* alleles share a common *fAc* terminus located at *p1* intron 2, and *Ac* elements (numbered) inserted at 3 different positions downstream of the *p1* gene, producing various ITS (inter-transposon segment) sizes. b) Phenotypes of *P1-rr::MTn/p1-ww; r1-m3::Ds* kernels. Red pigmentation in pericarp and cob is from the expression of *p1* gene in *P1-rr::MTn* allele. Kernels with colorless pericarp arise from loss of *p1* function, possibly caused by *MTn* excision. Purple aleurone sectors resulting from *Ds* excisions from *r1-m3* report active *Ac*. Kernels with colorless pericarp and purple aleurone sectors were screened as candidates of *Ac::MTn* excision and reinsertion.

*P1-rr458*, and *P1-rr460*. As shown in Fig. 1a, *Ac::MTn* is composed of the 3' terminus from *fAc* at *p1* intron 2, and the 5' terminus from a full-sized *Ac* element downstream of *p1*. The internal sequences (ITS) include partial intron 2 and the full sequences of exon 3 of *p1*. The size of each *Ac::MTn* is determined by the location of *Ac*: *P1-rr908* is 16 kb in total length, including a 9.4 kb ITS. The *Ac* in *P1-rr458* is 500 bp downstream from *Ac* in *P1-rr908*, resulting in a 16.5 kb *Ac::MTn* with a 9.9 kb ITS. Finally, *P1-rr460* contains an *Ac* inserted farther downstream, resulting in the largest *Ac::MTn* of 21 kb including a 14.4 kb ITS.

Before transposition, all 3 *Ac::MTn* alleles retain a functional *p1* gene and thus exhibit red pigmentation of both pericarp and cob. However, the excisions of *Ac::MTn* disrupt *p1* by removing exon 3, thus producing new *p1-ww* excision alleles. To identify cases in which the *Ac::MTn* had excised and reinserted into a new site, we crossed *P1-rr::MTn* plants with *Ac* tester line *p1-ww; r1-m3::Ds*. If *Ac* is active in *P1-rr::MTn* or *p1-ww::MTn*, then purple sectors on the aleurone can be observed due to reversion of the *r1* gene by somatic excision of *Ds* from the *r1-m3* allele (Kermicle 1980). The resulting ears were visually screened to identify candidate *Ac::MTn* transposition events from single kernel or multikernel sectors with colorless pericarp and purple-spotted kernel aleurone (Fig. 1b). The pedigree of the *p1-ww::MTn* alleles were summarized in Supplementary Fig. 1.

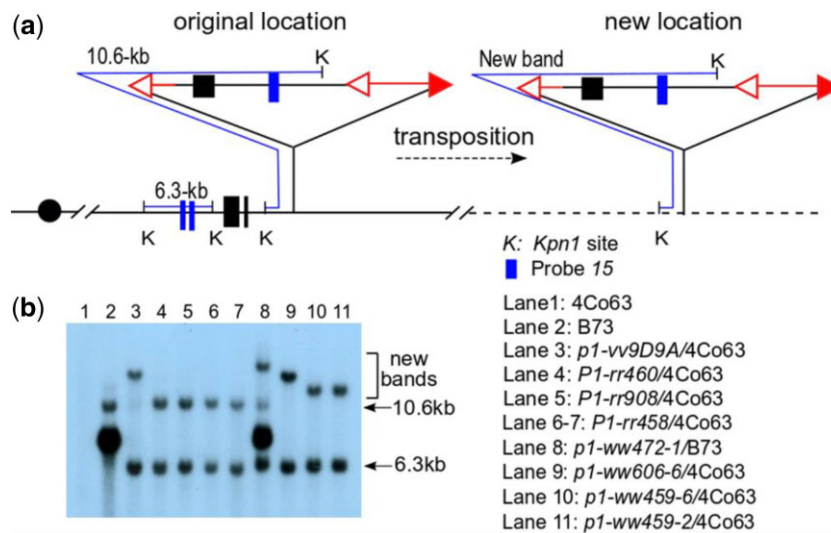
Molecular analysis was then performed on candidates of *Ac::MTn* transposition obtained from the visual screen (Fig. 2). We first used primers 1 and 2 flanking the *Ac::MTn* to detect its excision; candidates in which *MTn* had excised should produce strong bands. To confirm *Ac::MTn* excision and to exclude somatic excision events, a second PCR amplifying the junction between *Ac* 5' terminus and the flanking sequences (primers 2+5) was performed. Due to *MTn* excision, this junction was disrupted, and we expected the absence of any PCR products. The *MTn* reinsertion



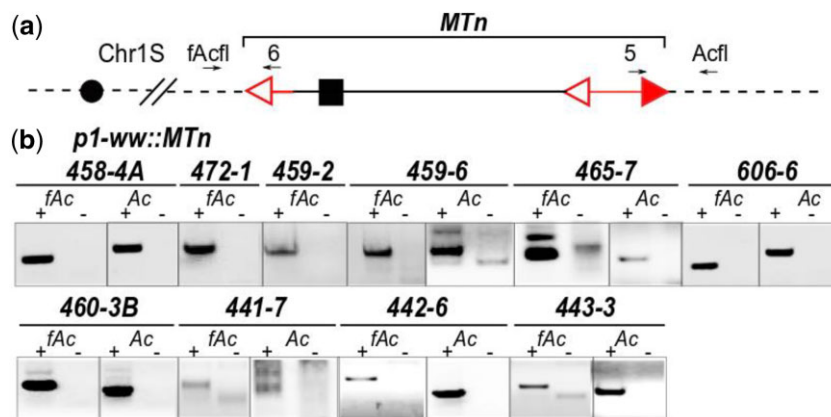
**Fig. 2.** PCR analysis of *Ac::MTn* transposition events. a and b) Schematic structure of the *P1-rr::MTn* allele and *p1-ww::MTn* allele with PCR primers labeled as arrows. Excision footprint was marked by "x" at the *Ac::MTn* donor site. Vertical lines are insertion target of *Ac::MTn*. c) Gel analysis of PCRs that identify the excision and reinsertion of *Ac::MTn*.

events were identified if the ITS sequences were detected by both primers 3+4 and primers 6+7. Using these diagnostic PCR tests, we identified 21 cases of *MTn* excision and reinsertion among 177 single or multikernel sectors with colorless pericarp and purple-spotted kernel aleurone from phenotypic screen (Supplementary Table 2). We named those alleles *p1-ww::MTn* because of the loss of function *p1* and the presence of *Ac::MTn* in the genome. We also identified 27 cases of *Ac::MTn* excisions without reinsertion, and 129 cases with no *Ac::MTn* excision. The latter 2 groups are not further studied because of the absence of *Ac::MTn* in the genome, or the disrupted *Ac::MTn* due to other mechanisms such as standard *Ac* insertion or recombination and segregation.

Southern blotting was performed to confirm the structures of the *p1-ww::MTn* alleles (Fig. 3). Genomic DNAs, including the grandparent allele *p1-uv9D9A*; the parental *P1-rr::MTn* alleles including *P1-rr458*, 460, and 908; and the progeny alleles *p1-ww::MTn* including *p1-ww472-1*, *p1-ww606-6*, *p1-ww459-6*, and *p1-ww459-2*, were digested with *Kpn1* and hybridized with *p1* probe 15. The 6.3-kb bands from upstream sequences of *p1* are common to all *P1-rr::MTn* and *p1-ww::MTn* alleles, indicating that *p1* upstream sequences are not involved in the *MTn* transposition. The 10.6-kb fragments generated from *fAc* of *Ac::MTn* and the *fAc* flanking sequences at *p1* locus were observed in all of the *P1-rr::MTn* alleles, confirming the location of *Ac::MTn* at *p1* locus



**Fig. 3.** Southern blot confirming the location of *Ac::MTn* reinsertion. a) Schematic structure of the *P1-rr::MTn* at *p1* locus (black line) and *p1-ww::MTn* at insertion site (dotted line). The expected fragments include a 6.3 kb fragment proximal to *p1* and a 10.6 kb fragment from *p1* sequences. The 10.6 kb bands are expected to be replaced by a band of new size in *p1-ww::MTn* lines. b) Result of Southern blot with bands labeled. Lane 2 contains DNA from maize inbred B73, genotype *p1-wr*, which has a tandem amplification of *p1* genes that produces intense band at ~7 kb (Sekhon et al. 2007), and a single copy band at about 10.6 kb. These bands are also observed in lane 8, which has the allele *p1-ww472-1* heterozygous with *p1-wr* [B73].



**Fig. 4.** PCR verification of *Ac::MTn* reinsertions. a) Schematic structure of *p1-ww::MTn* allele with PCR primers labeled. b) Gel analysis of verification PCR. Reinserted locations were confirmed by PCR with primers "AcfI" and "5," and primers "fAcfI" and "6." Primers "AcfI" and "fAcfI" are from newly obtained flanking sequences. Lanes "+" use templates from genomic DNA of each allele, and lanes "-" contain DNA from *p1-ww* [4Co63] as the negative control. Independent *Ac::MTn* insertions result in different locations in genome, thus primers "AcfI" and "fAcfI" are different in sequences and generate bands with different sizes as observed for each allele.

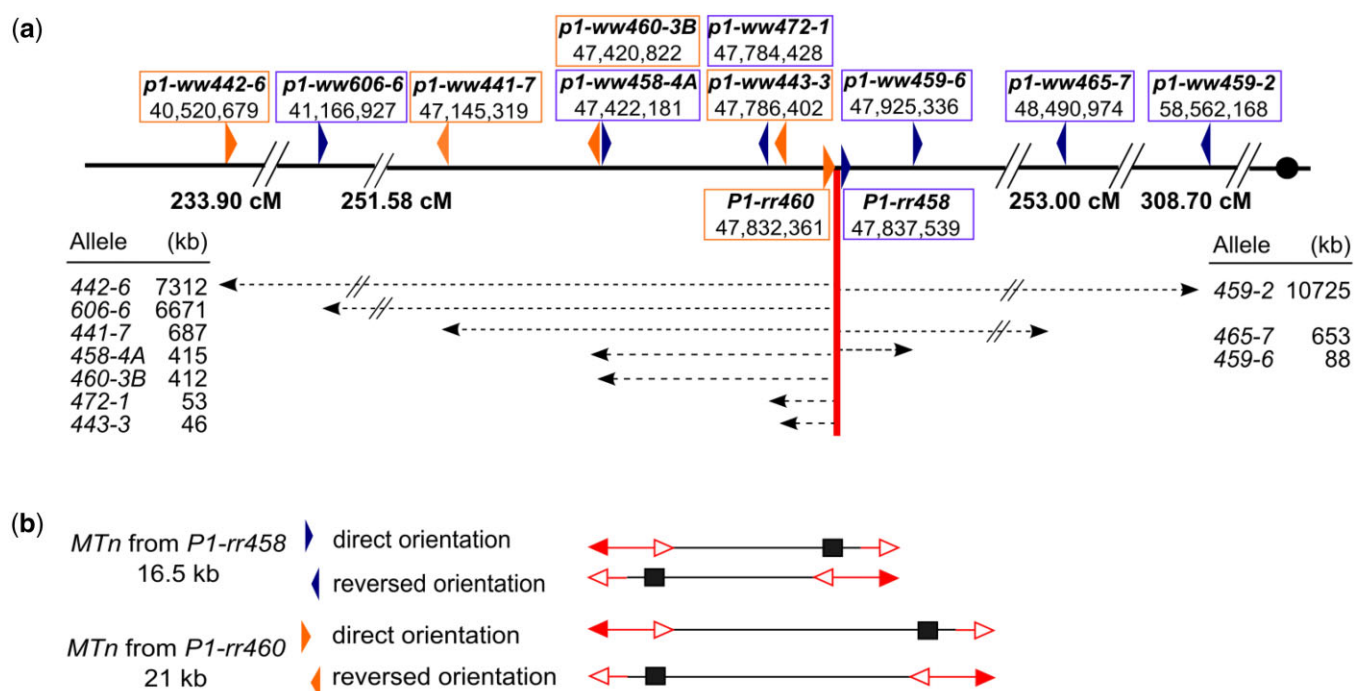
before transposition. However, this fragment is replaced by a new band of various sizes in *p1-ww::MTn* alleles including *p1-ww472-1*, *p1-ww606-6*, *p1-ww459-6*, and *p1-ww459-2*, indicating that the *Ac::MTn* was excised from *p1* and reinserted at new sites at various locations. The sizes of the new bands depend on the location of the new insertion.

To identify the reinsertion sites for the transposed *MTn* in the confirmed *p1-ww::MTn* alleles, we performed inverse PCR or *Ac* casting (Singh et al. 2003; Wang and Peterson 2013) and captured the sequences flanking each reinserted *MTn*. Putative *MTn* insertion sites were confirmed by PCR using one primer on the corresponding terminus of *MTn* and a second primer from the newly captured flanking sequences (Fig. 4). We have recovered reinsertion sites for 10 *p1-ww::MTn* alleles, all of which were confirmed, including 6 from *P1-rr458* and 4 from *P1-rr460*; none were derived from *P1-rr908*. With one junction between the *MTn* terminus (either *Ac* terminus or *fAc* terminus) and the flanking sequences confirmed, we extracted sequences from B73 genome assembly,

and designed primers to recover the other junction between the new flanking sequences and the remaining *MTn* terminus. We recovered a total of 8 junctions from the 10 confirmed reinsertions. In 2 cases, the junctions could not be amplified, possibly due to sequence polymorphisms between B73 and the mixed genetic background of the *Ac::MTn* lines. Among the 8 cases with both flanking junctions, 6 of these contained 8-bp TSDs flanking the transposed *Ac::MTn*; the 2 remaining cases had 6- and 3-bp TSDs, respectively. The complete flanking sequences of the reinserted *MTn* are listed in Supplementary Data 1.

### *Ac::MTn* preferentially inserts into nearby genic sequences

By aligning the *Ac::MTn* reinsertion target sequences with the maize B73 reference genome, we found that all insertions are located at the short arm of chromosome 1 (summarized in Fig. 5 and Table 1). The distances of the reinsertion sites from the *p1* locus range from 46-kb to 10.7-Mb of physical distance, including



**Fig. 5.** Summary of *Ac::MTn* reinsertions. a) The distribution of *Ac::MTn* reinsertion sites on chromosome 1S. The original location of *Ac::MTn* is at 47,832,361 (*P1-rr460*) or 47,837,539 (*P1-rr458*). The genetic mapping coordinate numbers labeled along the chromosome are extracted from IBM neighbors v.2.1. Arrows below the chromosome indicate the directions and physical distances of reinserted *Ac::MTn* in the recovered alleles. b) Orientations of the *Ac::MTn* reinsertions.

**Table 1.** Summary of *p1-ww::MTn* alleles.

Allele	Progenitor	Excision	Reinsertion				
			Footprint	Insertion site on chr1 <sup>a</sup>	TSD	Official gene model <sup>c</sup>	Location in the gene model
<i>p1-ww458-4A</i>	<i>P1-rr458</i>	Typical	47422181	GCGGGGAG	Zm00001eb014140	Exon	2
<i>p1-ww472-1</i>	"	Typical	47784428	ND <sup>b</sup>	Zm00001eb014210	2249 bp distal	4
<i>p1-ww459-2</i>	"	Typical	58562168	ND	NA	NA	1
<i>p1-ww459-6</i>	"	Typical	47925336	GCTAGC	Zm00001eb014260	Exon	5
<i>p1-ww465-7</i>	"	Deletion	48490974	TGCATGCA	Zm00001eb014490	5725 bp distal	3
<i>p1-ww606-6</i>	"	Typical	41166927	CAAATTAC	Zm00001eb012530	Exon	3
<i>p1-ww460-3B</i>	<i>P1-rr460</i>	Typical	47420822	CTCATCGC	Zm00001eb014140	Exon	2
<i>p1-ww441-7</i>	"	Typical	47145319	CCA	Zm00001eb014060	Exon	6
<i>p1-ww442-6</i>	"	Typical	40520679	CCCAAATC	Zm00001eb012360	Exon	2
<i>p1-ww443-3</i>	"	Typical	47786402	GGCCGGAG	Zm00001eb014210	275 bp distal	4

<sup>a</sup>Chromosome location was determined from Zm-B73-REFERENCE-NAM-5.0 assembly.

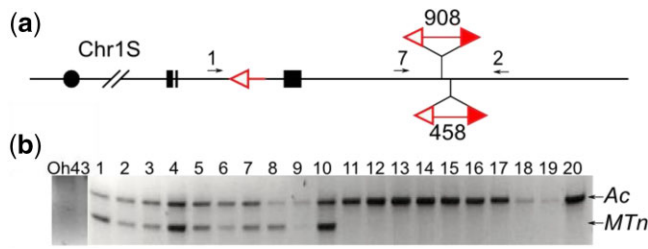
<sup>b</sup>ND for not determined.

<sup>c</sup>Official gene models from Zm-B73-REFERENCE-NAM-5.0.

8 distal sites and 2 proximal sites. Moreover, the reinsertions are all genetically linked to *p1*, including 7 cases that were mapped to the same genetic map position as *p1*.

To further understand the insertion preferences relative to the gene space, we extracted evidence-based gene models at the positions of reinsertions from the annotated B73 genome Zm-B73-REFERENCE-NAM-5.0 (Hufford et al. 2021). Nine out of 10 *Ac::MTn* insertions are located in or near genes, including 6 cases inserted within exon sequences, and 3 cases inserted distal to an evidence-based gene model. For example, the insertion of *p1-ww441-7* is located in the exon sequences of gene model Zm00001eb014060, which is 37.45 kb distal to Zm00001eb014070; this site is also 7.3, 11.20, 34.86, and 34.93 kb proximal to Zm00001eb014050, Zm00001eb014040, Zm00001eb014030 and Zm00001eb014020, respectively. Similarly, the *Ac::MTn* insertion of *p1-ww459-6* is located in the exon sequences of the evidence-based gene model

Zm00001eb014260; 4 other gene models are located within the flanking ±50 kb region. The *MTn* of allele *p1-ww443-3* is not directly inserted into a gene, but is located 275 bp distal of the gene model Zm00001eb014210. The insertion site is 5.12, 44.55, 45.93, and 50.49 kb distal to Zm00001eb014220, Zm00001eb014230, Zm00001eb014240, and Zm00001eb014250, respectively. In general, the *Ac::MTn* insertions show preference for genetically linked sequences. Most of the *Ac::MTn* insertions occurred into, or near, gene sequences. This insertion preference in the genic sequences is similar to that reported for standard *Ac/Ds* transposition (Chen et al. 1987; Bennetzen et al. 1994; Rabinowicz et al. 1999; Conrad and Brutnell 2005; Ahern et al. 2009; Vollbrecht et al. 2010). This conclusion is tempered somewhat by the possible differences between B73 and the mixed genetic background of the *Ac::MTn* lines; however, we did not observe any indication of differing insertion site preferences between *Ac/Ds* standard transposition and *Ac::MTn* insertion.



**Fig. 6.** Somatic transposition of Ac and Ac::MTn in *P1-rr458* and *P1-rr908* alleles. a) Schematic structure of the *P1-rr458* and *P1-rr908* alleles. Numbered arrows indicate primers designed to detect somatic transposition of Ac and Ac::MTn. b) PCR Gel analysis. DNA templates in lanes 1–10 are from sibling plants of *P1-rr458/Oh43*; Lanes 11–20 are from sibling plants of *P1-rr908/Oh43*. Lane “Oh43” contains genomic DNA from Oh43 as template serving as a negative control. The top bands are produced by primers “7” and “2,” from Ac transposition events; while the bottom bands are from primers “1” and “2,” from Ac::MTn excisions.

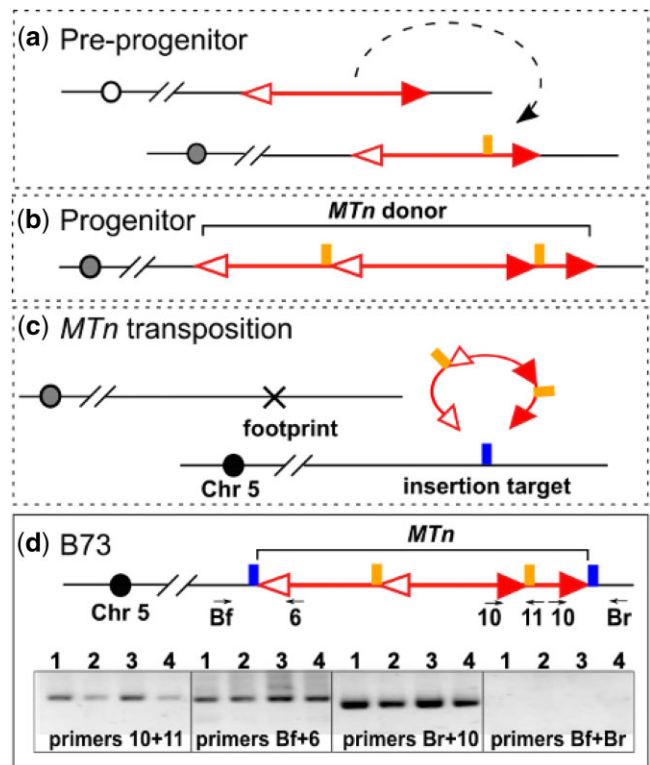
### Somatic transposition of Ac::MTn is not detected in *P1-rr908*

To study the frequency of Ac::MTn transposition, we compared alleles *P1-rr908* and *P1-rr458*. The 2 Ac::MTns differ by only 500bp, thus enabling use of the same PCR primers (primers 1 + 2, primers 7 + 2 in Fig. 6a) to detect excisions of the Ac::MTn and the component Ac for both alleles. Both *P1-rr908* and *P1-rr458* alleles were made heterozygous with inbred line Oh43, which does not produce a background band from either primer pair. Shown in Fig. 6b, bands from standard Ac transpositions were observed from both *P1-rr908* and *P1-rr458* alleles, indicating that the component Ac is somatically active in both lines. However, bands from Ac::MTn excision were absent in all of the sibling plants of *P1-rr908* and were only observed in plants of *P1-rr458*. The result also agrees with the zero recovery of Ac::MTn transpositions from *P1-rr908* described previously, indicating that Ac::MTn excision in *P1-rr908* is severely repressed.

### Identification of historic transposed MTn in B73 genome and NAM lines

We also investigated the maize B73 reference genome for evidence of possible MTn transposition events occurring during maize genome evolution. One such case is from chromosome 5 as shown in Fig. 7; corresponding sequences are presented in Supplementary Data 2. The potential MTn identified here consists of a 4564-bp Ds inserted in another 4,564-bp Ds at a location 637-bp from the 5' end of the hosting Ds in the same orientation. Both Ds elements share 99% sequence similarity with Ac sequence. Using PCR of genomic maize B73 DNA, we isolated the internal junctions between 2 Ds elements, as well as the junctions between MTn and flanking sequences, confirming the structure of the B73 MTn. By comparing the sequences flanking the internal Ds that is inserted in the host Ds, we observed 8-bp TSD flanking the insertion Ds (AGTGGAGG), indicating that this MTn was formed through Ac/Ds transposition. By comparing the MTn flanking sequences, we observed 8-bp TSD sequences (CCTACGAC) adjacent to the 5' and 3' termini of MTn, strongly indicating that this MTn was transposed to the current location in B73 from its original location in an ancient line. The ancient transposition would also leave an excision footprint sequence at the MTn donor site; however, this origin locus may have been segregated from the insertion site in the ancient line and would be difficult to trace.

However, somatic transposition of the existing B73 MTn was undetectable, indicating that this MTn is no longer active to transpose in B73. This was tested by crossing B73 with the active Ac line *p1-vv9D9A* (Athma et al. 1992) or *p1-ww-def1* (Zhang and Peterson 1999), then performing PCR using primers flanking the



**Fig. 7.** MTn in B73 genome. a) Predicted preprogenitor allele with 2 Ds elements inserted into one another at the target site (vertical line). b) The structure of MTn in the progenitor line of B73. TSDs (2 vertical lines) flank the internal Ds. c) The ancient transposition of MTn from the donor site to the target site (vertical line). d) MTn in B73. Upper panel: MTn is located on chromosome 5 of B73 genome, flanked by TSDs (2 vertical line). Numbered arrows indicate primers used in PCR to detect somatic transposition of MTn. Lower panel: PCR results testing the structure of MTn and its activity. Lane1-4: B73 carrying active Ac from *p1-vv9D9A*; B73 carrying active Ac from *p1-wwdef1*; B73; B73 (the latter 2 samples are from different DNA preparations).

MTn. No PCR products from MTn excision were observed in the progeny of either cross, indicating that somatic transposition of the B73 MTn is in some way repressed, even in the presence of an active Ac in *trans*. A search on the data from NAM Consortium (Hufford, et al., 2021) indicates that B73 MTn is heavily methylated at CG and CHG (Supplementary Fig. 2). A search on the data of B73 small RNAs (Zuo et al. 2016; Wang et al. 2020) also identified small RNAs that are homologous to the terminal sequences of the Ds elements in MTn (Supplementary Fig. 3). Therefore the MTn probably lost transposition activity due to epigenetic silencing mechanism (Leu et al. 1992; Brutnell and Dellaporta 1994).

We have also identified a highly conserved MTn in 5 NAM founder lines including B97, NC358, NC350, CML 247 and CML322. The sequences in MTn in NAM lines are >99.90% identical to the element observed in B73, all including the intact TSD sequences flanking the insertion Ds and TSD sequences flanking MTn (Supplementary Data 3 and 4). The occurrence of MTn elements in diverse lines suggests the ancient formation and transposition of MTn.

## Discussion

### Ac::MTn transposition shares similar features with Ac/Ds standard transposition

In this study, we used the *p1* gene as a visual marker to screen pigmented maize stocks containing *P1-rr::MTn* alleles for loss-of-

function alleles (*p1-ww*) carrying transposed *Ac::MTn*. The *P1-rr::MTn* alleles contain *fAc* inserted in *p1* and *Ac* inserted downstream of *p1*; transpositions of *Ac::MTn* separate *p1* exon 3 from the *p1* promoter and exons 1 and 2. To diagnose the transposition, we used 4 PCR assays to detect the presence or absence of external and internal junctions, and thereby identified 10 *Ac::MTn* transposition events. By using inverse PCR or *Ac* casting, we further cloned the reinsertion site for each event. Reinsertions of *Ac::MTn* were plotted and also annotated on the maize B73 genome assembly.

Two typical cases of *Ac::MTn* transposition are illustrated in [Supplementary Fig. 4](#). Due to the catalysis by *Ac* TPase, the transposition of *Ac::MTn* shares similar features with standard *Ac* transposition:

(1) *Ac::MTn* produces a similar pattern of excision footprint at *Ac::MTn* donor site. By sequencing the excision band in PCR using primers 1 and 2 in [Fig. 2c](#), we observed minor changes on nucleotides flanking the *Ac::MTn* donor site in 9 out of 10 excision cases, which is typical in an *Ac* TPase-induced transposition ([Supplementary Fig. 5](#)). The *p1-ww465-7* allele is an exception in that it shows no band from the footprint PCR assay. The *p1* sequences outside of *Ac::MTn* are also absent ([Supplementary Fig. 6](#)); these observations may due to a possible deletion at *p1*.

(2) Upon re-insertion, *Ac::MTn* generates 8-bp TSDs flanking the transposed *Ac::MTn* in 6 of 8 cases; a 6-bp TSD in one case, and a 3-bp TSD in one case. We compared the frequencies of perfect and imperfect TSDs generated by *Ac::MTn* insertions with that of 109 *Ds* transpositions from the *a1* locus of maize ([Vollbrecht et al. 2010](#)), and observed no significant differences between *Ac::MTn* and *Ds* transpositions; Fisher-Exact and Barnard's Test ( $P > 0.05$ ) ([Supplementary Data 5](#)).

(3) Similar to *Ac/Ds* transposition ([Athma et al. 1992](#); [Moreno et al. 1992](#); [Weil et al. 1992](#); [Vollbrecht et al. 2010](#)), *Ac::MTn* preferentially inserts into linked sites in or near genes. Nine of 10 *Ac::MTn* reinsertions occur at sites genetically linked to the *Ac::MTn* donor site in the *p1* locus, and 6 of 10 reinsertions are in the exons of a gene model.

Conclusively, the transposition of *Ac::MTn* shows a similar pattern with standard *Ac/Ds* transposition in regard of the patterns of excision footprint sequences, TSD sequences, and the preferential insertion targets.

## The mechanism that silences *Ac::MTn* in *P1-rr908* is unclear

It is interesting that the smallest *Ac::MTn* allele *P1-rr908* (16-kb) shows no detectable somatic *MTn* excision, even though its component *Ac* shows an apparently normal frequency of somatic transposition. In an attempt to understand the repression or silencing of *Ac::MTn* in *P1-rr908*, we first sequenced the *Ac::MTn* termini and found them to be intact and unchanged from the component *Ac* and *fAc* ([Supplementary Data 6](#)). Thus we eliminated the possibilities of *Ac::MTn* junction integrity for the silencing of *MTn* in *P1-rr908*.

We then compared the GC content of the 500bp sequences flanking the *Ac* of the Macrotransposons (*MTns*) *P1-rr908*, *P1-rr458*, and *P1-rr460* because GC content correlates with the possible CpG islands that can be the target of methylation. Both *P1-rr908* and *P1-rr458* contain a high GC% (GC% = 75%) in the sequences flanking *MTn* 5' terminus ([Supplementary Fig. 7](#), [Supplementary Data 7](#)), compared to the 46.5% overall GC content in maize genes ([Haberer et al. 2005](#)), making them possible to establish CpG islands. Due to the comparable GC contents flanking *Ac::MTn* in both *P1-rr908* and *P1-rr458*, we cannot explain the

varied *MTn* activity between *P1-rr908* and *P1-rr458*. Further tests on the methylation status at *MTn* locus of each line will be required to clarify this question.

Previous studies consistently showed an inverse relationship between the frequencies of AT-induced chromosome breakage/rearrangements and the distances separating the participating transposon termini ([Huang and Dooner 2008](#); [Yu et al. 2010](#)). Due to the inactive *Ac::MTn* in *P1-rr908*, we are unable to compare it with other active *Ac::MTn* to study the effect of *MTn* sizes on their activities. However, our results do indicate that, in addition to the effects of *MTn* size on transposition frequency, there are other factor(s) such as epigenetic modifications that may exert a dominant repressive effect on *MTn* transposition activity.

## Occurrence of *MTn* transposition events during maize genome evolution

Using bioinformatics searches we identified an *MTn*-like structure flanked by 8-bp TSDs in the maize lines B73, B97, NC358, NC350, CML247, and CML322. The *MTn* was derived from a progenitor locus containing one *Ds* inserted into another *Ds* in the same orientation. Another instance of *Ac/Ds* self-insertion comprises the chromosome-breaking *doubleDs*, which consists of one *Ds* element inserted into a second *Ds* in the reversed orientation ([McClintock 1948](#); [Döring and Starlinger 1984](#)). Similar structures have been characterized including *Sesqui-Ac* ([Martínez-Férez and Dooner 1997](#)) and *half-double Ds* ([English et al. 1995](#)), both of which show chromosome breaking activity similar to that of *doubleDs*. However, the chromosome breaking self-insertions that were described above have their transposition-competent termini in opposite orientations. In this configuration, chromosome breakage is thought to arise from sister chromatid fusions induced by transposition reactions that target termini located on sister chromatids following DNA replication ([Weil and Wessler 1993](#); [Yu et al. 2010](#)). In the *MTns* studied here, the involved *Ds* sequences are in the same orientation and thus transposition can occur at the 3' *fAc* and 5' *Ds* termini, resulting in mobilization of the entire compound structure. Tests have shown that these *MTns* cannot induce chromosomal breakage ([Yu et al. 2010](#)), and thus may be more stable over evolutionary time.

The identification of the *MTn* in diverse maize lines demonstrates that *MTn* transposition occurred very early during maize genome evolution. However, this *MTn* appears to be currently incapable of transposition, even in the presence of a known active *Ac* element introduced in *trans*. The *Ds* contained in the *MTn* share 99% identity to the corresponding regions in a canonical *Ac*, suggesting that the silencing is not likely due to sequence polymorphisms, but rather to epigenetic effects. For example, DNA methylation is correlated with silencing of *Ac* elements at *wx-m7* and *p1* loci ([Chomet et al. 1987](#); [Brutnell and Dellaporta 1994](#); [Conrad and Brutnell 2005](#)).

## *Ac::MTn* can serve as a potential genome-engineering tool

Some features of *Ac::MTn* transposition may be applied for purposes of genome engineering. First, the *Ac::MTn* shows a high capacity to carry plant genes: various studies have demonstrated the ability of *Ac::MTn* to transpose ITSs of 6.5-kb ([Huang and Dooner 2008](#)), 9.9 and 14.4-kb (this study), which is similar to the size range of typical plant genes. The tendency of *Ac::MTn* to undergo local transposition into nearby genic sequences demonstrated in this study suggests that engineered *MTns* could be useful tools for locus interaction and epigenetics studies. For example, *Ac::MTn* constructs could carry full-size genes, large and

complex regulatory elements, and hypo- or hyper-methylated sequence islands. Subsequent independent *Ac::MTn* transpositions could mobilize the foreign gene sequences into a variety of positions in or near a nearby gene, in both orientations. Alterations in expression of the target gene can reveal parameters of regulatory interactions such as the importance of distance and orientation, as well as epigenetic effects. A recent study showed that multiple recurrent inversions generated by independent *Ac* alternative transposition events induced varying levels of ectopic expression of a normally silent nearby gene (Sharma et al. 2021). Furthermore, it would be straightforward to modify the internal *Ac* termini to create “stabilized *Ac*” forms (Hehl and Baker 1989), that would eliminate single *Ac* transposition but retain full *Ac::MTn* transposition capability.

### MTn may contribute to the formation of dispersed transduplicated genes

Comparative studies of Arabidopsis and its relatives have shown that a large proportion (from one-fourth to three-fourths) of genes in Arabidopsis have been transposed during evolution in the Brassicales and Rosids (Freeling et al. 2008; Woodhouse et al. 2011). Maize is also known to contain a large proportion of transduplicated genes, some of which are carried by *MULE* and *helitrons* transposons (Jiang et al. 2004; Juretic et al. 2005; Lisch 2005; Morgante et al. 2005; ). Here, we show that an *Ac::MTn* can acquire and mobilize large segments of DNA capable of containing entire genes, including introns and flanking regulatory sequences. These results suggest that *MTn* transposition may have contributed to gene duplication and dispersal during the evolution of maize and other plants.

### Data availability

The *Ac::MTn* insertion data analyzed here are under NCBI accession numbers OM650318, OM650319, OM650320, OM650321, OM650322, OM650323, OM650324, OM650325, OM650326, OM650327, OM650328, OM650329, OM650330, OM650331, OM650332, OM650333, OM650334, and OM650335. Maize stocks are available upon request. All data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Supplemental material is available at GENETICS online.

### Acknowledgments

The authors thank Tao Zuo for extracting *Ac* termini sequences from maize B73 genome assembly. We thank Terry Olson, Alina Ott, and Pooja Gupta for technical assistance. They also thank Douglas Baker for field assistance.

DW, CY, JZ, and TP conceived and designed the experiments; DW performed the experiments; DW and TP wrote the paper.

### Funding

This study is supported by the USDA National Institute of Food and Agriculture Hatch project number IOW05282 and IOW05669, by State of Iowa funds, and by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under Grant #P20GM103476.

### Conflicts of interest

The authors declare no conflict of interest.

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