Ectopic Expression of a Maize Gene Is Induced by Composite Insertions Generated Through Alternative Transposition

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ABSTRACT Transposable elements (TEs) are DNA sequences that can mobilize and proliferate throughout eukaryotic genomes. Previous studies have shown that in plant genomes, TEs can influence gene expression in various ways, such as inserting in introns or exons to alter transcript structure and content, and providing novel promoters and regulatory elements to generate new regulatory patterns. Furthermore, TEs can also regulate gene expression at the epigenetic level by modifying chromatin structure, changing DNA methylation status, and generating small RNAs. In this study, we demonstrated that Ac/fractured Ac (fAc) TEs are able to induce ectopic gene expression by duplicating and shuffling enhancer elements. Ac/fAc elements belong to the hAT family of class II TEs. They can undergo standard transposition events, which involve the two termini of a single transposon, or alternative transposition events that involve the termini of two different nearby elements. Our previous studies have shown that alternative transposition can generate various genome rearrangements such as deletions, duplications, inversions, translocations, and composite insertions (CIs). We identified >50 independent cases of CIs generated by Ac/fAc alternative transposition and analyzed 10 of them in detail. We show that these CIs induced ectopic expression of the maize pericarp color 2 (p2) gene, which encodes a Myb-related protein. All the CIs analyzed contain sequences including a transcriptional enhancer derived from the nearby p1 gene, suggesting that the CI-induced activation of p2 is affected by mobilization of the p1 enhancer. This is further supported by analysis of a mutant in which the CI is excised and p2 expression is lost. These results show that alternative transposition events are not only able to induce genome rearrangements, but also generate CIs that can control gene expression.

KEYWORDS transposable elements; alternative transposition; composite insertion; enhancer

Transposable Elements (TEs) are DNA sequences that can move their positions and proliferate themselves in the genomes. Wicker et al. published a unified classification system for TEs in 2007 (Wicker et al. 2007). There are two types of TE: class I TEs are also called RNA elements, since their transpositions rely on RNA as intermediates; class II TEs do not need RNA for their transpositions, therefore they are also called DNA elements. Class II TEs can undergo standard transpositions: TE-encoded transposase binds to the termini of a single TE and facilitates the excision and insertion of the TE. In contrast, at least some class II TEs can also undergo alternative transpositions, which involve the termini of two TEs. This mechanism has been observed in various species and is mediated by different TE families (Gray 2000), including IS10/Tn10 in bacteria (Chalmers and Kleckner 1996), Tam3 in snapdragon (Martin and Lister 1989), P elements in Drosophila (Gray et al. 1996), and Ac/Ds elements in maize (Weil and Wessler 1993). In this study, we focused on characterizing the products of a specific type of alternative transposition reaction driven by maize Ac/Ds elements. Ac/Ds was the first TE system discovered by Barbara McClintock in the 1940s (McClintock 1947, 1950). Ac is the autonomous element, which encodes the transposase enzyme, and Ds is the nonautonomous counterpart that requires Ac transposase for transposition. Previous work in maize has shown that Ac/Ds can undergo two major types of alternative transposition:
reversed ends transposition (RET) involves the reversely ori-
ented termini of two different elements on the same chromo-
some, while sister chromatid transposition (SCT) targets the
termini of two TEs located on different sister chromatids
(Yu et al. 2010); while RET can generate deletions (Zhang and
Peterson 2005), direct duplications (Zhang et al. 2013), in-
versions, and translocations (Zhang and Peterson 1999, 2004;
Zhang et al. 2009). In addition, both SCT and RET can gen-
erate novel compound structures termed composite inser-
tions (CIs) (Zhang et al. 2014; Wang et al. 2020).

In addition to generating genome rearrangements, TEs can
affect gene expression in many different ways (Hirsch and
Springer 2017). For example, TE insertion in introns can alter
splicing patterns, leading to new transcripts and protein
products (Luehrsien and Walbot 1990). Many studies have
shown that TEs can provide novel promoters to drive expres-
sion of adjacent genes (Butelli et al. 2012). In certain condi-
tions, TEs may provide enhancer sequences that trigger
stress-induced gene expression (Makarevitch et al. 2015).
Additionally, TEs may exert epigenetic effects on nearby
genes, such as inducing the spread of DNA methylation from
TEs to flanking sequences, thereby suppressing expression of
neighboring genes (Hollister and Gaut 2009). Moreover, TEs
may alter chromatin states and thereby influence gene ex-
pression: Eichten et al. (2012) reported increased hetero-
chromatin and reduced gene expression in the vicinity of
TE insertions.

Enhancers are important cis-regulatory elements in eukary-
ocic genomes. Enhancers are typically short (50–1500 bp)
and bound by transcription factors to activate gene expres-
sion (Blackwood and Kadonaga 1998). They can be located
upstream or downstream of the target genes, and they may
function over long distances by forming chromatin loops
(Krivega and Dean 2012). In maize, only a small number of
enhancers have been identified and characterized (Oka et al.
2017). For example, the enhancer of the maize booster1
(b1) gene consists of multiple tandem 853-bp repeats located
~100 kb upstream of the b1 coding sequence (Stam et al.
2002). The enhancer of teosinte branched 1 (tb1), a maize
domestication gene (Doebly et al. 1995), is located ~60 kb
upstream of the tb1 target gene (Clark et al. 2006). The
gene pericarp color 1 (p1) controls biosynthesis of a red phlo-
baphe pigment in multiple maize organs such as pericarp,
cob, and silk. p1 expression is regulated by dual enhancer
sequences that are repeated at sites upstream and down-
stream of the p1 coding sequence. Fragment 15 (f15) is lo-
cated downstream of the p1 coding region (Lechelt et al.
1989) and acts as a floral organ enhancer (Sidorenko et al.
1999). In this study, we show that p1 enhancer f15 can be
mobilized by alternative transposition events to activate ec-
topic expression of a second maize gene. These results dem-
onstrate the potential impacts of terminal inverted repeat
(TIR) TEs and alternative transposition events on maize ge-
nome evolution.

Materials and Methods

Maize genetic stocks and screen

The progenitor allele p1-wwB54 has p1 loss-of-function due
to the deletion of the first two exons of p1, therefore, it yields
white pericarps and white cobs. To screen for new RET events
resulting in pericarp color 2 (p2) expression, ~4000 plants of
genotype p1-wwB54 heterozygous with a p1 null allele
(p1-ww [4Co63]) were grown in an isolation field and
allowed to pollinate with p1-ww[4Co63] pollen parents.
The resulting ears were screened, and kernels with red perici-
carps were selected and propagated. The potential heritabil-
ity of each red sector is roughly proportional to the area of
kernels covered by that sector (Emerson 1917). Moreover,
one-half of all new potentially heritable mutations will not
be recovered due to segregation in the female meiosis. From
~4000 p1-wwB54/p1-ww ears, we identified ~400 half-
kernel red sectors, ~40 whole-kernel events, and several multi-
kernel sectors and whole-ear events (Figure 3). Following
propagation of these cases, we obtained ~50 heritable new
alleles with red kernel pericarps that were further analyzed
for insertions in p2 (Supplemental Material, File S1). Geno-
ocic DNA (gDNA) samples were screened by PCR using pri-
mers located in Ac and fractured Ac (fAc), paired with primers
from the p2 gene sequence. Samples giving positive results
for both 5′Ac/p2 and 3′fAc/p2 junctions were considered to
be candidate CI alleles. The candidate CI alleles were then
planted and self-pollinated to generate homozygotes for
analysis. To screen for further mutations of CI S7 and E3
alleles, plants carrying these alleles were self-pollinated or
crossed with p1-ww [4Co63] in the isolation field; in resulting
ears, kernels with white (S7M) or light red pericarps (E3M)
were selected as mutants derived from the respective CIs.

DNA extraction and PCR

Total gDNA was prepared by using a modified cetyltrimethyl-
lammonium bromide extraction protocol from leaves of
3-week-old plants. Promega (Madison, WI) GoTaq Green
Master Mix was used for PCR reactions. The PCR was initi-
ated by a 2-min denaturation at 95°, then 30 sec of annealing step
at a temperature of 5° below the melting temperature of
the primers, then 1 min extension per kb at 72°; these steps were
repeated for 30 cycles and a final extension at 72° for 5 min
was applied.

RT-PCR

Total RNA was extracted by Invitrogen (Carlsbad, CA) TRIzol
Reagent from maize pericarp 20 days after pollination and
treated with New England Biolabs (Beverly, MA) DNase I to
treat gDNA. Complementary DNA (cDNA) was prepared by
Invitrogen SuperScript II Reverse Transcriptase kit and used
as the RT-PCR template.
When a paralog located on the short arm of maize chromosome 1, separated by ~70 kb (Zhang et al. 2000). Phenotypes of p1 alleles are commonly identified by a two-letter suffix that indicates the color of kernel pericarp and cob. For example, p1-ww indicates white pericarp and white cob, and p1-wr indicates white pericarp and red cob (Anderson 1924). The P1-rr11 allele conditions red pigmentation of kernel pericarp and cob. It contains an intact p1 gene with a full-length (4565 bp) Ac element inserted upstream of p1 exon 1, and an fAc (only 2039 bp 3′ of Ac) inserted in p1 intron 2 (Figure 1) (Zhang and Peterson 2004). In a previous study, Yu et al. (2011) showed that the Ac and fAc termini in P1-rr11 could undergo RET to induce deletions of the DNA between the Ac/fAc termini. In one case, deletion of p1 exons 1 and 2 produced a mutant allele termed p1-wwB54 (hereafter referred to as B54) with colorless pericarp and cob. The B54 allele retains the Ac and fAc elements in reversed orientation, with the 5′ terminus of Ac and 3′ terminus of fAc separated by a segment of 331 bp (Figure 1). In this configuration, the Ac and fAc termini in B54 can generate sister chromatid fusions and chromosome breaks (Yu et al. 2011).

Using a different p1 allele, a previous study showed that a pair of reverse-oriented Ac/fAc in p1 can undergo RET and induce DNA rereplication to generate flanking duplications and novel structures termed CIs (Zhang 2013, 2014) (because the formation of duplications was previously described in detail, here we focus on the formation and action of the CIs). We hypothesized that p1-wwB54 may also produce CIs via RET during DNA replication, as shown in Figure 2. In this model, the Ac transposase excises the 3′ end of fAc and 5′ end of Ac from a region of replicated DNA, and inserts these termini into an unreplicated target site. This insertion generates a rolling circle replicon to rereplicate Ac and flanking sequences, while fAc and its flanking sequence will be rereplicated by elongation of the impinging replicon. At some point, rereplication spontaneously aborts to produce two broken ends with double-strand breaks (DSBs). The fusion of these two DSBs will rejoin the two chromosome fragments and generate a CI at the new junction (Zhang et al. 2014). If the rereplication fork through fAc is sufficiently extended, the CI is expected to include a copy of p1 exon 3 and transcriptional enhancer element f15.

**Results**

**CIs produced from B54 via RET during DNA replication**

The p1 gene encodes an R2R3 Myb transcriptional factor (Grotewold et al. 1991), and regulates phlobaphene biosynthesis in maize floral organs including kernel pericarp and cob glumes (Dooner et al. 1991). p2 is a paralog of p1, but is not expressed in pericarp and cob. Both p1 and p2 are located on the short arm of maize chromosome 1, separated by ~70 kb (Zhang et al. 2000). Phenotypes of p1 alleles are commonly identified by a two-letter suffix that indicates the color of kernel pericarp and cob. For example, p1-ww indicates white pericarp and white cob, and p1-wr indicates white pericarp and red cob (Anderson 1924). The P1-rr11 allele conditions red pigmentation of kernel pericarp and cob. It contains an intact p1 gene with a full-length (4565 bp) Ac element inserted upstream of p1 exon 1, and an fAc (only 2039 bp 3′ of Ac) inserted in p1 intron 2 (Figure 1) (Zhang and Peterson 2004). In a previous study, Yu et al. (2011) showed that the Ac and fAc termini in P1-rr11 could undergo RET to induce deletions of the DNA between the Ac/fAc termini. In one case, deletion of p1 exons 1 and 2 produced a mutant allele termed p1-wwB54 (hereafter referred to as B54) with colorless pericarp and cob. The B54 allele retains the Ac and fAc elements in reversed orientation, with the 5′ terminus of Ac and 3′ terminus of fAc separated by a segment of 331 bp (Figure 1). In this configuration, the Ac and fAc termini in B54 can generate sister chromatid fusions and chromosome breaks (Yu et al. 2011).

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**Unexpected reversion of deletion allele p1-wwB54**

Initial observations of maize ears produced by plants containing p1-wwB54 showed that many kernels contained red sectors resembling the red revertant sectors typical of somatic reversion of p1-vv to P1-rr (Emerson 1929). This was surprising, considering that both exons 1 and 2 of the p1 gene were deleted in p1-wwB54. These two exons contain most of the coding sequence for the Myb DNA-binding domain that is essential for p1 function (Grotewold et al. 1991). We hypothesized that these sectors may result from ectopic expression of the p2 gene, a P1 paralog located ~70 kb proximal to p1 (Zhang et al. 2000). The p1 and p2 genes encode highly similar (95% identical) proteins (Zhang et al. 2000), and previous studies have shown that p2/p1 chimeric genes are capable of producing pericarp pigment (Zhang et al. 2006;
Therefore, we hypothesized that the red sectors observed on p1-wwB54 ears represented activation of p2 expression, possibly by CIs carrying and inserting a copy of the p1 enhancer element in or near p2. The insertion of the p1 enhancer would induce ectopic expression of p2, resulting in the red pericarp sectors observed on p1-wwB54 ears. To test this hypothesis, we screened ears produced from plants carrying p1-wwB54 and selected kernels with red pericarp sectors ranging in size from around one-half of a kernel to the whole ear (Figure 3 and File S1). Because the maize kernel pericarp is derived from the ovary wall that gives rise to the female gametophyte, premeiotic mutations in the developing ear tissues can produce clonal sectors that are expressed in the pericarp and also inherited by the kernel embryo (Emerson 1917). However, due to the intervening meiosis, each new mutant allele has only a 50% chance of being transmitted to the embryo. About 450 kernels from independent red sectors (Materials and Methods) were grown and propagated to establish a new allelic series of 50 orange and red pericarp types derived from the p1-wwB54 allele (File S1).

**Identification of CIs at the p2 locus**

The 50 new revertant alleles obtained from the screen described above were analyzed for structural changes in the p2 gene. First, using genomic PCR and Southern blot analysis (not shown), we determined that a large majority of alleles tested do indeed carry new CIs inserted in or near the p2 gene. For 24 cases, we mapped the sites of CI insertion by PCR using primers specific for Ac or fAc sequences, paired with primers in p2 (File S2). Reversed primers in Ac (Ac-r) were paired with reversed primers in p2 (p2-r) to amplify the Ac junction, followed by a second PCR using p2-f plus fAc-f primers to amplify the fAc junction (Figure 4A and File S3).

Figure 4B shows the PCR results from 10 CI alleles as examples. PCR products were sequenced and compared with p2 genomic sequence to identify the precise insertion sites in 24 CI alleles: 10 cases contained CIs in the p2 promoter region, while 14 cases had CIs in p2 intron 2. Among these 24 CIs, 21 of them had the same orientation as shown in

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**Figure 2** Model of CI formation from p1-wwB54. (A) The structure of the allele p1-wwB54. The hexagons indicate replicons. α and β indicate two replication forks. Other symbols have the same meaning as in Figure 1. (B) Transposase binds to the fAc and Ac and the two termini insert into the target site αβ, which is not yet replicated. (C) The insertion of Ac generates a rolling circle replicon and the insertion of fAc joins with target site β. (D) Ac and its insertion into the rolling circle replicon. (E) The replication aborts and the two double-strand breaks (indicated by > and <) fuse together. (F) A CI is generated containing Ac, fAc, p1 exon 3, and enhancer f15, and a portion of the flanking sequences. CI, composite insertion; fAc, fractured Ac.

**Figure 3** Screening for new CI alleles derived from p1-wwB54. (A) Maize ear with typical p1-wwB54 phenotype with predominantly colorless pericarp, and small, infrequent red revertant sectors. (B) Ear grown from p1-wwB54 kernel, with a large multikernel red sector (upper) on an ear with otherwise typical p1-wwB54 phenotype (lower portion of ear). (C) Ear grown from p1-wwB54 kernel with whole-ear red pericarp. Infrequent colorless sectors suggest ongoing instability of this novel allele, most likely due to Ac activity. In all ears, solid-colored and spotted kernels reflect Ac-induced excision of Ds element from r1-m3::Ds allele, resulting in sectors of purple kernel aleurone. CI, composite insertion.
Figure 4A, with the Ac 5’ end closest to p2 exon 3; and 3 cases had the opposite orientation, in which the fAc 3’ end was closest to p2 exon 3 (Figure 4C). By comparing the sequences of the Ac and fAc junctions in p2, we determined that each CI is flanked by an 8-bp target site duplication (TSD), which is a characteristic feature of Ac/Ds insertion (File S2). This finding confirms that the CIs are indeed generated by an Ac/fAc transposition event, consistent with the model proposed in Figure 2. Finally, we also identified three alleles in which the Ac element had excised from the CI, leaving behind a partial CI containing fAc and the p1 sequences including the enhancer f15. This indicates that following CI formation, the Ac TE is still active and capable of subsequent independent transposition (Figure 4C and File S2).

According to the model shown in Figure 2, DNA rereplication resulting from alternative transposition should generate CIs with varying sizes and sequence compositions. However, all CIs should contain sequences flanking the original Ac donor site, with p1 5’ sequences (upstream of Ac) fused to p1 3’ sequences (downstream of fAc) as shown in Figure 5A. Moreover, p1 forward and p1 reverse PCR primers, which are divergent in p1-wwB54, should converge in each CI across the internal junction. To test this, we analyzed the internal structures of 10 independent CIs. The internal junction products were amplified by combinations of primers including p1-r + p1-f as shown in Figure 5A, and Ac-f + p1-f for those cases in which the internal junction was sufficiently close to the Ac 3’ end (File S4). Due to the heterogeneity of CI length and structure, PCR was performed using a series of p1 forward and reverse primers to scan the region. In this way, we isolated and sequenced the internal junctions of 10 independent CIs (Figure 5B and File S5); based on the internal junction sequences, we could surmise the structure of each case (Figure 5C). The 10 CIs range in size from 12.8 to 23.6 kb, including the Ac and fAc elements flanking each CI. In 6 out of the 10 alleles analyzed, the internal junctions contained microhomologies of...
Evidence that CI insertion drives p2 expression

Importantly, all of the 10 CI cases examined in detail contain 3’ p1 sequences, including transcriptional enhancer fragment 15 (indicated as red “E” box in Figure 5). This is consistent with the hypothesis that ectopic expression of p2 in kernel pericarp in the CI-containing alleles is driven by the p1 enhancer. A corollary to this hypothesis is that excision of the CI should result in loss of p2 expression and reversion to the progenitor p1-wwB54 phenotype. Excision of the CI as a macrotransposon may be expected, considering that it contains suitably oriented Ać and fAć transposons at each end (Huang and Dooner 2008). Indeed, many of the CI alleles exhibited variably sized sectors of colorless and or less-pigmented pericarp (e.g., Figure 3C).

To test this hypothesis, we examined ears produced by p2-S7, an allele containing a 17.2 kb CI inserted upstream of p2. As shown in Figure 6, p2-S7 conditions red kernel pericarp with some colorless sectors. Among ~50 ears grown from p2-S7 progenies, we identified one ear that had a large clonal sector of ~20 kernels with near-colorless pericarp. Kernels from this sector gave rise to the stable mutant called p2-S7M, which has a phenotype of colorless pericarp with some red sectors, similar to the p1-wwB54 allele (Figure 6A). We analyzed the structure of p2-S7M by PCR using primers to amplify the original CI insertion site in p2, both Ać and fAć junctions with p2, and the internal CI junction (Figure 6B). The results (Figure 6, B and C) show that in p2-S7M, the CI excised from the target site as a macrotransposon, leaving behind the 8-bp TSD from the original insertion. These results show that p2 expression was indeed a result of CI insertion, and that removal of the CI eliminates the expression of p2 and restores the phenotype of the progenitor B54 allele.

To further test p2 expression in the CI alleles, we measured p2 transcript levels in p1-wwB54, p2-S7, and p2-S7M by RT-PCR (Figure 6D). Total RNA was prepared from developing kernel pericarp, reverse-transcribed into cDNA, and amplified with PCR primers located in p2 exons 1 and 2 (p2-e1 and p2-e2 in Figure 6B and File S6). Primers complementary to the GPD gene were included as an internal control. The RT-PCR results showed that p2 transcripts were detected only in the CI allele p2-S7, and were undetectable in progenitor p1-wwB54 and descendent p2-S7M in which the CI had excised. PCR products were sequenced to confirm their origin from the p2 gene (File S7). These results confirm that the red pericarp phenotype was caused by the expression of p2, and that p2 expression is dependent on the presence of a CI.

Figure 5 Identification of CI internal structures. (A) Structure of representative chromosome containing the original p1-wwB54 structure and a new CI insertion into the p2’ region. p1-r and p1-f represent sets of forward and reverse primers that are divergent in CI (right). (B) Results of PCR to amplify internal junctions of 10 CIs using p1-f and p1-r primers shown in (A). The samples tested here correspond to the same 10 CI examples shown in Figure 4B. Bands vary in intensity due to different PCR efficiencies using primers specific for each CI junction. (C) CI structures in 10 representative alleles; the first column indicates CI names, with CI sizes in parenthesis; the second column indicates the DSB repair mechanism inferred from the junction sequences. CI, composite insertion; DBS, double-strand break.
**P2-CI epiallele has altered DNA methylation**

As noted above, some p2-CI alleles exhibited sectors and progeny ears with reduced pericarp pigment intensity. One case analyzed was derived from the p2-CI-E3 allele, which contains a 15.9-kb CI inserted in the 5’ region of p2. This variant (termed E3M) was isolated from a single kernel in a small sector of light orange pericarp on an otherwise red E3 ear (Figure 7A). Progeny plants grown from this kernel have distinctly lighter orange kernel pericarp, indicating a heritable reduction in p2 expression in E3M. However, unlike the CI-excision allele S7M, PCR analysis of the Ac, fAc, and internal junctions showed that E3M does not have any structural variations in the CI target site (Figure 7, B and C). We hypothesized that the E3M dilute-pigment phenotype was caused by epigenetic change(s) rather than structural variation. Epigenetic variations such as DNA methylation are known to be correlated with changes in gene expression (Assaad et al. 1993). Therefore, we conducted bisulfite sequencing of seven targeted regions in p1 and p2 to analyze DNA methylation at single-base resolution (File S8). We examined methylation of the f15 enhancer fragment, Ac and fAc junctions in the p1 background, and the Cs of E3 and E3M, as well as the p2 sequences flanking both Ac-CI and fAc-CI (File S9 and File S10). The results showed that in the tested enhancer fragment and the p2 flanking sequences, cytosines are unmethylated and there is no detectable difference between B54, E3, and E3M (File S9 and File S10). In contrast, some methylation changes were observed in Ac and fAc sequences. In the first 100 bp of the Ac 5’ end, there are 23 cytosines. In the background Ac, B54 has four methylated cytosines in this region; while E3 and E3M have 12 and 9 methylated cytosines, respectively (File S9 and File S10). In the CI Ac, E3 has a net +1 additional methylated cytosine compared to the background Ac; this results from one demethylation and two de novo methylations. In E3M, the CI Ac has three de novo methylated cytosines compared to the background Ac. In the first 100 bp of the fAc 3’ end, there are 18 cytosine residues; in the background fAc, B54 has 10 methylated cytosines in this region, while E3 and E3M have 10 and 12 methylated residues, respectively. In the CI fAc, E3 has the same methylation pattern as the background, while E3M has one demethylated cytosine and one de novo methylation (File S9 and File S10). These results showed that methylation does not change dramatically between the E3 and E3M alleles in the Ac and fAc segments analyzed. However, a recent report indicates that changes in methylation at a single CpG can influence transcription factor binding (Yang et al. 2020). Although methylation of Ac sequences is known to affect Ac transcription (Kunze et al. 1988), further work will be required to determine whether the observed differences in methylation of E3 and E3M are causally associated with differential expression of p2.

**Discussion**

TEs are usually considered to be selfish DNA providing little or no benefit to the host genome (Orgel et al. 1980). Many studies have shown that TEs often have deleterious effects such as disrupting gene structures and modifying the
epigenetic features near their insertion sites (Hollister and Gaut 2009; Zuo et al. 2016). However, recent reports have shown that TEs can modify coding sequences and regulate gene expression to potentially increase the fitness of the host (Chuong et al. 2017). For example, TEs can perform enhancer-like functions in eukaryotic genomes. In the human genome, widespread enhancers overlap with TEs (Cao et al. 2019), and experimental data have confirmed that a subset of TE enhancers play important roles in gene regulation in early mouse development (Todd et al. 2019). These studies support the idea that TE domestication is important in eukaryotic genome evolution; however, most of these reports were focused on class I TEs in animal and human systems (Sundaram and Wysocka 2020). In this study, we identify a new mechanism by which class II TEs can regulate genes in maize. We demonstrate that, in addition to evolving into regulatory elements over time, TEs can induce sudden changes in gene expression by acquiring and mobilizing existing genomic enhancer elements.

In previous studies, we have described the mechanism of RET-induced DNA rereplication in maize (Zhang et al. 2014). This rereplication process is initiated by Ac/fAc transposition, which generates a rolling circle replica to replicate the TE and flanking sequences during an additional round in the same cell cycle. This can produce a CI at the Ac/fAc insertion site. Here we show that, during their formation, CIs can acquire a regulatory element, enhancer f15 of the p1 gene, and activate expression of the p2 gene, which is normally not expressed in kernel pericarp. By screening maize ears from plants of genotype p1-wwB54, which contains reverse-orientated Ac/fAc termini, we obtained a series of red pericarp alleles containing CIs inserted in or near the p2 gene: among 24 mapped CIs, 10 were inserted in the upstream sequences of p2, while 14 inserted in p2 intron 2. CI insertions upstream of p2 can induce transcription of the intact p2 gene; while CI insertion into p2 intron 2 can generate a chimeric p2/p1 gene (Zhang and Peterson 2005; Wang et al. 2015). A few insertion hotspots were observed: five CIs inserted into a <200-bp region upstream of p2 (positions −3188 to −3364) and four CIs inserted into a <100-bp region in p2 intron 2 (positions 8017–8102). Moreover, some CIs insert very closely to each other, or even at the same site (L12 and S7; TZ3-1 and TZ3-12; and S10 and TZ3-17). We did not detect any clear sequence signatures in these hotspots (Vollbrecht et al. 2010) (data not shown); it is possible that insertion site preference is influenced by epigenetic modifications.

We analyzed the detailed structures of 10 CIs ranging in size from 12.8 to 23.6 kb. All were composed of Ac and fAc elements enclosing duplications of p1 sequences flanking the original donor elements. These duplications were joined together at internal junctions with sequences characteristic of fusion by NHEJ, accompanied by the presence of filler DNA sequences in one-half of the cases. These structures are all consistent with a model of CI formation by DNA rereplication induced by RET (Zhang et al. 2014). Notably, all the CIs included copies of enhancer fragment f15 derived from the p1 gene 3′ region.

Activation of p2 expression by the enhancer-containing CI was confirmed by analysis of a particular case, S7M, in which the complete CI excised as a macrotransposon. CI excision resulted in heritable loss of kernel pericarp pigmentation and elimination of p2 RNA, proving that the red pericarp phenotype was caused by CI-induced p2 expression. Another variant allele (E3M), which specified orange pericarp phenotype, was analyzed and found to have some DNA methylation changes in the terminal sequences of the CI Ac and fAc elements. Although it is not clear whether these methylation differences are responsible for the differences in p2 expression, it is known that TE DNA methylation can impact the expression of nearby genes (Wittmeyer et al. 2018).

RET is one type of alternative transposition, a transposition mechanism involving the termini of two different TEs (Gray...
2000). A second type of alternative transposition is termed SCT, in which the two TEs are located on sister chromatids (Weil and Wessler 1993; Zhang and Peterson 1999). Recently, SCT has been shown to generate CIs containing inverted duplications of TE sequences that can induce silencing of Ac (Wang et al. 2020). Together, these results indicate that alternative transpositions are able to copy and mobilize regulatory elements and thereby regulate gene expression patterns, while other alternative transposition events can initiate TE silencing. These mechanisms are meaningful in plant development and genome evolution. Depending on the length of the rereplication, CIs enlarge the genome by various sizes. Furthermore, the target p1/p2 locus plays a central role in regulating phlobaphene biosynthesis in maize tissues (Grotewold et al. 1994). Because phlobaphene pigment accumulation is correlated with kernel pericarp thickness and reduced mycotoxin contamination on maize kernels (Landoni et al. 2020), the ectopic expression of p2 induced by CI alleles could be beneficial.

Although TEs proliferate and contribute to a large portion of repetitive sequences in the evolutionary process, most TEs are epigenetically silenced and heavily methylated in both plant and animal genomes (Aravin and Bourc’his 2008; Hollister and Gaut 2009; Panda et al. 2016). Moreover, the silencing signal can spread beyond the TE and affect the flanking sequence and nearby genes (Noshay et al. 2019). These silenced TEs are immobile or reduced in transposition potential, and thus are hardly able to generate large genome rearrangements. In maize, Mu and Ac/Ds elements have been characterized as active TE families that tend to land in low-methylation regions with open chromatin structures (Springer et al. 2018). In this study, we show that after CIs insert to the target sites, the Ac element is still active and can induce transposition of itself and nonautonomous Ds elements. Furthermore, the S7M mutant derived from the S7 allele indicates that the complete CI can move as a macrotransposon. Although we did not detect reinsertion of the S7 CI, it is quite possible that a macrotransposon of this size (17.2 kb) can excise and reinsert in the genome (Huang and Dooner 2008). Mutation or loss of the CI Ac 3’ end would prevent independent excision of the Ac element, converting the complex CI macrotransposon into a single mobile element. This provides one plausible mechanism for sequence acquisition by TIR elements. For CIs that contain functional enhancers as described here, such cases may be considered as authentic controlling elements, as originally described by McClintock (1956).

In this study, we used Ac/fAc and the p1/p2 loci as examples to reveal the potential regulatory role of alternative transpositions in genome evolution. Because our screen was based on the recovery of pericarp pigment, we detected only CI insertions into the p2 locus. In fact, CIs can insert into any location in the genome (Zhang et al. 2014; Wang et al. 2020), not necessarily producing a readily observable phenotype. The occurrence of a pair of active TEs (Ac/fAc) inserted into one copy of closely linked paralogs (p1/p2) controlling a visible, nonessential trait provides an ideal system in which to detect such events in real time, in relatively small experimental populations. It may be argued that similar haplotypes are so rare in natural stocks that their true impact is very small. However, because maize genomes contain >50,000 full-length TIR TEs (Su et al. 2019), as well as many more copies of partial and fractured elements (Su et al. 2020), RET events involving some TE systems and affecting a variety of genes may have occurred frequently over evolutionary time. Alternative transpositions have also been identified in snapdragon Tam3 elements (Martin and Lister 1989) and Drosophila P elements (Gray et al. 1996), suggesting that this mechanism could potentially be an important source of regulatory modification in both plant and animal genomes.

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