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Abortive Gap Repair: Underlying Mechanism for *Ds* Element Formation

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The mechanism by which the maize autonomous *Ac* transposable element gives rise to nonautonomous *Ds* elements is largely unknown. Sequence analysis of native maize *Ds* elements indicates a complex chimeric structure formed through deletions of *Ac* sequences with or without insertions of *Ac*-unrelated sequence blocks. These blocks are often flanked by short stretches of reshuffled and duplicated *Ac* sequences. To better understand the mechanism leading to *Ds* formation, we designed an assay for detecting alterations in *Ac* using transgenic tobacco plants carrying a single copy of *Ac*. We found frequent de novo alterations in *Ac* which were excision rather than sequence dependent, occurring within *Ac* but not within an almost identical *Ds* element and not within a stable transposase-producing gene. The de novo DNA rearrangements consisted of internal deletions with breakpoints usually occurring at short repeats and, in some cases, of duplication of *Ac* sequences or insertion of *Ac*-unrelated fragments. The ancient maize *Ds* elements and the young *Ds* elements in transgenic tobacco showed similar rearrangements, suggesting that *Ac-Ds* elements evolve rapidly, more so than stable genes, through deletions, duplications, and reshuffling of their own sequences and through capturing of unrelated sequences. The data presented here suggest that abortive *Ac*-induced gap repair, through the synthesis-dependent strand-annealing pathway, is the underlying mechanism for *Ds* element formation.

Dissociation, or *Ds*, is the first discovered transposable element (TE). It was identified as a maize locus on chromosome 9, where breaks occur in the presence of *Activator* (*Ac*), a second gene found at a separate locus (33, 34). Subsequent studies showed that *Ac* can transpose autonomously whereas *Ds* moves only in the presence of *Ac* (35, 36). In addition, *Ac* activity can turn into a *Ds* type of instability, while no occurrences were found of *Ds* turning into *Ac* (37, 38). On the basis of these observations, McClintock proposed that *Ds* nonautonomous elements are derived from *Ac* through mutations (39). The proposal that *Ac* and *Ds* are phylogenetically related has been supported by molecular analysis, as described below, but the mechanism responsible for the conversion of *Ac* into *Ds* is still unknown.

Ac is a 4.6-kb-long element flanked by 11-bp terminal inverted repeats (TIRs) (12). It encodes an 807-amino-acid protein, the transposase, necessary for both *Ac* and *Ds* transposition (28). *Ds* elements, on the other hand, do not encode a functional transposase but retain regions which are essential for their transposition (6). There are six fully sequenced *Ds* elements, all of which share with *Ac* nearly identical TIRs and fall into the following four categories: (i) those with nearly no similarity to *Ac*, like *Ds1* (57); (ii) elements with highly similar subterminal regions but with internal deletions, like *Ds9* (46); (iii) double *Ds* elements where one internally deleted *Ds* is inserted into another identical *Ds* in an inverted orientation (9); and (iv) *Ds* elements that contain both deletions and insertions in the internal part of the element, like *Ds2* (40) and the *Ds* element in *WxB4* (60). While double *Ds* elements can be interpreted as transposition of one element within the other (9), the mechanism of formation of other types of *Ds* elements is not known.

The organization of TEs as two-element systems, with a full-length autonomous element and several defective nonautonomous elements, is a general feature of elements transposing via a conservative (cut-and-paste) pathway (51). For example, several internally deleted TEs were characterized for the maize *Spm* (13), the nematode *Tc1* (18), and the *Drosophila S* (42) and *P* (48) elements. A mechanism responsible for the formation of nonautonomous elements has been proposed for the *Drosophila P* element. Upon *P* excision, gap repair was shown to be initiated from the donor site, leading to the replacement of the excised element at its original locus (11, 43). Based on these findings, internally deleted elements were suggested to originate from premature interruption of gap repair (10) and insertions were proposed to occur during gap repair as a result of template switch to an ectopic site by the synthesis-dependent strand-annealing (SDSA) gap repair pathway (43). In plants, a series of observations with the maize *Mutator* (*Mu*) elements suggest that abortive gap repair (AGR) might also be involved in the formation of nonautonomous *Mu* elements (21, 32), but a detailed study of AGR has not been carried out for any species.

To test whether AGR is the underlying mechanism of *Ds* formation, we have used two approaches, i.e., sequence analysis of native maize *Ds* elements and determination of the requirements for de novo alterations in *Ac* DNA. Sequence analysis of the maize *Ds* elements indicates that *Ds* formation involves complex patterns of deletions and insertions of *Ac*-unrelated sequence blocks, two of which are homologous to known maize genomic sequences. *Ac*-related and *Ac*-unrelated blocks alternate, and short stretches of *Ac* sequences are sometimes reshuffled or duplicated at the insertion sites. In addition, using transgenic tobacco plants, we have developed an assay aimed at isolating and characterizing newly formed *Ds* elements. De novo formation of *Ds* elements was shown to be excision rather than sequence dependent: it occurs with *Ac* but not with an almost identical *Ds* element, and it does not occur with a stable transposase-producing gene. The deletion breakpoints usually occur at short repeats. Taken together, sequence analysis of *Ds* elements and the experimental data are in agree-

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ment, suggesting that *Ac*-induced AGR via the SDSA pathway is the underlying mechanism for *Ds* element formation. A model for AGR is described in this paper. This model explains the data presented here as well as several reports on DNA rearrangements in plants.

MATERIALS AND METHODS

Sequence analysis. Sequence analysis was performed with the Genetics Computer Group (GCG; Madison, Wis.) sequence analysis package, version 8.0, with the previously described simPCR program (50) or with specially designed programs (available upon request). *Ac*-related sequences were identified in the public sequence database GenBank (release 79) by the following search methods: (i) iterative blast searches with *Ac9* as a query; (ii) STRINGSEARCH with combinations of the keywords *Ac*, *Ds*, transposon, and maize; and (iii) simPCR analysis (50) using the *Ac* TIRs and allowing one mismatch. A nonredundant list of *Ac*-related entries was prepared by performing all-against-all analysis with BESTFIT and GAP, eliminating sequences which were 100% identical.

A special program, LocalFit, was written for the finely detailed analysis of deletion-insertion elements. This program utilizes BESTFIT with its built-in Shuffle test to identify highly localized similarities between two sequences. The program supplies information which can be used to estimate the statistical significance of each similarity region. The program and all other relevant material are available upon request from the authors.

Plant material. Transgenic tobacco plants (*Nicotiana tabacum* var. *xanthi*) were made via *Agrobacterium*-mediated transformation from leaf discs with constructs pJJ4411 (26), pAGS4081 (24), and Bam35S-Ac (14). Constructs pJJ4411 and pAGS4081, kindly provided by H. Dooner, are described below (see Fig. 4A). Both constructs are identical except that in pAGS4081, the 5′ *Hin*dIII site of *Ac* was filled in. The resulting frameshift mutation in the transposase open reading frame created a nonautonomous *Ds* element (26). The activity of *Ac* and the immobility of *Ds* could be confirmed by germinating seeds of transgenic plants on streptomycin-containing media and looking for the presence of green revertant sectors in cotyledons as an indication of element excision (25). Seeds were collected from selfed primary transformants and germinated on medium containing 15 μg of hygromycin per ml. pJJ4411 or pAGS4081 plants with a single insertion were selected based on the segregation of hygromycin-resistant seedlings. The presence of a single-copy insert was confirmed in some of the transgenic plants (data not shown). The genotype of each plant, whether homozygote or hemizygote, was determined by segregation of hygromycin resistance in selfed progeny.

Construct Bam35S-Ac, kindly provided by N. Fedoroff, contains the 35S promoter instead of the 5′ terminus of *Ac* up to the *Bam*HI site (14). Bam35S-Ac plants were shown to mobilize *Ds* elements (data not shown), which indicates that they produce a functional transposase. In this article, the Bam35S-Ac construct will be referred to as a stable transposase.

DNA extraction. DNA was extracted from two to three axillary or apical buds (5 to 10 mm long) by a modified Dellaporta extraction protocol (7) with two phenol-chloroform extractions. DNA was resuspended in 50 to 100 μl of double-distilled water (DDW), and the concentration was determined.

PCR and sequencing. All PCRs were performed with Promega *Taq* polymerase as described in the manufacturer's recommendation, with 1.5 mM $MgCl_2$ and 200 mM deoxynucleoside triphosphates in an MJ thermocycler. For amplification of the 1.6-kb fragment (see Fig. 4A), the following program was used: 2 min of denaturation at 94°C, 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and a final step of 5 min at 72°C. For the 2.9-kb fragment amplification (see Fig. 4A), the reaction was performed with *Taq* Extender (Invitrogen) as described in the manufacturer's instructions, and the cycles were modified to 2 min at 96°C, 30 cycles of 1 min at 96°C, 1 min at 55°C, and 3 min at 72°C, and a final step of 5 min at 72°C. For a second round of amplification with more internal primers (see Fig. 4A), 2 μl of the first-round product was used.

Bands of interest were extracted from agarose gels, either with GeneClean as described in the manufacturer's instructions or by the freeze-squeeze method (i.e., 200 μl of DDW was added to the gel slices, and the slices were frozen in liquid nitrogen and centrifuged for 5 min at 10,000 × *g*). Freeze-thaw treatments were repeated twice. Two microliters of the GeneClean product or the freeze-squeeze eluant was used as the template in the PCR as described above. PCR products were ethanol precipitated and directly sequenced.

RESULTS

General structure of *Ac* and *Ds* elements. To find out how *Ds* elements originate from *Ac*, we performed a sequence analysis on all database entries related to the *Ac-Ds* family (see Materials and Methods). All nonredundant *Ac*-related entries were aligned with the COMPARE, BESTFIT, GAP, and PILEUP programs in the GCG package. *Ac-Ds* family members (schematically described in Fig. 1) have in common some sequences

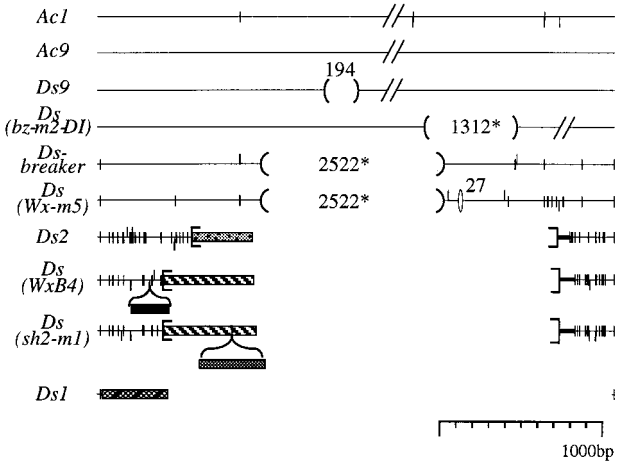


FIG. 1. Overall structure of *Ac-Ds* family members. Sequences of all *Ac* and *Ds* elements found in the public sequence databases were aligned with the sequence of *Ac9*. Regions with a high degree (>90%) of similarity to *Ac9* (thin lines), with complex similarity (thick lines), or with no significant similarity (blocks) are shown. Brackets are at the borders of deletion-insertions, and parentheses are at the borders of deletions, with the length in base pairs given relative to the sequence of *Ac*. Vertical lines indicate sites of minor mutations, with substitutions crossing the horizontal similarity line, and with insertions indicated above and deletions indicated below the line. Some large regions which contain no mutations relative to *Ac9* were omitted from the alignment and are indicated by slashes. The following database entries were used for comparison to *Ac9*: *Ac9* (accession no. K01964, bases 4682 to 122); *Ac1*, X05424; *Ds-breaker*, X00269; *Ds(Wx-m5)*, X62655; *Ds2*, X65746; *Ds(WxB4)*, X51636; *Ds(sh2-m1)*, L33921; *Ds1*, K03283; the sequence of *Ds9* was reconstituted from the description in reference 46; the sequence of *Ds(bz-m2-DI)* was reconstituted from the description of the *Ds* element in allele *bz-m2(DI)* (8).

such as the TIRs and the transposase binding motif and fall into five groups, as follows.

(i) *Ac* elements. There are only two nonredundant *Ac* entries, *Ac1* and *Ac9*. These two sequences differ from each other by nine point mutations, of which four are possibly the result of sequencing errors (i.e., reversed order of consecutive nucleotides). Since the two elements are very similar, *Ac9* was chosen to serve as a reference sequence in Fig. 1.

(ii) *Ds1*-like elements. Many database entries are either annotated as *Ds1*-like elements or have a very high degree of sequence similarity to *Ds1*, including a small group of *rUq* elements (16, 44). These elements are short (~400 bp) and have in common with *Ac* only the TIRs and one or two AAA CGG transposase binding motifs. Since all *Ds1*-like elements are very similar to each other, we chose to include only *Ds1* from the *Adh1-Fm335* allele for comparison to *Ds* elements (Fig. 1).

(iii) Simple deletion elements. Four *Ds* elements have high similarity to *Ac9* in the terminal regions but have internal deletion(s) (shown in parentheses). Two elements, *Ds-breaker* and *Ds(Wx-m5)*, have a deletion of 2,522 bp with precisely the same position but differ by an additional 29-bp deletion in *Ds(Wx-m5)*. For *Ds9* and *Ds(bz-m2-DI)*, genetic and molecular evidence indicates that these elements were formed by de novo deletions in *Ac* (8, 12, 37, 38). Their sequence is not available in databases; it was reported to be identical to their *Ac* progenitor sequence except for a 195-bp internal deletion in *Ds9* (12) and a 1,312-bp deletion in *Ds(bz-m2-DI)* (8).

(iv) Deletion-insertion elements. Three elements which have been sequenced have both deletions and *Ac*-unrelated insertions. Two elements, *Ds(sh2-m1)* and *Ds(WxB4)*, are closely related: they have a highly similar deletion and insertion, but each has an additional insertion relative to the other. *Ds2* has

an insertion and a deletion unrelated to that of *Ds(sh2-m1)* and *Ds(WxB4)*.

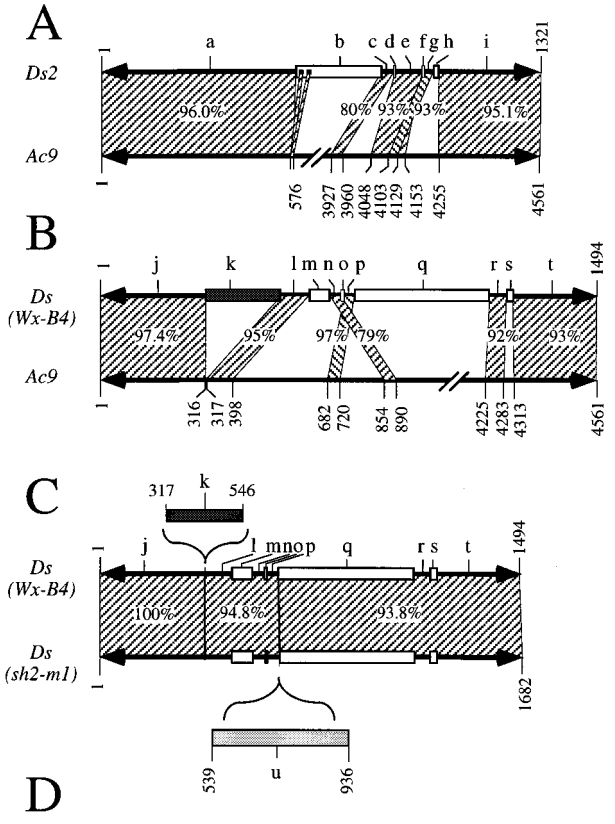
(v) Composite *Ds* elements. Composite *Ds* elements are made of more than one *Ds*. The only fully sequenced composite *Ds* is the chromosome breaker element in allele *sh-m5933* (9, 33). It is composed of two completely identical, simple deletion *Ds* elements in reverse orientation and of the 8-bp host duplication which flanks the internal element. Each element is nearly identical to *Ds(Wx-m5)*, with minor differences, and is further referred to as *Ds-breaker*.

The phylogenetic relationship among the various *Ac-Ds* elements was studied by comparing their *Ac*-related terminal regions, which are important for transposition (6). *Ac9* nucleotides 1 to 257 for the left side, nucleotides 4246 to 4561 for the right side, and the corresponding regions in *Ds* elements were used to draw a maximum likelihood tree. Each end was analyzed separately, yielding two similar (but not identical) trees, which can be described as follows: {[*Ac1, Ac9, Ds9, Ds(bz-m2-DI)*], [*Ds(Wx-m5)*, *Ds*-breaker]}, {*Ds(sh2-m1)*, *Ds(WxB4)*}, *Ds2*. *Ac1* and *Ac9* are very similar and clearly form one group. *Ds9* and *Ds(bz-m2-DI)* were included in this group based on author description (8, 12). Interestingly, both *Ac1* and *Ac9* have imperfect TIRs, while all fully sequenced *Ds* elements except for *Ds9* and *Ds(bz-m2-DI)* have perfect TIRs. *Ds1*-like elements form a separate group which has very little similarity with *Ac* except in the TIRs.

Fine-structure analysis of *Ds* elements. (i) Deletion-insertion elements. Three *Ds* elements, *Ds(sh2-m1)*, *Ds(WxB4)*, and *Ds2*, have extensive homology with *Ac* in the subterminal region (Fig. 1) but have deletions of *Ac* sequences and insertions of *Ac*-unrelated sequences in their internal part. A sequence comparison of *Ac* with *Ds2* (Fig. 2A) indicates that *Ds2* formation involved complex mutation patterns rather than simple deletions and insertions. The following rearrangements were found: alternation of *Ac*-related and unrelated sequences; triplication of *Ac* sequences as shown on the left of sequence block b and as detailed in the sequence of this region (Fig. 2D); and reshuffling and duplication (block g of Fig. 2A, coordinates 4103 to 4129, is duplicated in *Ds2* and occurs in an order different from that of *Ac*).

Comparison of *Ds(WxB4)* to *Ac* also shows complex rearrangements. Interestingly, block k (Fig. 2B) is a clean insertion, not associated with deletions. This block was previously described by Varagona and Wessler (60) and was suggested to be a transposon based on the presence of inverted repeats at its termini. In agreement with this proposal, database searching indicates multiple homologies: block k has 75% identity with 182 bp at the 5′ region of GenBank entry zmprmsg, designated maize PR promoter, and 73% identity with a 107-bp-long region of entry zmcata1, designated maize catalase 1. Next to block k, a region of complex alternation of *Ac*-related and unrelated sequences is found (blocks l to t). Two *Ac*-related blocks, n and p, occur in an order which is not collinear to the order in *Ac*. Interestingly, these two reshuffled blocks contain deletions in a region which is triplicated in *Ac* (see sequence in Fig. 2D).

The third deletion-insertion element, *Ds(sh2-m1)*, is closely related to *Ds(WxB4)* in its subterminal regions (Fig. 1) (see phylogenetic grouping described above). It also has in common with *Ds(WxB4)* the similar *Ac*-unrelated blocks m, o, q, and s inserted between *Ac* sequences (Fig. 2C). They differ in two *Ac*-unrelated insertions, blocks k and u (Fig. 2C). Block u has 62% sequence identity with a 279-bp region of GenBank entry zmh2b3a, designated maize histone H2B. This block does not show the features expected for a transposon (i.e., TIRs and host duplications).



Block n

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Ac9          854- AAAACAAGGCGACAGACAACAGCCCATGCATCAAAAC -890
Ds(Wx-B4)    689- AAAGCAAGGCAACA---CGCAGCTTATGCACCAAAAC -722
Ds(sh2-m1)   463- AAAGCAAGGCAACA---AGCAGCTCATGCACCAAAAC -496
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Block p

```
Ac9          682- ACGGCTGGGAGCTAGGCAGCTAGCAGCTAG--CGCCTAACA -720
Ds(Wx-B4)    733- ACGGCTGGGAGCTA--------GCAGCTAG--CGTCTAACA -763
Ds(sh2-m1)   507- ATGGCTGGGAGCTA--------GCTGCTAGAGCGCCTAAGA -539
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Block a-b junction

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Ds2  574- TTAATACACATACATTCTCATTTAATACACATACATTCT
          CATGATTAAATACATATACATTCTCAT -641
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FIG. 2. Fine-structure analysis of deletion-insertion *Ds* elements. (A to C) The structure of deletion-insertion elements is shown. *Ds2* (A) and *Ds(WxB4)* (B) are compared to *Ac9*, and *Ds(sh2-m1)* is compared to *Ds(WxB4)* (C). *Ac9* is shown as a bold line flanked by arrows representing the TIRs. Hatched areas represent similarity regions, with the percent similarity shown within the area. Numbers below the *Ac9* sequence indicate the borders of similarity regions, with nucleotide 1 representing the terminus near the 5′ end of *Ac* transposase. *Ac*-unrelated sequences are indicated as blocks. Open blocks do not have sequence homology with any non-*Ds* entry in any sequence database. Blocks k and u differ from each other and have homology to different database entries. Regions related to *Ac* (thick lines) or unrelated to *Ac* (blocks) alternate and are indicated by letters. Similar regions are indicated by the same letter. (C) The structure of *Ds(sh2-m1)* is shown in comparison to *Ds(WxB4)* because they differ only by two insertions, blocks k and u. (D) The sequences of selected blocks are shown. Blocks n and p contain three copies of a small tandem repeat (indicated within a box) in *Ac* and only two copies in *Ds(WxB4)* and *Ds(sh2-m1)*. At the junction between blocks a and b, *Ds2* contains a region of three 21-bp-long repeats (framed boxes). Each repeat begins with the terminal 10 nucleotides from block a in *Ac* (shaded box, coordinates 567 to 576).

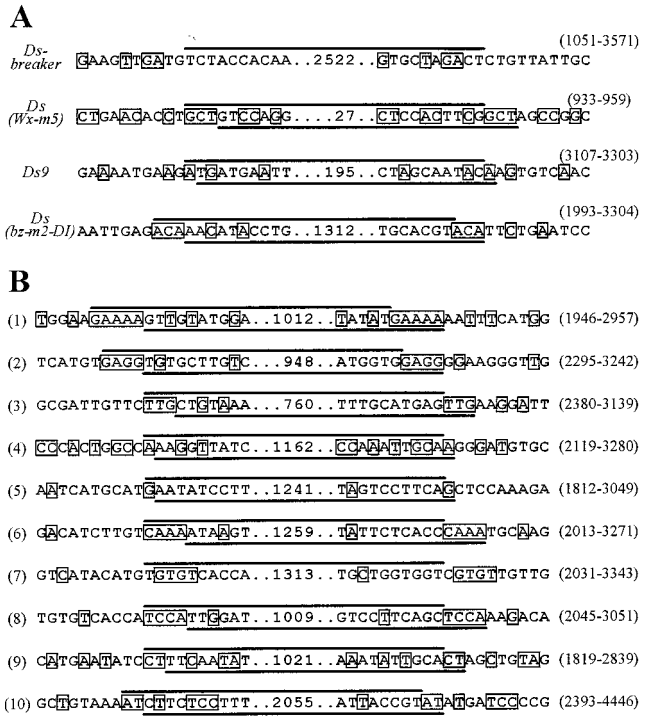

FIG. 3. Analysis of breakpoints in simple-deletion *Ds* elements. Deletion breakpoints, determined by sequence comparison to *Ac9*, are shown in the maize *Ds* elements (A) and in de novo deletions in *Ac* isolated from transgenic tobacco plants (B). The deleted regions are indicated by bold lines. Staggering of bold lines (above and beneath the deleted region) indicates that the exact breakpoint location cannot be determined because of the presence of short repeats at the deletion borders. In such cases, nucleotides could have been deleted from either staggered side. Directly repeated bases adjacent to the breakpoint are boxed. The size of the deletion is given within the deleted region, and its location is given as coordinates in parentheses.

(ii) Simple deletion elements. Four *Ds* elements, *Ds9*, *Ds(bz-m2-DI)*, *Ds(Wx-m5)*, and *Ds-breaker*, are highly similar to *Ac* (more than 95% identity throughout their sequence) but lack stretches of *Ac* sequences in their internal part. Deletion size and position vary in the different elements (see Fig. 3A). Unlike *Ds9*, *Ds(bz-m2-DI)*, and *Ds-breaker*, which have a single deletion, *Ds(Wx-m5)* contains two deletions. The large one, spanning nucleotides 1051 to 3572, is identical to that in *Ds-breaker*. The sequence at the deletion breakpoints of all three elements is shown in Fig. 3A. Since direct repeats were previously proposed to play a role in the formation of deletions (1, 21, 29), such features were looked for in *Ds* elements. Short 3-bp repeats flank both the deletion in *Ds(bz-m2-DI)* and the small deletion (nucleotides 933 to 959) in *Ds(Wx-m5)*. The deletion in *Ds9* (nucleotides 3107 to 3303) is flanked by a single base repeat. The deletion in *Ds-breaker* (nucleotides 1051 to 3571) is not flanked by repeats.

De novo rearrangements in *Ac*. The complexity of *Ds* sequences might be explained by a mechanism capable of creating deletions, insertions, duplication, and reshuffling of the *Ac* sequence. Alternatively, the conversion of *Ac* into *Ds* might have occurred over a long evolutionary period and could result from a series of successive independent mutations. To determine the mechanism responsible for *Ds* formation, we have screened and analyzed de novo rearrangements in *Ac* in transgenic tobacco plants whose genome is free of native *Ds* elements. We have tested whether *Ac*-induced AGR could result in the formation of a nonautonomous element (see the introduction). The predicted characteristics of defective element formation via AGR are the following: (i) excision dependence, (ii) occurrence at the original donor site, (iii) possible homolog sensitivity, and (iv) occurrence at short direct repeats (1, 21, 29).

These requirements were tested by using tobacco plants carrying a single copy of an active *Ac* element or a nonmobile *Ds* inserted between the 35S promoter and the streptomycin resistance gene (Fig. 4A, SPT::*Ac* or SPT::*Ds*) and plants car-

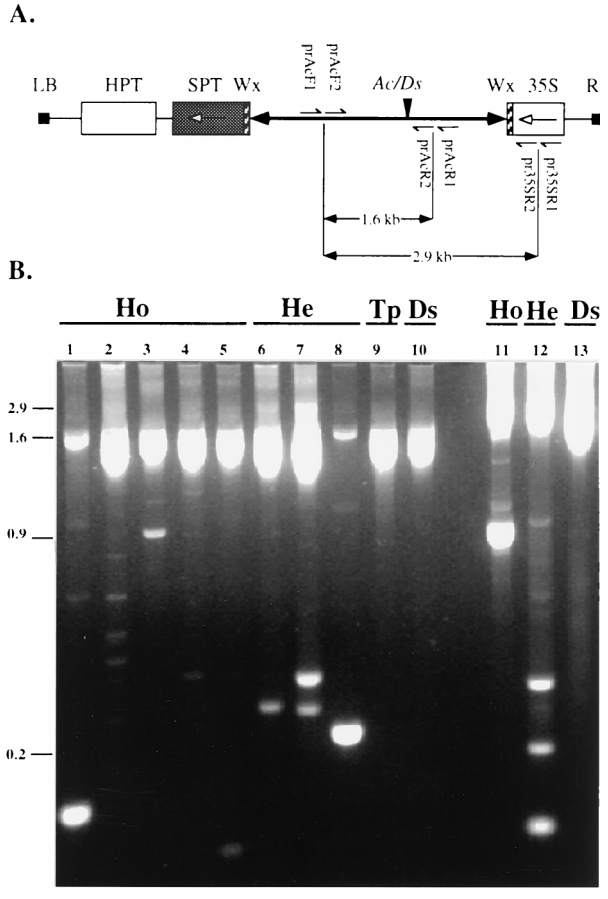

FIG. 4. Requirements for de novo rearrangements in *Ac*. (A) The constructs used in the assay for de novo rearrangements in *Ac* (SPT::*Ac* [pJJ4411] or SPT::*Ds* [pAGS4081]) constructs (25, 26) are schematically described. They contain a nearly identical *Ac* or *Ds* element inserted between the 35S cauliflower mosaic virus promoter (empty box with an arrow showing the direction of transcription) and a gene conferring resistance to streptomycin (SPT). The *Ds* element differs from *Ac* by a 4-bp insertion into the 3′ *Hin*dIII site (indicated by a black triangle). Primers were designed to amplify an internal 1.6-kb fragment by nested PCR, with two forward primers (prAcF1, 5′-AGATCCGAGTCAAG ATGATATG-3′; prAcF2, 5′-GTACGATGAAGTGGTTAGCC-3′) and two reverse primers (prAcR1, 5′-TCTCATGATTTGTTGCAGC-3′; prAcR2, 5′-CAT ATCAGAGCTCCAACAATC-3′). A second pair of primers (pr35SR1, 5′-GA CGCACAATCCCACTATC-3′; pr35SR2, 5′-TCATTTCATTTGGAGAGGAC-3′) from the donor site was used to amplify a 2.9-kb fragment with prAcF1 and prAcF2 in nested PCR. The left and right borders (LB and R, respectively) of the binary vector are shown as small black boxes; the transformation marker gene conferring hygromycin resistance (HPT) is shown as an empty box. The *Waxy* sequences (Wx) flanking *Ac* are shown as narrow hatched boxes. *Ac* is shown as a thick line flanked by arrows which indicate the TIRs. (B) An ethidium bromide-stained gel of the PCR products obtained with the 1.6-kb primers (lanes 1 to 10) or with the 2.9-kb primers (lanes 11 to 13) is shown. DNA was extracted from the plants with the following characteristics: homozygous for SPT::*Ac* (Ho; lanes 1 to 5 and 11), hemizygous for SPT::*Ac* (He; lanes 6 to 8 and 12), stable transposase (Tp; lane 9), and SPT::*Ds* (*Ds*; lanes 10 and 13). Molecular sizes (in kilobases) are indicated on the left.

rying a stable transposase gene, 35S-transposase (14). Total genomic DNA was extracted from leaves of lines described in the legend to Fig. 4, and 600 ng of this DNA, equivalent to approximately 40,000 genomes (3), was used as a template in nested PCR with primer pairs schematically described in Fig. 4A. Several (tens) plants were analyzed for each genotype, and a representative sample is shown in Fig. 4B.

Mutations affecting the length of the element are detected in *Ac*-containing plants, both homozygous and hemizygous for the SPT::*Ac* construct (Fig. 4B, lanes 1 to 8, 11, and 12). With primers prAcF2 and prAcR2, a 1.6-kb band is expected. Bands of varying length, ranging from 190 to 1,000 bp, with a weaker intensity than that of the full-length 1.6-kb fragment, were observed. Since the 1.6-kb fragment covers a significant portion of the reading frame, rearrangements in this region are expected to represent a conversion of *Ac* into *Ds*. Under similar conditions, no mutations affecting the element length were detected in SPT::*Ds* plants (lane 10) or in stable *Ac* plants (lane 9). The lack of mutations in *Ds*-containing plants indicates that the occurrence of mutations in *Ac* is transposition dependent rather than sequence dependent (*Ac* and *Ds* differ by only 4 bp). The lack of mutations in 35S-stable transposase plants indicates that the occurrence of mutations in *Ac* is not dependent on the presence of the transposase protein per se but rather on the transposition it catalyzes.

Rearrangements in *Ac* could occur in a transposition intermediate during insertion or, as expected from the AGR model, at the donor site after excision. To test whether rearrangements in *Ac* occur at the original donor site, *Ac* primers were used in combination with primers from the flanking 35S promoter to amplify the 2.9-kb fragment shown in Fig. 4A. The full-length 2.9-kb fragment was amplified in both SPT::*Ac* and SPT::*Ds* plants (Fig. 4B, lanes 11 to 13). Multiple reduced-size bands occurred in both SPT::*Ac* homozygotes and hemizygotes (lanes 11 and 12, respectively) but not in SPT::*Ds* plants (lane 13). This indicates that alterations in *Ac* are excision dependent and occur at the donor site.

Sequence analysis of de novo rearranged *Ac* elements. The sequence of a sample of the bands whose size was smaller than that of the expected full-length PCR product (Fig. 4B) was determined. All reduced-size bands obtained with primers prAcF2 and prAcR2 contained simple internal deletions (Fig. 3B, sequences 1 to 9). With primers prAcF2 and pr35SR2, only one of eight sequenced bands showed a simple internal deletion (Fig. 3B, sequence 10). Deletions varied in size (from 758 to 2,056 bp) and in position. Direct repeats ranging from 1 to 5 bp were observed either immediately adjacent or very close to the breakpoint. The other seven bands amplified with primers prAcF2 and pr35SR2 showed a variety of events (Fig. 5, bands 2 to 8). Most events resulted in the elimination of the 3′ end of *Ac*, with (Fig. 5, bands 2, 3, and 8) or without (bands 4 and 7) deletion in flanking 35S sequences. In two reduced-size bands (bands 7 and 8), the presence of both an insertion and a deletion was found: 96 bp (band 7) and 52 bp (band 8) of *Ac*-unrelated sequences were inserted within 599-bp and 2,574-bp deletions, respectively. In two cases (bands 2 and 6), the 3′ end of *Ac* was deleted and a region of *Ac* was duplicated at the breakpoint, similarly to the duplications in natural *Ds* elements (Fig. 2). For band 6, 2,369 bp of *Ac* were deleted, but two *Ac* regions were duplicated following the breakpoint (100% identity to *Ac* bases 2116 to 2125 and 2118 to 2136). Surprisingly, two events (bands 5 and 7) contained typical excision footprints (52, 54) in the *Waxy* host duplication (data not shown) in addition to the deletions and insertions described above.

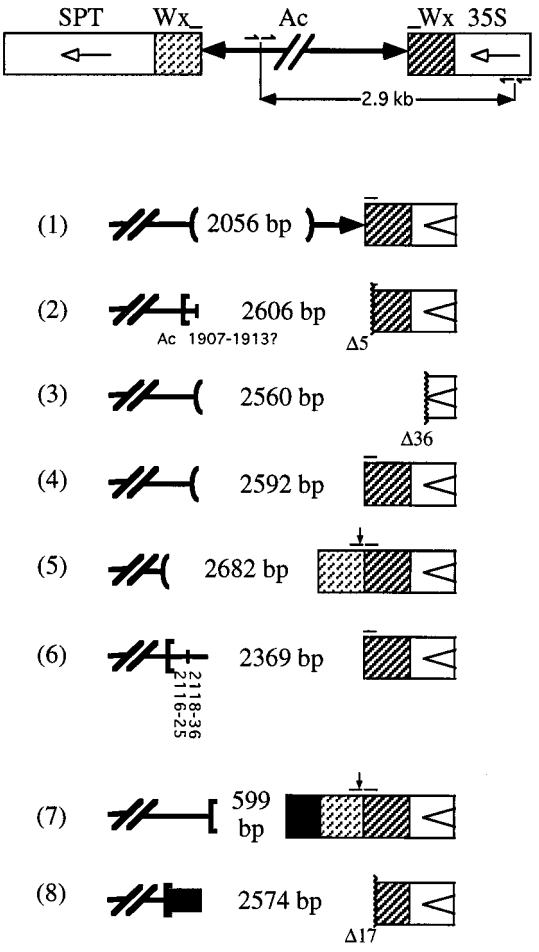

FIG. 5. Analysis of complex rearrangements in *Ac*. Eight bands with a reduced size compared to the expected 2.9-kb PCR fragment (see Fig. 4A) were isolated and sequenced. A schematic description of the donor site with the *Ac* insert is shown at the top of the figure. *Ac*-related sequences are shown as thick horizontal lines, with arrows representing the TIRs. Blocks represent the streptomycin resistance gene (SPT), the short maize *Waxy* sequences which were introduced during the cloning of *Ac* (Wx; hatched blocks), the CaMV 35S promoter (35S), or sequences of unknown origin (solid black blocks). Thin lines above the Wx blocks represent the host duplication in *Waxy*. Primers are indicated by small one-sided arrows. Deletions are indicated by parentheses with the size given in base pairs within the parentheses. Wavy vertical lines in the *Waxy* or 35S box indicate a deletion (△) whose size in base pairs is given. The downward vertical arrow above the *Waxy* region in bands 5 and 7 indicates a single base substitution in the sequence of the host duplication, which is similar to a typical excision footprint for this locus. Brackets indicate deletions and insertions (solid blocks) or duplication of *Ac* sequences whose coordinates are shown below the brackets in bands 1 and 6.

DISCUSSION

The mechanism of *Ds* formation was addressed by performing a sequence analysis of the maize *Ds* elements and by studying de novo alterations in *Ac*. Rearrangements were found for maize native *Ds* elements (compared to *Ac*) and de novo for *Ac* elements in transgenic tobacco, indicating that *Ac-Ds* elements evolve structurally more rapidly than stable genes. *Ds* formation involves deletions, capturing of *Ac*-unrelated genomic sequences, reshuffling and duplication of *Ac* regions, and alternation of *Ac*-related and *Ac*-unrelated blocks. These rearrangements can be accounted for by AGR through the SDSA pathway. We propose that SDSA is a general mechanism for double-strand break (DSB) repair in plants and for capturing of filler DNA at deletion sites.

Structure of native *Ds* elements. In terminal regions known to be essential for transposition (6), all *Ds* elements are homologous to *Ac*, with the exception of *Ds1*-like elements (13). The sequence variability among *Ds* elements in those regions (Fig. 1) indicates that in the maize genome, the *Ac-Ds* family is ancient and not a recent acquisition by horizontal transmission as found for the *Drosophila P* element (20). This is in agreement with the findings of Huttley et al. (22), indicating the antiquity of *Ac* in grasses.

The internal part of *Ds* elements contains either simple deletions or deletions and insertions of *Ac*-unrelated sequences. We have identified in the sequence databases homologs of two *Ac*-unrelated insertions, designated blocks k and u in Fig. 2. Block k in *Ds(WxB4)* has homology with two maize database entries (pathogenesis-related promoter and catalase 3′UTR). The same block was previously proposed to be a DNA transposon, based on the presence of inverted repeats and host duplication at its borders (60). Data presented here support this proposal because (i) there is a multiple copy number of block k homologs in the maize genome; (ii) there is a lack of association of this insertion with a deletion; and (iii) in both homologous maize entries, the homology to block k ends at or near one of the inverted repeats (data not shown). Thus, one of the mechanisms involved in *Ds* formation seems to be insertion of *Ac*-unrelated transposons. Such nested transposon structures for inverted repeat-flanked elements is reminiscent of the nested structure of retroelements reported recently for maize (53). One difference, however, with respect to retroelements is that mobility was shown for *Ds(WxB4)* (60) while mobility was not shown for composite retroelements.

The other *Ac*-unrelated insertion with an identified homolog in the database is block u. This insertion is not homologous to any known transposon, neither in sequence nor in structure. It is similar (65% identity) to a region in the promoter of a maize histone gene, suggesting that it might have been captured from a genomic sequence. Capturing of genomic sequences by transposons has been previously shown for DNA-DNA transposons such as *P* (19, 59). To our knowledge, capturing of genomic sequences by transposons in plants was shown only for retroelements (4, 23). Genomic capturing represents a new type of *Ac*- or *Ds*-induced mutation which can create gene duplication and pseudogenes and might generate mobile promoters and enhancers.

The remaining *Ac*-unrelated insertions do not have homology to any database entry as of January 1997. These insertions are not simple insertions such as block k. They all occur in association with deletions and are often flanked by duplicated or reshuffled *Ac* sequences. Inserts occurring at deletion sites have already been found in maize (10, 62) and in mammalian nonmobile genes (49) and were termed filler DNA. We discuss below a possible mechanism for the origin of these inserts.

Alterations in *Ac* occur via AGR. The conversion of *Ac* into *Ds* in real time (8, 12, 37, 38) and the frequent alterations in *Ac* described here suggest that mutations in *Ac* occur at a higher rate than they do in stable genes. Furthermore, our assay, based on detection of reduced-size PCR products, is biased in the spectrum of mutations it can detect. Small deletions, or deletions-insertions which do not cause an observable shift in PCR product size, will not be identified. Deletions or inversions which span a primer annealing site or other rearrangements that prevent amplification will also be unnoticed. These biases can also account for the different type of mutations we found when using primers within *Ac* (Fig. 3) or out of it (Fig. 5). Taken together, the limitations of the assay cause an underestimation of the actual frequency of alterations in *Ac*. With our assay, we compare a number of mechanisms and assess their ability to explain the rapid and complex rearrangements within *Ac*.

Slippage during DNA replication was proposed to be responsible for both deletions and deletions-insertions at the *Waxy* locus (62). If such a mechanism is to explain *Ds* formation, it would be expected to be sequence dependent and to occur at the same rate in stable and mobile genes of similar structure. The fact that a *Ds* element, which is almost identical in sequence to *Ac*, failed to yield mutations indicates that this is not the case, i.e., that slippage is not inherent to *Ac* sequence. It could be speculated that transposase induces slippage through binding to *Ac* sequences (27). No mutations were found with a stable transposase source. Thus, the controls done in this work show that alterations in *Ac* sequences are excision rather than sequence dependent, ruling out slippage as a mechanism for *Ds* formation.

Alterations in *Ac* could also occur by an as-yet-unknown mechanism during transposition, for example, in an extrachromosomal intermediate or upon integration at a new position. This possibility, however, cannot fully explain *Ds* formation since de novo *Ds* elements were identified in the original donor site (Fig. 4, lanes 11 to 13, and Fig. 5, band 1).

The findings described above, namely, that alterations in *Ac* are not sequence dependent and that they occur at the donor site and only with *Ac*, not with *Ds*, imply that *Ds* formation requires excision and does not occur in a transposed element. The most plausible mechanism to explain how an alteration in *Ac* can be formed at a site lacking *Ac* (following excision) is that these alterations occur in a newly synthesized element. According to this scenario, *Ac* excision initiates gap repair at the empty donor site through invasion of a homologous template carrying an unexcised element. Errors in this process would lead to the replacement of the excised element by a mutated copy, i.e., a new *Ds* element. We therefore conclude that, typically, *Ds* formation occurs through homologous recombination between the broken donor site and its homologous region, and we rule out DSB repair mechanisms that do not involve DNA synthesis, such as the single strand annealing (SSA) model (31) or end-joining of broken ends at the donor site (52, 54).

Direct evidence for *Ac*-induced homologous recombination has been recently reported for a system based on reactivation of an inactive reporter gene through ectopic recombination (55). In our assay, recombination at the donor site could occur between homologous chromosomes or sister chromatids. Rearrangements in *Ac* were found in both homozygous and hemizygous plants (Fig. 4). This provides evidence that a sister chromatid carrying an unexcised element can be used as a template for gap repair. Such a possibility was previously mentioned to explain untwined light-variegated sectors in maize (5). In *Drosophila* and in *Caenorhabditis elegans*, the frequency of gap repair via homologous recombination was shown to be homolog sensitive (11, 45). Such sensitivity was not detected in our assay. This could be due to its nonquantitative nature, or alternatively, it might be that the preferential transposition of *Ac* during DNA replication (5) provides a sister chromatid as a template for gap repair and there is no need to use the homolog.

Two repair mechanisms involving DNA synthesis have been proposed to explain the conversion of autonomous *P* elements into nonautonomous ones (11, 43). According to the yeast DSB repair model (58), protruding 3′ single-strand DNA ends are generated upon DSB formation, invade a homologous template, start DNA synthesis, and generate a stable heteroduplex (Holliday junction structure). Premature interruption of DNA synthesis in such a model would generate internal deletions but

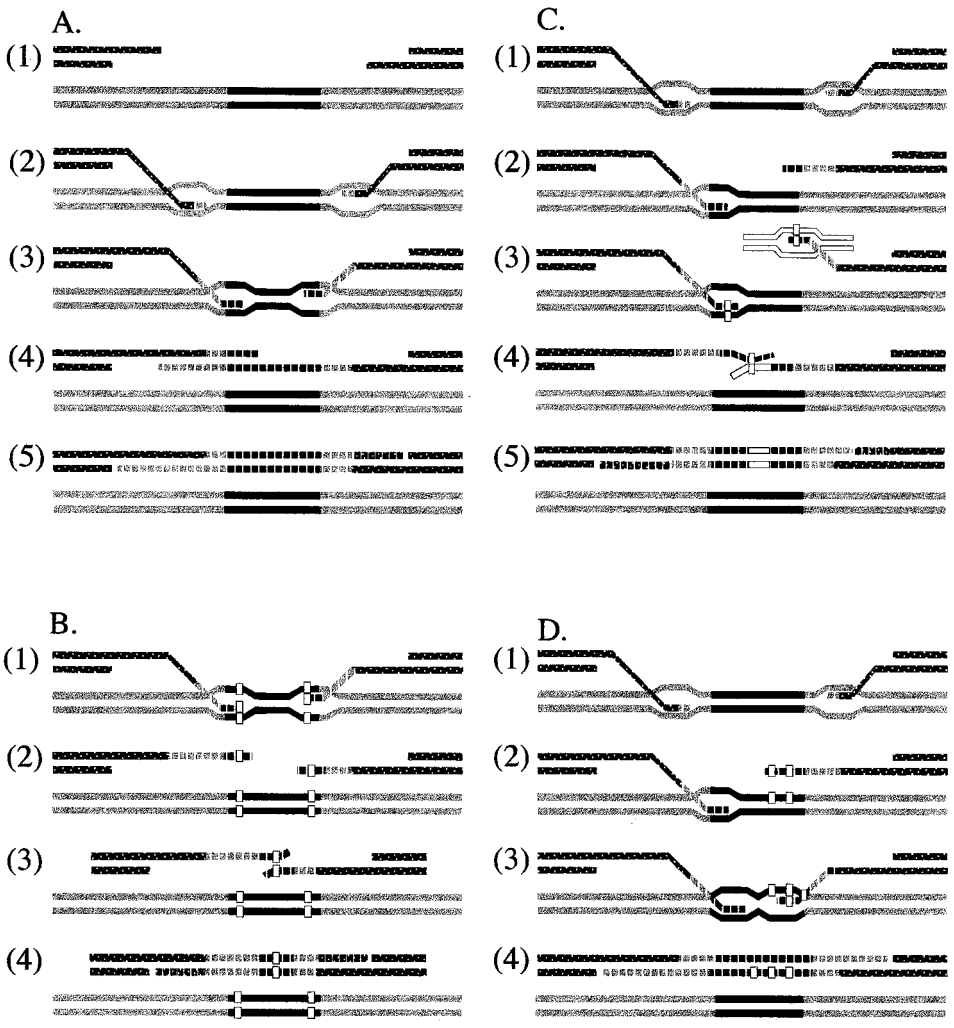

FIG. 6. Model for AGR through the SDSA pathway. Each line represents a single DNA molecule. Two recombination partners are shown, the double-stranded DNA (gray line) with an *Ac* insertion (black line) and a homologous DNA (stippled line; e.g., a sister chromatid or a homolog chromosome) from which *Ac* was excised. A number of gap repair scenarios and outcomes are shown, i.e., complete gap repair (A), deletion caused by premature abortion of gap repair (B), insertion of unrelated sequences through invasion of a non-*Ac* template (C), and duplication of *Ac* sequences through replication slippage (D). Newly synthesized DNA is shown as broken lines. (A) Following *Ac* excision, the ends of the donor site are degraded and 3′ protruding ends are exposed (stage 1). These ends invade the homologous gray template, and DNA synthesis is initiated (stage 2) in synthesis bubbles which migrate as DNA synthesis proceeds (stage 3). Single-stranded DNA is released from the bubble, stretches of newly synthesized complementary DNAs anneal (stage 4), and completion of gap repair results in the replacement of *Ac* at its original site by a newly synthesized copy (stage 5). (B) If gap repair (stage 1) is prematurely aborted, i.e., before long stretches of complementary DNA are produced (stage 2), and a template reinvasion does not occur, nonhomologous ends need to be joined to seal the DSB. Nonhomologous end joining occurs usually at short repeats (stage 3) shown as small empty boxes (one to a few bases). The outcome of such an event is an internal deletion in *Ac* (stage 4). (C) If gap repair (stage 1) is aborted in one of the two sides of the DSB (stage 2) and a sequence not related to the homologous template is invaded (white bubble; stage 3), end joining of the two noncomplementary ends (stage 4) will result in an insertion of a non-*Ac*-related sequence in *Ac* (stage 5). (D) If gap repair (stage 1) is aborted in one of the two sides of the DSB (stage 2) and the homologous template is reinvaded imprecisely, i.e., DNA synthesis proceeds from an upstream location relative to the abortion site, a duplication of *Ac* sequences will occur (stages 3 and 4). This kind of slippage is expected to occur preferentially at sites of short repeats.

can hardly explain the occurrence of insertions and the complex reshuffling observed in maize elements. SDSA (15), on the other hand, best fulfills the requirements for *Ds* formation as discussed below.

SDSA: an error-prone mechanism for gap repair. The SDSA model was originally demonstrated for bacteriophage T4 (15), and some of its genetic implications have been discussed (15, 43). According to the SDSA model (Fig. 6A), 3′ protruding ends formed upon DSB or transposon excision (Fig. 6A1) invade a homologous template and prime DNA synthesis. DNA synthesis proceeds together with migration of a synthesis "bubble" and release of newly synthesized DNA (Fig. 6A2 and 3). Completion of gap repair can result in reconstitution of the excised element at its original site if the template is identical in sequence to the original allele, e.g., a homologous chromosome or a sister chromatid (Fig. 6A4 and 5). One important difference between the SDSA and the yeast DSB repair pathway is the formation of a migrating bubble(s) rather than of a large and stable heteroduplex (Holliday junction) between the newly synthesized strand and the template. The implications of such unstable interaction between the newly synthesized DNA and the template have been proposed to facilitate bypass of DNA lesions and to explain multiple template switches in bacteriophages (15). Similarly, the unstable association between the newly synthesized DNA and the template can facilitate AGR and explain the complexity of

DNA rearrangements observed in *Ds* elements and in other cases of DNA recombination in plants.

Deletions can be caused by premature release of the newly synthesized single-strand DNA (Fig. 6B2). In that case, two DNA fragments which do not have extensive homology need to be joined for DSB repair. Nonhomologous end joining (also called illegitimate recombination) has been shown in several species to occur preferentially at very short (1- to 6-bp) repeats (see references in reference 41). Therefore, one expects to obtain internal deletions preferentially at sites of short direct repeats (Fig. 6B3 and 4). This is consistent with our finding that short repeats are present at deletion breakpoints (Fig. 3). If only one end was engaged in strand invasion and gap repair was prematurely aborted, an element deleted at one end would have been formed. Examples of such mutations are presented here (Fig. 5, bands 3 and 4), and similar one-sided deletions were previously reported with the *Mutator* element (30). These events support the proposal by Nassif et al. (43) that each side of the DSB might carry a template invasion independently (Fig. 6C). Such one-sided invasion is reminiscent of the one-sided invasion pathway, which has been proposed as a mechanism for DSB repair in mammals (2) and in plants (47), with one important difference; i.e., while in SDSA, the bubble migration releases single-strand DNA, whereas with one-sided invasion, a large and stable heteroduplex is formed.

Insertions can be interpreted as DNA release from the original homologous template and reinvasion into an unrelated template (Fig. 6C2 and 3), as suggested by Nassif et al. (43). Reinvasion probably does not require extensive homology between the invading single-stranded DNA and the new template. This mechanism could explain the capturing of *Ac*-unrelated sequences as found in *Ds* elements (Fig. 2) and as reported here (Fig. 5, bands 7 and 8). Capturing was also identified when studying end-joining of linearized plasmid DNA transformed into plant cells (17a). A similar mechanism was proposed to explain the origin of filler DNA associated with the illegitimate integration of transfected DNA in a mammalian genome (41).

Duplications occurring at direct repeats could also be explained by AGR and inaccurate reinvasion into the same template as described in the legend to Fig. 6D2 to 4. Abortion can occur in one or both sides of the DSB (Fig. 6D2), and if reinvasion occurs upstream of the point where DNA synthesis was interrupted, a duplication of *Ac* sequences will occur (Fig. 6D3 and 4). If reinvasion occurs downstream of the abortion point, a deletion will be generated (data not shown). This could provide an alternative mechanism for deletion formation to that proposed in Fig. 6B.

Mutations associated with AGR, as described in this work, could in principle occur during normal DNA replication. The fact that stable genes show a low rate of deletions, insertions, and duplications suggests that SDSA is more error prone than regular DNA replication. This might result from the involvement of a different set of enzymes for SDSA-mediated DNA replication. In particular, SDSA, unlike normal replication, requires displacement of the newly synthesized DNA, probably by a RecA-like, or UvsX-like SDSA-specific enzyme. In addition, the release of single-strand DNA and the timing during the cell cycle when DSB repair occurs might affect the outcome of the repair process.

In summary, AGR should be considered as a process involving the following steps: (i) gap repair, initiated from one or both ends, with as a template a sequence which shares sufficient homology; (ii) abortion of gap repair, with or without reinvasion into the same or an ectopic template; (iii) nonhomologous end-joining, with the presence of short repeats as a hallmark. AGR via SDSA might provide a general mechanism to explain previously observed data of DNA rearrangements in plants. Filler DNA in spontaneous deletions (62), complex rearrangements flanking T-DNA insertions (17), and deletions, insertions, and inversions observed following repair of ionizing radiation-induced lesions (56) or nonhomologous end-joining of linear DNA (17a) could all be explained by an error-prone repair mechanism such as, or similar to, SDSA. Furthermore, the maize R-r complex locus was proposed to have evolved through transposon-induced rearrangements similar to those described here, i.e., deletions, insertions, and reshuffling of DNA segments (61). An SDSA-like gap repair mechanism could readily explain these rearrangements and thus affect patterns of gene expression during evolution.

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