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Reversed end *Ds* element: a novel tool for chromosome engineering in Arabidopsis

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Abstract The maize *Ac/Ds* transposable element (TE) transposes by a "cut and paste" mechanism. Previous studies in maize showed that when the TE ends are in reversed orientation with respect to each other, alternative transposition reactions can occur resulting in large scale genome rearrangements including deletions and inversions. To test whether similar genome rearrangements can also occur in other plants, we studied the efficacy of such alternative transposition-mediated genome rearrangements in Arabidopsis. Here we present our analysis of 33 independent chromosome rearrangements. Transposition at the reversed ends *Ds* element can cause deletions over 1 Mbp, and inversions up to 2.4 Mbp in size. We identified additional rearrangements including a reciprocal translocation and a putative ring chromosome. Some of the deletions and inversions are germinally transmitted.

Keywords *Ac/Ds* · Alternative transposition · Chromosome engineering · Deletion · Genome rearrangement · Inversion · Transposable element

Introduction

Barbara McClintock's study on the *Ac/Ds* transposable system is marked by two notable observations (reviewed by Jones 2005). One, the *Dissociation* (*Ds*) element at the 'standard' location proximal to the *waxy* locus on the short arm of maize chromosome 9 caused frequent chromosome breakage precisely at the "*Dissociation*" locus. Two, the *Ds* element could transpose from the standard locus to other loci such as *C*, *Wx* and *Bz*, giving rise to new alleles including c^{m-1}, wx^{m-1} and bz^{m-1}, respectively. McClintock described two different 'states' of the *Ds* element: state I *Ds* elements caused chromosome breakage frequently but underwent transposition rarely. In contrast, state II *Ds* elements transposed frequently but seldom caused chromosome breakage.

Molecular analysis of several *Ds* elements revealed that state II elements have simple structures with 11 bp Terminal Inverted Repeats (TIRs) in standard orientation, plus approximately 250 bp subterminal sequence (Kunze and Weil 2002). In contrast, state I elements are complex structures with two or more transposon termini in non-standard orientation (Courage-Tebbe et al. 1983; Weck et al. 1984; Ralston et al. 1989; Weil and Wessler 1993; English et al. 1995; Martinez-Ferez and Dooner 1997; Zhang and Peterson 1999). When multiple *Ds* termini are present in close proximity, it is thought that the 3′ and 5′ ends of different TEs could serve as transposase substrates and undergo aberrant transposition (English et al. 1993; Weil and Wessler 1993; Martinez-Ferez and Dooner 1997). For example, several *shrunken* alleles generated by McClintock show frequent chromosome breakage and carry a double *Ds* element—one *Ds* inserted into another (Courage-Tebbe et al. 1983; Burr and Burr 1982; Chaleff et al. 1981; Doring et al. 1981, Weck et al. 1984). English et al. (1995) showed that double-*Ds* elements can undergo transposition reactions that involve the *Ds* 3′- and 5′-ends on sister chromatids. In addition to chromosome breakage, such complex transposon structures may cause other kinds

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of genome rearrangements including deletions, duplications and inversions. Zhang and Peterson (1999) analyzed a twin sector in the maize ear formed by sister chromatid transposition (SCT) in the *p1-vv9D9A* allele and reported a deletion and corresponding duplication of the flanking DNA. Moreover, independent SCT reactions at this allele generate 'nested deletions'—a series of deletions extending from one of the TIRs to a flanking chromosomal site representing the transposition target site (Zhang and Peterson 2005). In a screen for mutants defective in female gametogenesis, Page et al. (2004) identified large genomic deletions flanking normal *Ds* elements at a frequency of 5–10%; they proposed that these deletions are a product of transposition of a hybrid element involving one TIR of a newly transposed element and another TIR from the donor element.

Complex genome rearrangements can also occur when the transposon ends are in reversed orientation (Fig. 1). In the maize *P1-rr11* allele, a full-length *Ac* element and a fractured *Ac* element (2,039 bp of the 3′ portion of *Ac*) are approximately 13 kb apart and inserted in such a manner that the 5′ end of the *Ac* element and the 3′ end of the *fAc* element are oriented towards each other. Alternative transposition reactions involving the reversed *Ac* ends in the maize *P1-rr11* allele could generate deletions, inversions, and local rearrangements in the genome (Zhang and Peterson 2004).

Here we examined the efficacy of reversed *Ds* ends to cause chromosome rearrangements in Arabidopsis. Our results show that transposition at reversed *Ds* ends can generate both deletions and inversions that can range from 2 kb to several megabases in size. In addition, reversed *Ds* ends transposition can generate rearrangements that contain the two transposon ends fused to each other, which is, apparently, a result of aborted transposition events.

Materials and methods

NIPB3—the reversed *Ds* ends construct

Plasmid pNIPB3 (Fig. 2) was constructed using T-DNA plasmid pCB302 (Xiang et al. 1999) as the backbone vector. The *Ds* 5′- and 3′-ends (255 and 217 bp, respectively), were amplified by PCR from plasmid pSLJ7C3 (Carroll et al. 1995) and cloned in reverse orientation, between the right border and the nosP-Bar-nosT of pCB302. Coupland et al. (1989) had shown that 209 bp of the *Ac* 3′-end and 238 bp of the 5′-end are sufficient to enable *Ds* excision at frequencies comparable to that of longer *Ac* ends, in tobacco. The negative selection marker gene 2′-*iaaH* from plasmid pAJ6 (Panjabi et al. 2006) was cloned between the reversed ends of the *Ds* element. An *Eco*RI–*Bam*HI fragment containing a bacterial origin-of-replication (*ori*) and the *β-lactamase* (*bla*) gene from pBR322 (New England Biolabs, Beverly, MA) was cloned adjacent to the 5′-*Ds* end to facilitate plasmid rescue of the T-DNA insertion locus in the plant genome. A *Kpn*I–*Cla*I fragment of *nosP-nptII* from pDW418 (D. Wright and D. Voytas, unpublished) was cloned between the 3′-*Ds* end and the right border of the T-DNA. The construct was sequenced (at the DNA Sequencing and Synthesis Facility, Iowa State University) and the orientation and order of all cloned fragments was confirmed.

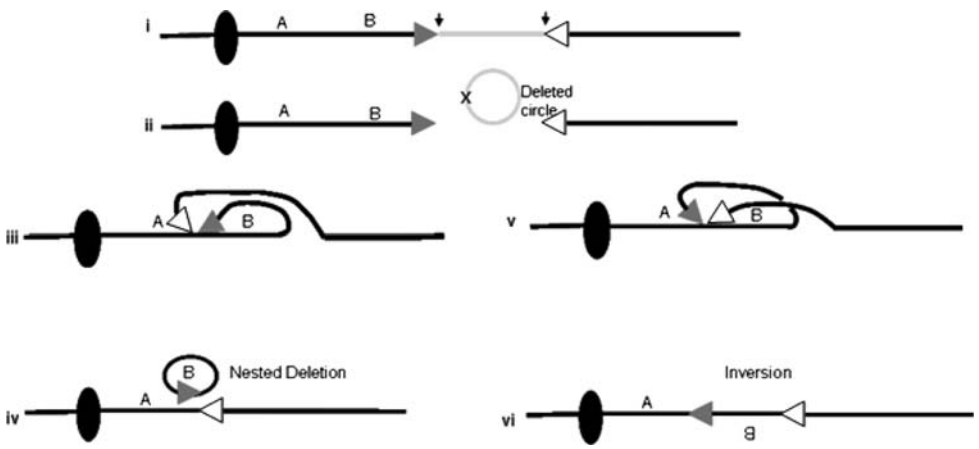

Fig. 1 Transposition at reversed-ends *Ds* element: solid and open triangles indicate the 5′ and 3′ *Ac/Ds* termini, respectively. (i) Transposase makes cuts at the junctions of the transposon termini and the flanking DNA. (ii) The intervening DNA circularizes and is presumably lost. Insertion of the transposon termini at new sites can result in a variety of rearrangements. Insertion of the transposon ends as shown in (iii) results in deletion of the 'B' region (iv). Insertion of the transposon ends as shown in (v) results in inversion of the 'B' region (vi). Other rearrangements are possible, depending on the site and orientation of transposon insertion

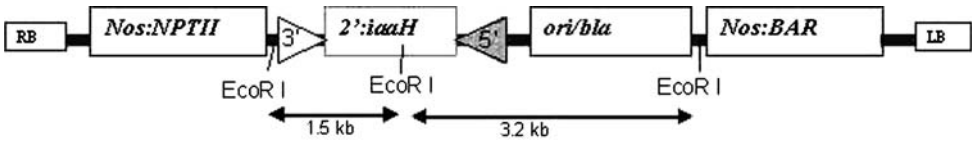

Fig. 2 The pNIPB3 construct: the open and solid triangles represent the *Ds* 3′- and 5′-ends, respectively. The *iaaH* gene is the negative selection marker that confers sensitivity to naphthalene acetamide. The *NPTII* and *BAR* genes confer resistance to kanamycin and bialaphos, respectively. The *ori/bla* segment (bacterial origin of replication and *β-lactamase* gene conferring resistance to ampicillin) is used for plasmid rescue of the T-DNA insertion locus. LB and RB are the left- and right-border T-DNA sequence. Positions of *Eco*RI sites and sizes of expected fragments are indicated

Plant transformation

Arabidopsis (*NoO*) plants carrying *rbcS*:*Ac*-1017 (Honma et al. 1993) were transformed by floral dip method (Clough and Bent 1998) using Agrobacterium (GV3103) carrying the pNIPB3 construct. The T_1 seeds were selected on bialaphos (20 μg/ml) media, and DNA blot hybridization performed to identify single copy insertion lines.

Plant growth conditions

Seeds sown on MS media and appropriate selection were cold-stratified for 2–3 days and moved to a growth room with the following conditions: 24 h light, 25°C. After 10–12 days, selected plants were transplanted to soil and moved to a growth chamber (16 h light, 18°C). Upon flowering, the bolts were supported with a bamboo stake and enclosed in a paper tube to facilitate seed harvest and minimize pollen and seed contamination.

DNA gel blot hybridization

Southern blot analysis was carried out according to Sambrook et al. (1989). Genomic DNA was isolated from 2–8 mg of leaves, and 10 μg of genomic DNA was digested with *Eco*RI. The digested DNA was electrophoresed on 0.8% agarose gels (SeaKem LE, FMC, Rockland, ME) and transferred to nylon membrane (Zeta probe GT, BioRad, Hercules, CA). Southern hybridization was performed using [α-^{32}P]-radioactively labeled probe DNA (RPN1607, GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The membrane was washed under high-stringency washing conditions in a buffer consisting of 0.1× SSC and 0.5% SDS at 65°C and exposed to X-ray film.

Plasmid rescue

For plasmid rescue, genomic DNA was digested with *Eco*RI or *Xba*I restriction enzyme in a volume of 50 μl in an overnight reaction. The digested DNA was purified using QIAprep spin column (Qiagen, Valencia, CA), and eluted in 180 μl of water. The DNA was ligated with T4 DNA ligase (Promega, Madison, WI) in a volume of 200 μl at 16°C overnight. Then the DNA was ethanol precipitated and resuspended in 30 μl of water. Aliquots (4–5 μl) of the ligation mixture were electroporated into 20 μl of *Escherichia coli* DH10B (Cat#18290-015, Invitrogen, Carlsbad, CA, USA). Transformed *E. coli* was cultured on LB + Ampicillin agar medium overnight at 37°C, and colonies were inoculated into LB + Ampicillin liquid medium. Plasmid preparation was performed using QIAprep Spin miniprep kit (cat.# 27104, Qiagen, Valencia, CA). Plasmids containing genomic DNA were sequenced using the Ac5-2 primer 5′-GTATATCCCGTTTCCGTTCCGTT-3′, which is complementary to the 5′*Ac* subterminal sequence. The sequence of the site of insertion obtained from the rescued plasmid was compared to the Arabidopsis genome sequence available at Plant Genomes Central (http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html).

PCR

Taq DNA polymerase (Promega, Madison, WI) and dNTPs (Eppendorf, Westbury, NY) were used in PCR with 100 ng of genomic DNA as template. Annealing temperatures of 55–64°C were used, depending upon primer sequences. A typical reaction consisted of initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation for 35 s, annealing for 30–40 s, extension at 72°C for 30–90 s, followed by a final 10 min extension at 72°C.

Results

Generation of Arabidopsis lines containing a single copy of the reversed ends *Ds* construct

The T1 generation plants that were resistant to bialaphos selection were analyzed by Southern blot hybridization to identify those carrying single-copy insertions of construct NIPB3 (Fig. 2). Three single copy insertion lines—C15, C17 and C42—were identified and plasmid rescue was used to determine the sites of T-DNA insertion in these

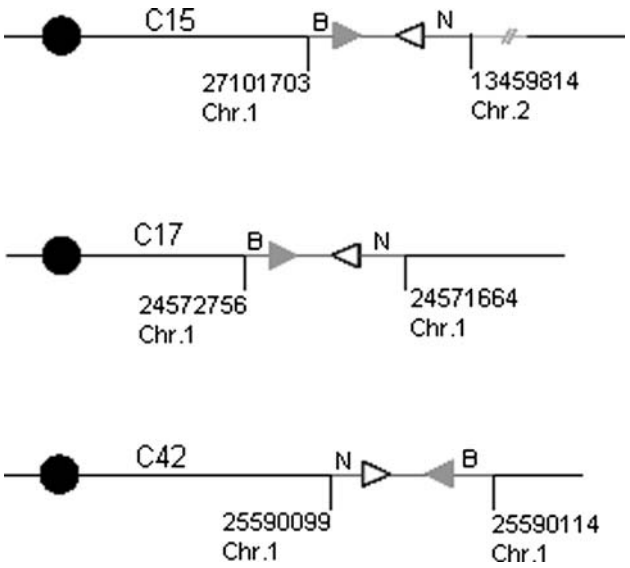

Fig. 3 pNIPB3 insertion sites in three transgenic lines—C15, C17 and C42: the solid and open triangles indicate the *Ds* 5′- and 3′-ends, respectively. Numbers refer to the positions of insertion of left and right borders of T-DNA, as determined by plasmid rescue. 'B' indicates the position of the *bialaphos resistance* gene and 'N' indicates the position of the *neomycin phosphotransferase* gene. The RB of the T-DNA in C15 is flanked by a duplication of part of chromosome 2. Chromosome and T-DNA lengths are not drawn to scale

plants (Fig. 3). It has been reported previously that the T-DNA insertion process sometimes introduces genome rearrangements flanking the T-DNA borders (Nacry et al. 1998; Laufs et al. 1999; Tax and Vernon 2001). In the C15 line, the right border of the T-DNA is associated with a rearrangement in which a large segment of chromosome 2 is copied onto chromosome 1. Limited molecular analysis suggests that the segment of chromosome 2 sequence located adjacent to the T-DNA is about 3.5 Mb; however, the precise junction of chromosome 1–chromosome 2 could not be determined. Southern hybridization and PCR results show that the segment inserted into chromosome 1 is also present at its normal location on chromosome 2 (data not shown).

Screening for transposon-induced rearrangements

The T_2 seeds were sown on media containing NAM (5 μM) in combination with bialaphos (20 μg/ml) or kanamycin (50 μg/ml). Most selections were done using bialaphos plus NAM in order to identify lines that retained the *ori/bla* sequence for plasmid rescue. The *iaaH* gene encodes indole acetamide hydroxylase which converts naphthalene acetamide (NAM) to naphthalene acetic acid (NAA), an auxin. When plants carrying the *iaaH* gene are grown on media containing NAM, the NAA produced by indole acetamide hydroxylase will induce a hairy or knotted root phenotype (Britton et al. 2008). Resistance to the effects of NAM can facilitate the identification of plants in which transposition of the reversed *Ds* ends has resulted in loss of the *iaaH* gene. The NAM selection served only to enrich for plants with rearrangements, and we relied on DNA blot hybridization to confirm loss of the *iaaH* gene from apparently 'NAM resistant' plants. Figure 4a–c show representative autoradiograms hybridized with probes for the *ori/bla*, *nptII* and *iaaH* sequences, respectively. Based on the plasmid map, the unrearranged transgene should give an *ori/bla*-hybridizing *Eco*RI fragment of 3.2 kb. In Fig. 4a (membrane probed with *ori/bla* fragment) most of the plants tested show a clear alteration in the size of the *ori/bla*-hybridizing fragment, indicating transposition at the reversed-ends *Ds* and concomitant loss of the *iaaH* gene in that sample. Lanes with multiple bands may indicate plants containing multiple independent rearrangements within the tissue sampled. The *nptII* probe hybridized with a 2.1 kb *Eco*RI fragment in the unrearranged transgene. Figure 4b (membrane probed with *nptII* probe) shows that the *nptII* sequence has been deleted in some plants (e.g. lanes 17F, 17L and 17M). Finally, the *iaaH* probe should hybridize to 1.5 and 3.2 kb *Eco*RI fragments (Fig. 2); as shown in Fig. 4c (membrane hybridized with *iaaH* probe) the absence of these fragments in most plants indicates rearrangement at the reversed-ends *Ds* and subsequent loss of the *iaaH* gene. In several cases (e.g. 17G, 17J, 17P, 17S), the *iaaH* fragment is absent whereas both *ori/bla* and *nptII* fragments are present; some of these cases were found to contain rearrangements such as inversions, translocations or fusion of the two *Ds* ends (see following section).

Deletion, inversion, translocation and local rearrangement are generated by reversed ends transposition

Samples which gave Southern blot results suggesting the presence of rearrangements at the reversed-ends *Ds* element were further characterized by plasmid rescue and sequencing of the junctions of the *Ds* termini and associated DNA. In most cases the type of genome rearrangement could be inferred based on the site and orientation of insertion of the *Ds* 5′ TIR. The types of rearrangements detected in the progeny of NIPB3-carrying plants are shown schematically in Fig. 5, and are listed in Table 1.

Deletions

Several deletions (Fig. 5, ii), including both somatic and germinal events, were identified. The deletions obtained from a single transgenic line comprise a nested series; for example, the C17 line produced deletions of 338 bp, 742 bp, 3.9 kb, 8.8 kb, 11.6 kb, 15.4 kb, 17.5 kb, 23 kb,

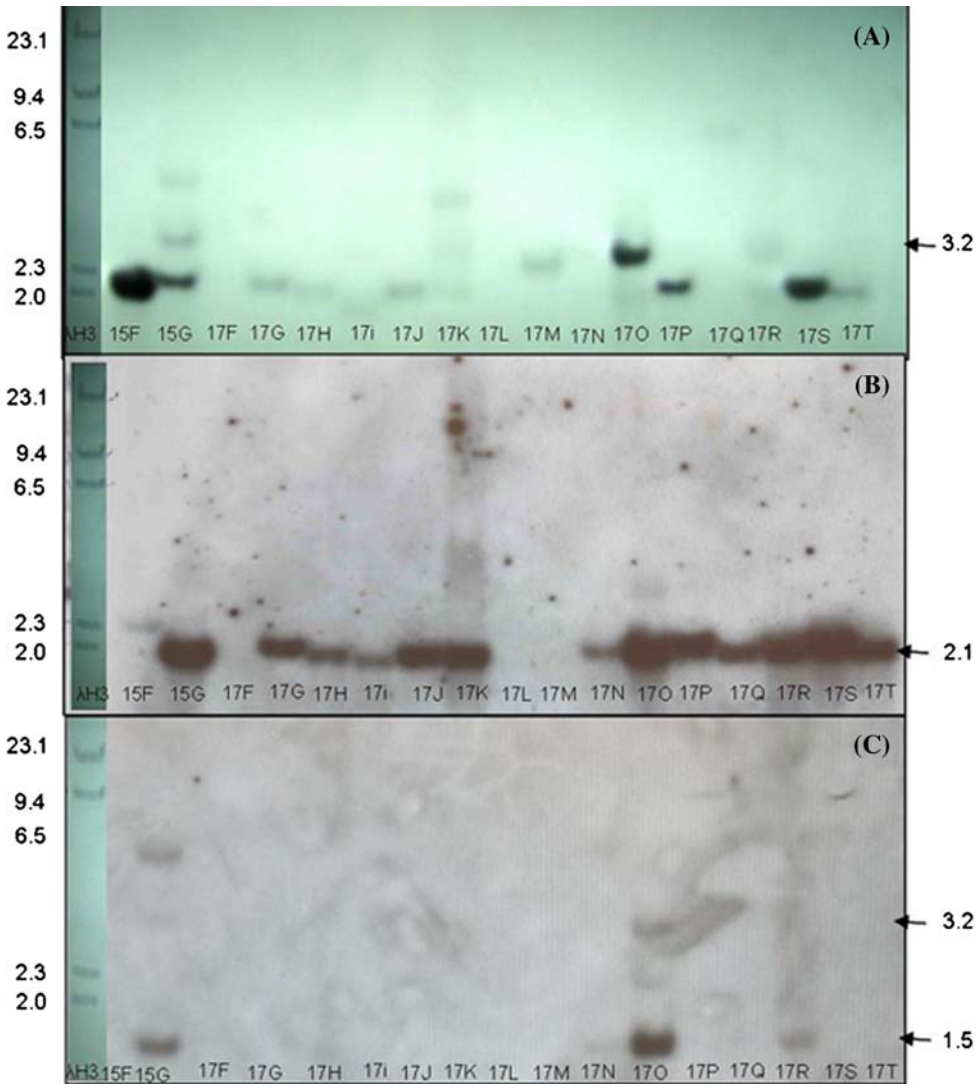

Fig. 4 Southern hybridization to identify rearrangements at the reversed-ends *Ds*: genomic DNA from T2 plants was digested with *Eco*RI; **a**, **b** and **c** are autoradiograms of the same blot probed with *ori/bla*, *nptII* and *iaaH* DNA fragments, respectively. λ-*Hin*DIII DNA ladder was used as a size marker on the gel. Plants carrying rearrangement(s) are identified by alterations in the sizes of bands hybridizing with *ori/bla* (**a**) and by absence of the 1.5 kb band hybridizing with *iaaH* (**c**). The multiple bands observed when probed with *ori/bla* indicate the presence of multiple independent somatic rearrangements

61 kb, 236 kb, 405 kb, 435 kb, and 1.13 Mb (Fig. 6). The lines C17I and C17K carry 23 and 8.8 kb germinal deletions of genomic DNA flanking the pNIPB3 insertion, respectively. To confirm the presence of deletions, a PCR analysis for the deleted region in the progeny of these lines was performed. In both of these lines, the deleted sequences were not amplified, while the regions distal to the deletions are amplified (Fig. 7).

Inversions

In 10 cases, the sequences of the rescued plasmids indicated the presence of inversions of the flanking DNA (Fig. 5, iii). The inversions range in size from 17.5 kb to 2.4 Mb. To test for the presence of inversions, we designed PCR primers complementary to genomic sequences near the predicted site of insertion of the *Ds* 3′ end (based on the site of insertion of the *Ds* 5′ end obtained from plasmid rescue); these were used in PCR together with a primer annealing to the *Ds* 3′ terminus. PCR products were obtained for nine of the 10 putative inversions; following PCR, sequence analysis of the products confirmed that all nine PCR-positive cases contained inversions. Among the nine inversions for which we obtained both the 5′- and 3′-TIR insertion sequences, three cases had 8 bp Target Site Duplications (TSD) flanking the 5′ and 3′ TIR, two cases had only 1 bp duplications, and the other four cases lacked any TSD, i.e. were flush insertions (Table 2).

Fused-ends

Using a PCR-based screen, we found that approximately 50% of NAM-resistant plants carry a rearrangement in which the *Ds* 5′ and 3′ ends joined together forming 'fused-ends' (Fig. 5, iv), with concomitant deletion of the *iaaH* gene.

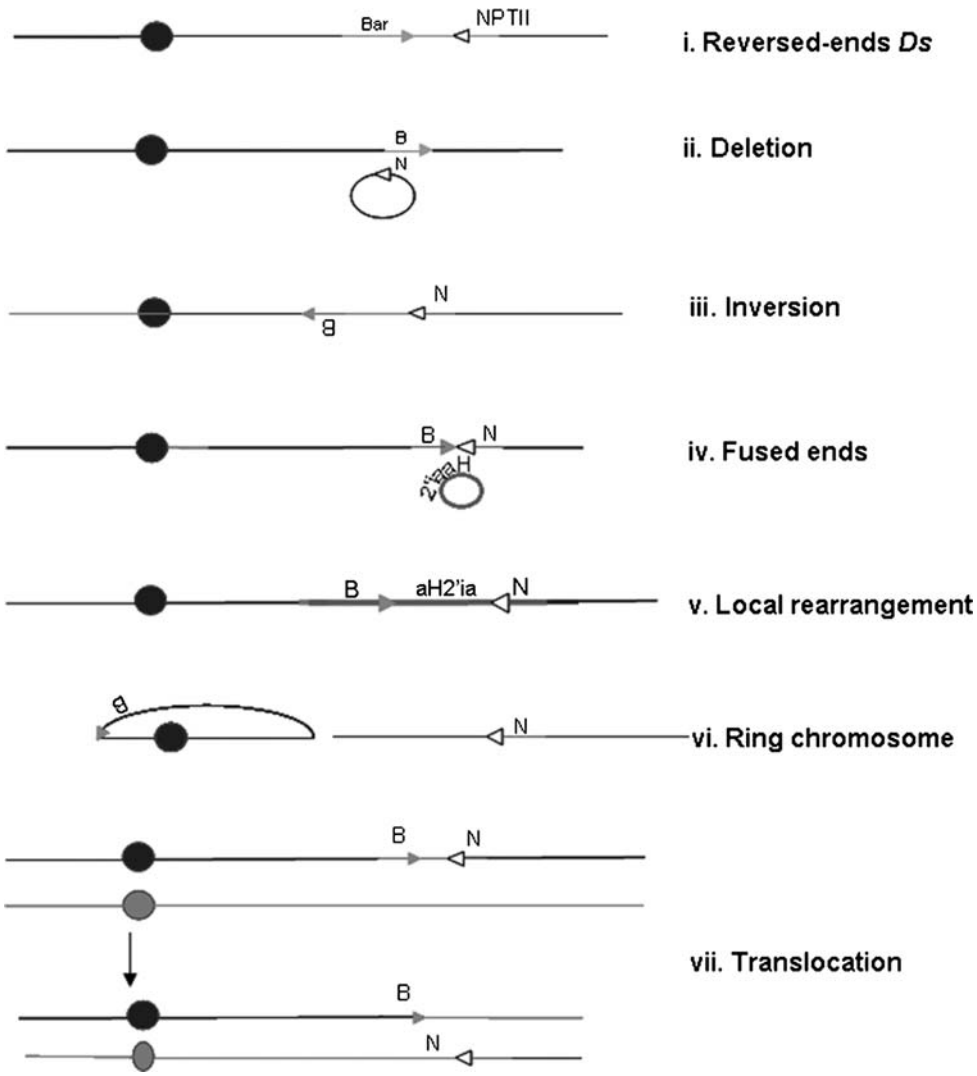

Fig. 5 Different types of genome rearrangements observed at the reversed-ends *Ds* element: i. The NIPB3 construct inserted in the Arabidopsis genome. ii. Deletion of the NPTII gene (N) and a distal segment of the chromosome. iii. Inversion of the BAR gene (B) and a proximal segment of the chromosome. iv. Fusion of the reversed *Ds* ends and loss of the *iaaH* gene. v. Insertion of the *Ds* ends into the sequence that lies between them, resulting in a rearrangement of the *iaaH* gene. vi. Insertion of the *Ds* ends into a site on the opposite chromosome arm, generating a ring chromosome. A complementary acentric chromosome is also predicted, but was not detected in our experiment. vii. Insertion of the *Ds* ends into a non-homologous chromosome, resulting in a reciprocal translocation

Other rearrangements

The other types of rearrangements observed at the reversed-ends *Ds* element included one local rearrangement (Fig. 5, v), in which the transposon ends inserted into the *iaaH* gene; one putative ring chromosome (Fig. 5, vi) formed by pericentric insertion of the *Ds* 5′ end; and a chromosome-1–chromosome-2 translocation (Fig. 5, vii). The translocation and the ring chromosome were detected only in somatic tissues, whereas the local rearrangement was germinally transmitted.

Ac transposase can use *Ds* 5′ and 3′ ends on sister-chromatids to effect transposition

In the line C17, the T-DNA is inserted in chromosome 1 in such a way that the *ori/bla* sequence and the *bar* gene are proximal (i.e. closer to the centromere) with respect to the *Ds* 5′ terminus of the construct. In two of the rearrangement events derived from C17 (#13 and #14; Table 1), the *Ds* 5′ end is inserted proximal to the T-DNA insertion site, and the sequence reads towards the T-DNA. Theoretically, this result could be explained by 'same chromatid transposition': i.e., the transposase excised the *Ds* 5′ and 3′ termini on the same chromatid, and inserted the 5′end at a proximal target site. This would generate an acentric circular DNA molecule (see iv in Fig. 1) which would most likely be lost. The likelihood of detecting such a transient circular molecule by plasmid rescue seems small; therefore, we considered an alternative 'sister chromatid transposition' model in which *Ac* transposase acts on *Ds* 5′and 3′ termini on sister chromatids, and the excised *Ds* 5′end inserts into the opposite chromatid at a site proximal to the T-DNA locus (Fig. 8). Such an insertion would result in two distinct sister chromatids: one carries a duplication of the DNA proximal to the T-DNA, and the other contains a corresponding deletion (Fig. 8). This result suggests that, in transposition reactions at a

Table 1 Various rearrangements generated by the reversed-ends *Ds* construct

	Parent line and plant ID	5′ TIR insertion site	Nature of rearrangement	Size of the rearrangement	# Of genes in the region
1	C15 Ecl1	13459814 + chr.2	Deletion (germinal)	157 bp	0
2	C15 RER137	13459948 + chr.2	Deletion (germinal)	290 bp	0
3	C17 PH89cl2	24572418 + chr.1	Deletion (germinal)	338 bp	0
4	C17 PRJcl1	24573498 + chr.1	Deletion (germinal)	742 bp	0
5	C17 NK13cl4	24576671 + chr.	Deletion (germinal)	3.9 kb	1
6	C17 PRKcl1	24581566 + chr.1	Deletion (germinal)	8.8 kb	4
7	C17 Rcl2	24584359 + chr.1	Deletion	11.6 kb	4
8	C42	25575593 − chr.1	Deletion	14.5 kb	5
9	C17 NK6cl6	24588238 + chr.1	Deletion	15.5 kb	6
10	C17 K	24593885 + chr.1	Deletion	17.5 kb	8
11	C17 Icl2 and I4cl1	24595755 + chr.1	Deletion (germinal)	23 kb	8
12	C17 PROh1cl1	24634219 + chr.1	Deletion	61.4 kb	18
13	C17 Qcl3	24458978 + chr.1	Deletion	113 kb	27
14	C17 Icl4	24456006 + chr.1	Deletion	116 kb	28
15	C17 Rcl1	24809216 + chr.1	Deletion	236 kb	54
16	C17 RER237	24977350 + chr.1	Deletion	404.6 kb	105
17	C17 RER277cl1	25008039 + chr.1 or 24976576 + chr.1	Deletion	435 kb or 404 kb	111
18	C17	23441421 chr.1	Deletion	1.13 Mb	305
19	C15 PH65	24581185 + chr.1	Deletion	2.5 Mb	637
20	C17 PH89cl3 and PH95cl2	24590304 − chr.1	Inversion (germinal)	17.5 kb	8
21	C17 PRIi3cl1	24511173 − chr.1	Inversion	61.5 kb	12
22	C17 Qcl2	24720311 − chr.1	Inversion	147.5 kb	36
23	C15 PH65colG	24777089 − chr.1	Inversion	2.3 Mb	47
24	C17 Qcl6	24806015 − chr.1	Inversion (germinal)	233 kb	54

Table 1 continued

	Parent line and plant ID	5′ TIR insertion site	Nature of rearrangement	Size of the rearrangement	# Of genes in the region
25	C17 PH95col3	24127891 − chr.1	Inversion	444.8 kb	114
26	C17 PROh6cl1	23567105 − chr.1	Inversion	1 Mb	270
27	C15 RER43cl3	14459689 − chr.2	Inversion or reciprocal translocation	999 kb	289
28	C15Q RER108cl4	24806015 − chr.1	Inversion	2.3 Mb	586
29	C15 RER108cl3	24669513 − chr.1	Inversion	2.4 Mb	616
30	C15 RER151	Insertion into the *iaaH* gene	'Local rearrangement'	1.4 kb	0
31	Several	Fused ends	Fused ends	Fused ends	0
32	C17	540311 + chr.1	Ring chromosome	24 Mb	–
33	C17 Ohcl2	16122026 chr.2	Reciprocal translocation	chr1–chr.2 chimera	–

The number refers to the position on the chromosome. + or − refers to the + and − strand of chromosome 1, respectively, as inferred from Arabidopsis genome sequence database

reversed ends *Ds* element, the 5′- and 3′-ends on sister chromatids can be used as transposition substrates. This finding expands the types of genome rearrangements that may be generated by alternative transposition reactions.

Discussion

Previous studies in maize have shown that the 5′ and 3′ ends of *Ac*/*Ds* elements in reversed orientation with respect to each other can undergo alternative transposition reactions, resulting in a variety of gross chromosomal rearrangements (Zhang and Peterson 2004; Zhang et al. in preparation). To evaluate the potential of using alternative *Ac*/*Ds* transposition to create genome rearrangements in Arabidopsis, we generated transgenic Arabidopsis lines carrying *Ac*/*Ds* termini in reversed orientation and screened the progeny for alterations in the transgene and flanking DNA. Our results clearly indicate that reversed *Ac*/*Ds* ends can efficiently generate deletions and inversions, as well as chromosomal translocations and ring chromosomes.

Deletions and inversions are common

Of the 33 rearrangements characterized in this study, 19 are deletions and 10 are inversions. We observed deletions up to 1 Mb and inversions up to 2.4 Mb in size. In standard *Ac*/*Ds* element transposition, the frequency of *Ac*/*Ds* element insertion is highest at sites linked to the original donor site, and decreases with increasing distances from the donor site (Bancroft and Dean 1993; Zhang et al. 2003). Similarly, in our analysis of constructs containing reversed-ends *Ac*/*Ds* elements, we find that smaller rearrangements are more frequent than larger rearrangements (Fig. 6).

One important application for genome rearrangements at reversed *Ac*/*Ds* transposon ends is for the generation of deletions. Deletions are highly useful for gene mapping, generation of null mutants, analysis of dosage effects, and for dissection of complex loci. A series of overlapping deletions can be used to remove one or several copies of tandemly repeated genes. Additionally, deletion heterozygotes can be used in mutant screens to identify non-lethal recessive mutations in a single generation. Despite their potential usefulness, there are relatively few well-characterized deletions available in plant genetic stock centers. Ionizing radiation is very effective in generating deletions, but the stochastic nature of irradiation can result in mutations at multiple loci that can complicate the recovery and analysis of deletion mutants. Therefore, tools that can generate deletions targeted to specific chromosomal regions are preferred. The cre/*lox* recombination system has been successfully used in mice (Ramirez-Solis et al. 1995; Li et al. 1996; Wagner et al. 1997; Zeh et al. 1998), tobacco (Dale and Ow 1990; Bayley et al. 1992; Russell et al. 1992; Medberry et al. 1995) and Arabidopsis (Russell et al. 1992; Osborne et al. 1995) to generate locus-specific genome rearrangements. A combination of the cre/*lox* recombination system and *Ds* transposable element (*Ds*-*lox*/cre) has been used in chromosome engineering

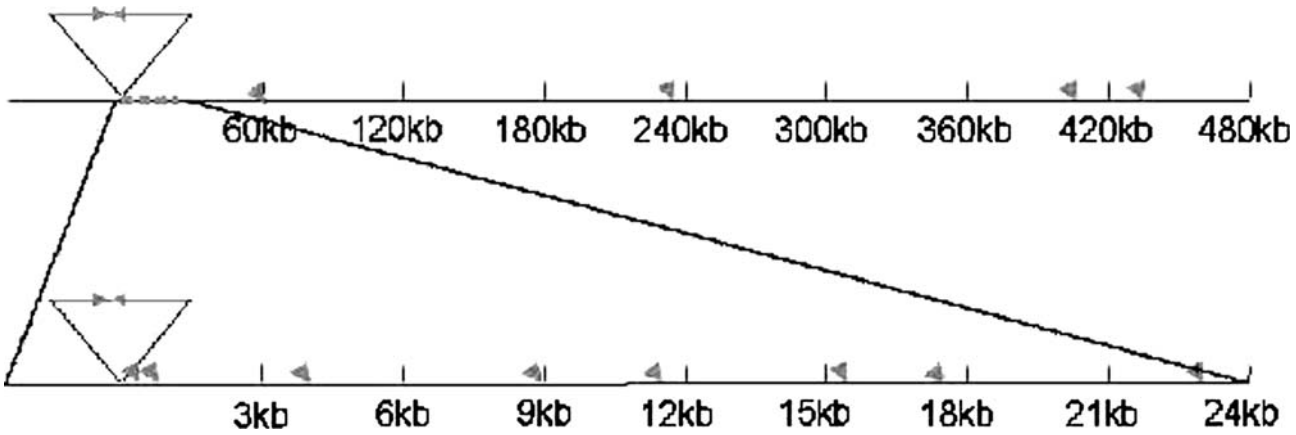

Fig. 6 Deletion series in the C17 line: Rearrangement at the reversed-ends *Ds* can generate a nested deletion series. Here, deletions distal to the C17 insertion site are shown. The upper scale shows all deletion end-points; in the lower scale, the 24 kb region flanking the T-DNA insertion is enlarged. The solid arrow-heads represent the sites of insertion of the *Ds* 5′ end. The sizes of deletions depicted here are as follows: 338 bp, 742 bp, 3.9 kb, 8.8 kb, 11.6 kb, 15.4 kb, 17.5 kb, 23 kb, 61 kb, 113 kb, 116 kb, 236 kb, 405 kb, 435 kb, and 1.13 Mb. These numbers indicate the bases deleted from the genomic sequence. Each deletion includes an additional 2 kb of T-DNA sequences

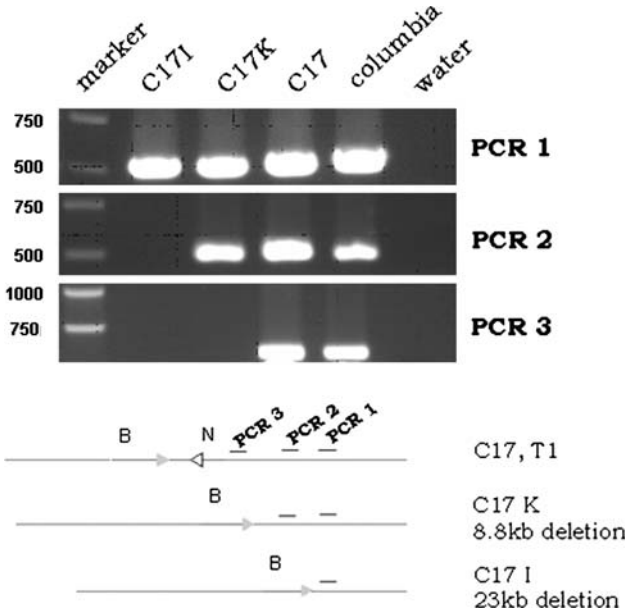

Fig. 7 PCR analysis confirms the presence of deletions in lines C17I and C17K: Transposition at the reversed ends *Ds* element resulted in deletion of 8.8 and 23 kb in C17K and C17I, respectively. Therefore, C17I amplifies only region PCR1, but not regions PCR2 and PCR3; C17K amplifies regions PCR1 and PCR2, but not region PCR3. The progenitor plant C17 and the columbia positive control amplify all three regions tested. Lane marked "water" is negative control of PCR sample lacking DNA template. PCR primers: PCR1F: 5′-CGGAAA GTGCTGAGTCTTC-3′; PCR1R: 5′-GCAATGCACTTCACAGAA AG-3′; PCR2F: 5′-GATCCATTCTAATCAATAGATG-3′; PCR2R: 5′-GTACCATCTAGCAACCAAAG-3′; PCR3F: 5′-AATGGCAC CACAGTGACTG-3′; PCR3R: 5′-CAGGCTCACAGCATCAAGT-3′

(Osborne et al. 1995). In this approach, transgenic plants are generated that carry a construct with two *lox* sites, one within a *Ds* element and a second outside the *Ds* element. *Ac*-encoded transposase induces transposition of the *Ds-lox* element to another site, most often nearby. In subsequent generations, the expression of CRE recombinase will induce recombination between the 2 *lox* sites resulting in either deletion or inversion of the intervening DNA segment. A collection of over ten thousand Arabidopsis T-DNA insertion lines carrying a *Ds-lox* element has been generated by Woody et al. (2007).

The reversed *Ds* ends transposition system described here has certain distinct advantages over the *Ds-lox*/Cre system. First, to generate a series of sequential deletions using the *Ds-lox* system, several lines of *Ds* transposed lines have to be generated, and each such line can give rise to only one deletion or inversion depending on the relative orientation of the *lox* sites. In contrast, the reversed *Ds* ends system can generate a series of deletions and inversions from a single line, as clearly demonstrated by our experiments. Second, in Arabidopsis, many of the germinal excision events occur late in plant development (Bancroft and Dean 1993), likely after the determination of cell lines leading to the formation of the pollen and ova. As a result a majority of the germinal events observed are independent events. Therefore, it is possible to generate a series of nested deletions or inversions even from a single plant. Third, the *Ds-lox* system requires several steps of crossing-in/crossing-out or inducing/silencing the expression of the *Ac* transposase and cre recombinase genes. Whereas, the reversed end *Ds* system is a single step process, and once a transgene insertion locus is identified, one can screen for rearrangements within a single generation. Our study confirms the efficacy of this system as a functional genomics tool for chromosome manipulation in planta.

Inversions generated by transposition at the reversed ends *Ds* element could serve as genetic balancers for the

Table 2 Reversed ends *Ds* element generated inversions: inversion breakpoint locations, and variation in target site duplication sequence lengths

Parent line and plant ID	5′TIR insertion site (p. rescue)	3′TIR insertion site (PCR)	Target site duplication
C17 Qcl6 and Ii4cl1	24806015 − chr.1	24806016 + chr.1	Flush insertion
C17 PH89cl3 and PH95cl2	24590304 − chr.1	24590305 + chr.1	Flush insertion
C17 PH65colG	24777089 − chr.1	24777090 + chr.1	Flush insertion
C15Q RER108cl4	24806015 − chr.1	24806016 + chr.1	Flush insertion
C15Q RER43-C13	14459689 − chr.2	14459689 + chr.2	1 bp
C17 PH95col3	24127891 − chr.1	24127891 + chr.1	1 bp
C17 PRIi3cl1	24511173 − chr.1	24511166 + chr.1	8 bp
C17 PROh6cl1	23567105 − chr.1	23567098 + chr.1	8 bp
C15Q RER108cl3	24669513 − chr.1	24669506 + chr.1	8 bp

The numbers in column 2 and 3 refer to the position of TIR insertion on the chromosome. + or − refers to the + and − strand of chromosome 1, respectively, as inferred from Arabidopsis genome sequence database

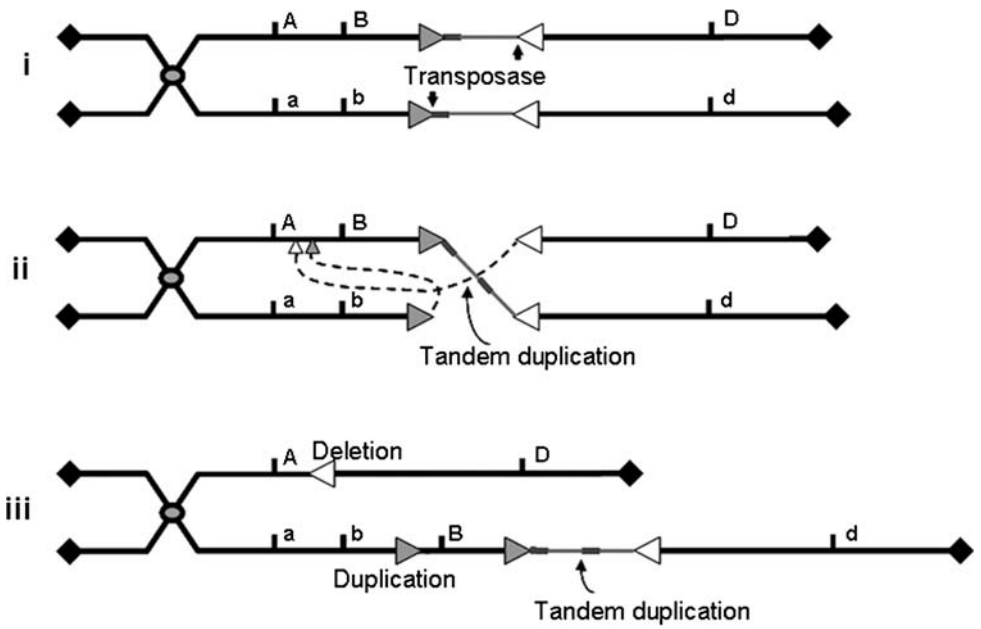

Fig. 8 Sister chromatid transposition at reversed-ends *Ds*: i. Chromosome containing reversed-ends *Ds* after DNA replication. Markers A, B, D are in upper- and lower-case font on the upper and lower chromatids, respectively, in order to identify their origin in subsequent rearrangements. Transposase makes cuts at the *Ds* 5′ and 3′ ends of the lower and upper chromatids, respectively. ii. Intermediate structure of reversed-ends *Ds* transposition. Upon transposase mediated excision of the *Ds* 5′ and 3′ ends, the 3′of the *2′iaaH* gene in the upper chromatid is joined with the 5′ end of the *2′iaaH* gene from the lower chromatid to form a tandem duplication. The dotted lines indicate the new site of insertion of the excised transposon ends. iii. Resolution of the two chromatids following transposition

inverted region. Meiotic recombination between a balancer chromosome and its homologue within the inverted segment results in nonviable gametes; this blocks the recovery of genetic recombinants. Balancer chromosomes are useful for maintaining heterozygous stocks of mutants of genes in the inverted region that are homozygous lethal. Ever since their use by Muller (1918), balancer chromosomes have found extensive application in Drosophila and mice (Hentges and Justice 2004).

Mechanistic variation in *Ds* insertion in Arabidopsis

The generally accepted model for transposon insertion postulates that the *Ac*/*Ds* transposase makes a staggered cut at the target site, which is filled-in to form a Target Site Duplication (TSD) flanking the transposon (Kunze and Weil 2002). The size of the TSD is characteristic of each transposon family: for *Ac*/*Ds* elements, insertion generates an 8-bp TSD in maize (Müller-Neumann et al. 1984; Pohlman et al. 1984) and tobacco (Hehl and Baker 1990). However, it is not known what features of the transposase determine TSD size, nor whether host factors are involved in the insertion mechanism. Recent high-throughput studies on *Ds* transpositions in Arabidopsis by Kuromori et al. (2004) and Ito et al. (2005) indicate that only about 40% of *Ds* insertions are flanked by an 8-bp TSD. However, these two studies did not report the sequences flanking *Ds* in the remaining 60% of insertions. Among the nine inversions generated by transposition at the reversed *Ds* ends in our study, three cases have an 8-bp TSD, two have one base pair duplications, and four are flush insertions. Taken together, these results suggest that the mechanism and/or host factors involved in *Ac*/*Ds* transposition in Arabidopsis generates a higher proportion of insertions without the 8-bp TSD that is typically found in maize. One possible explanation for the flush and single nucleotide duplications at the insertion sites is that these were repaired by a non-homologous end joining (NHEJ) reaction between the excised transposon ends and the target site. However, NHEJ often involves deletion of a few nucleotides from the broken ends. The absence of such deletions at the target site in the inversions does not favor this model.

Another interesting observation is the high frequency of "fused ends" products, in which the *Ds* 5′ and 3′ ends joined together with concomitant deletion of the *iaaH* gene (Fig. 5, iv). These "fused ends" products are reminiscent of the fused terminal junctions of circular *Ac* elements identified in transgenic tobacco and proposed to represent products of aborted transposition (Gorbunova and Levy 1997). A detailed analysis of the "fused-ends" products and their origin during *Ac*/*Ds* transposition will be presented elsewhere (Krishnaswamy and Peterson, in preparation).

Both sister-chromatid rearrangement and same-chromatid rearrangement are observed

Genetic studies in maize have shown that *Ac* elements commonly transpose during or immediately after DNA replication, and that one of the two replicated elements is more competent to transpose (Greenblatt and Brink 1962; Greenblatt 1984; Chen et al. 1987, 1992). This phenomenon, termed chromatid selectivity, is thought to be controlled by the methylation state of the transposon ends. According to the model proposed by Wang et al. (1996), following DNA replication, the 5′-ends on both sister chromatids are equally transposition competent; whereas, only one of the two 3′-ends are transposition competent due to the effects of the strand-specific methylation pattern on the binding affinity of *Ac* transposase. English et al. (1995) observed that transposition at a "half double *Ds*" element (a 5′-end and a 3′-end present as direct repeats) predominantly used the transposon ends on sister chromatids as substrate. In contrast, when the *Ds* ends are in reversed orientation, the methylation pattern of the DNA strands should be similar to the methylation pattern on a standard transposon; therefore it is expected that transposition at reversed ends would involve the ends on the same chromatid. This prediction was supported by analysis of the products of transposition events involving *Ac*/*Ds* termini present in reversed orientation at the maize *p1* locus (Zhang and Peterson 2004).

In contrast, our analysis of rearrangements in the C17 line suggests that transposition at reversed-ends *Ds* in Arabidopsis could involve termini on sister chromatids (Fig. 8). However, the same line was also shown to generate both inversions and local rearrangements, which can only arise from transposition reactions involving *Ds* termini on the same chromatid. Together, these results indicate that both same- and sister-chromatid transposition reactions can occur at a reversed-ends *Ds* element in Arabidopsis. Because the methylation patterns of the termini in reversed-ends and standard *Ds* elements are expected to be similar, we suggest that sister chromatid transposition may also be possible at standard *Ds* elements in Arabidopsis. To our knowledge, no such transposition has been reported, although it might have been mischaracterized as standard transposition associated with complex genome rearrangements of flanking DNA.

Both somatic and germinal rearrangements are observed

Among the 33 rearrangements characterized here, only 10 cases were shown to be heritable based on their detection in sibling or progeny plants. There are several possible

reasons that the other rearrangements were not detected in multiple progeny. First, many of the rearrangements may have occurred in the somatic tissue from which genomic DNA was isolated, and these rearrangements were not included in the cells that gave rise to gametophytes. Second, some rearrangements may have occurred late during the development of the gametophyte and therefore contribute to a small fraction of the seed pool. Because our screen did not include all seeds from a plant, we may not have detected rearrangements that were infrequent among the sibling plants. Third, rearrangements that result in gametophyte lethality would not be transmitted.

In Arabidopsis, the frequency of germinal *Ds* transposition events depends on several factors including, but not limited to, the promoter driving the expression of the *Ac* transposase, and the positions of the *Ac* gene and the *Ds* elements in the genome (Swinburne et al. 1992). Honma et al. (1993) compared the frequency of germinal transposition of Ds^{ALS} when expression of the *Ac* transposase (Ac^{st}) is driven by the 35S, *rbcS*, or *CHS* promoters. *CHS*-Ac^{st} resulted in relatively low germinal excision frequencies (0.4–0.9%); whereas, 35S-Ac^{st} and *rbcS*-Ac^{st} lines exhibited maximal germinal excision frequencies of 64 and 67%, respectively. However, high variation in germinal transposition frequency was observed among different lines and among individual F2 plants derived from the same cross. In our experiments we used the *rbcS-Ac* C-1017 line generated by Honma et al. (1993). The germinal frequency of *Ds* excision in this line was not reported, but it appears to be less than the 28% germinal *Ds* excision frequency reported by Honma et al. for the *rbcS*-Ac^{st} B1056 line. Therefore, the lower frequency of germinal rearrangements observed in our experiments may not be an inherent limitation of reversed ends *Ac*/*Ds* transposition. Possibly, introducing the NIPB3 construct into an *Ac* transposase-expressing line that exhibits a high frequency of germinal *Ds* excision would enable more frequent generation of germinal rearrangements.

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