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High rates of *Ac/Ds* germinal transposition in *Arabidopsis* suitable for gene isolation by insertional mutagenesis

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ABSTRACT **Overexpression of the Activator (*Ac*) transposase gene in *Arabidopsis thaliana* resulted in a minimal germinal transposition frequency of 27% in which independent Dissociation (*Ds*) transposition events were observed. Molecular analysis of 45 F_1 generation *Ac/Ds* plants indicated that high rates of somatic excision had occurred, and independent germinal insertions were identified in F_2 generation progeny plants. A tandem cauliflower mosaic virus (CaMV) promoter fused to two different *Ac* coding sequences significantly increased the rate of *Ds* transposition. The CaMV–Ac fusions activated single and multiple copies of two different *Ds* elements, *DsDHFR* and *Ds35S-1*, and reciprocal crosses resulted in similar transposition frequencies. The improved rate of independent germinal transposition observed makes *Arabidopsis* an ideal system for insertional mutagenesis.**

Arabidopsis thaliana is an excellent model plant for classical and molecular genetic studies. Current efforts toward characterizing the genome of *Arabidopsis* include restriction fragment length polymorphism analysis (1, 2), production and physical mapping of yeast artificial chromosome libraries (3–5), and *Agrobacterium tumefaciens* T-DNA insertional mutagenesis (6–8). An alternative means of isolating genes, which has been effective in many other model systems, is the use of transposable elements. In this paper, we report the development of a two-element Activator/Dissociation (*Ac/Ds*) transposable element system suitable for gene tagging in *Arabidopsis*.

The *Ac/Ds* transposable element system was originally discovered in maize by McClintock (9). It has since been shown that *Ac* transposes in *Nicotiana tabacum* (10) and numerous other dicotyledonous plant species (for review, see ref. 11). Its behavior in heterologous plant species has been most intensively studied in tobacco (10, 12–21), tomato (22–24), and *A. thaliana* (25–28). Many transposition-related properties associated with *Ac* activity in maize are also found in heterologous systems. For instance, the 4.6-kilobase (kb) autonomous element forms 8-base-pair duplications upon insertion (10), is capable of mobilizing receptor *Ds* elements (12, 13, 25, 29), and preferentially transposes to nearby locations (16, 28).

To develop an efficient transposon tagging system for *Arabidopsis* and other plants, we have constructed a two-element *Ac/Ds* system containing plant selectable markers designed to monitor the presence of *Ac*, *Ds*, and *Ds*-related excision events (25, 30). A marked *Ds* element (*DsDHFR*) was constructed by inserting a plant selectable marker cassette, including a dihydrofolate reductase (*DHFR*) gene under the control of a cauliflower mosaic virus (CaMV) 35S promoter, into the central region of a *Ds* element. We have previously shown that the *DsDHFR* element transposes in tobacco and *Arabidopsis*, and the DHFR cassette does not decrease the frequency of *Ds* transposition (25). Furthermore, it was determined that approximately two-thirds of excised *DsDHFR* elements reinserted in transgenic tobacco protoplasts while the other one-third failed to reinsert (25). In addition, *DsDHFR*-induced mutations can be genetically segregated from *Ac*; thus, the marked *DsDHFR* element allows for more efficient transposon mutagenesis.

Although *Ac* transposes in *Arabidopsis* (25–28), its transposition frequency is generally lower and more variable than that found in tobacco and tomato. Schmidt and Willmitzer (26) found that the germinal transposition frequency of the autonomous *Ac* element in *Arabidopsis* is 0.2–0.5%, while Dean *et al.* (28) have observed higher and more variable rates of *Ac* germinal activity. Although a direct comparison is not possible with the two-element system, we have observed that transgenic *Arabidopsis* plants, containing the *DsDHFR* element and a stable, nonexcising *Ac* element, had very low rates of *DsDHFR* transposition (R.M. and C.G., unpublished results), even though the same *Ac* construct (pKU19) was able to promote high rates of *Ds* excision in transgenic tobacco protoplasts (19).

In this paper, we show that overexpression of the *Ac* transposase gene in *Arabidopsis* results in a significant improvement in the rates of independent *DsDHFR* and *Ds35S-1* transposition events. We have introduced to *Arabidopsis* T-DNA constructs containing complete and truncated *Ac*-coding sequences under the control of a tandem CaMV 35S promoter. Molecular and genetic analyses indicate that the level of independent germinal transposition events obtained with these CaMV–*Ac* fusions is more than sufficient for *Ds* transposon mutagenesis in *Arabidopsis*.

MATERIALS AND METHODS

Bacterial Strains and Plant Material. Binary vectors were directly transformed into *A. tumefaciens* strain LBA4404 (31). *A. thaliana* (L.) Heynh ecotype Columbia was used for *Agrobacterium*-mediated plant transformations (32). Transgenic *Arabidopsis* plants containing pGV3850::pDsDHFR (25) and pGV3850::pDs35S-1 (V.F. and R.M., unpublished data) were used in crosses with *Ac*-containing plants.

Vector Constructions. An abbreviated version of the *Ac* vector cloning steps is described; further details can be obtained from the authors. Part of a tobacco mosaic virus (TMV) leader sequence (ref. 33; 5′-TCGAGCTGCAGAATTACTATTTACAATTACAAG-3′) was cloned downstream of a double CaMV 35S promoter cassette (kindly provided by Andreas Bachmair, University of Vienna) as an *Xho* I/*Bam*HI

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Abbreviations: CaMV, cauliflower mosaic virus; TMV, tobacco mosaic virus.

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fragment to enhance translation initiation (TMV sequence); the resulting plasmid was termed pK19-8530. Genomic *Ac*-coding sequences Ac-101 (3.26 kb; 5′ is 22 base pairs before ATG-1 and 3′ is a *Ban* II site) and Ac-102 (3.17 kb; 5′ is at an *Acc* I site adjacent to ATG-2 and 3′ is an *Acc* I site) were cloned as *Bam*HI fragments into pK19-8530, which resulted in pK19-Ac101 and pK19-Ac102. An *Eco*RI/*Xho* I/*Sal* I linker (5′-TCGACTCGAG-3′) was inserted upstream of the double CaMV 35S cassette in pK19-Ac101 and pK19-Ac102; subsequently, a *Sal* I fragment containing the double CaMV promoter region, the TMV sequence, and the *Ac*-coding sequence were inserted into the unique *Sal* I site of pBIB-HPT (34); this resulted in the binary vectors pB-Ac101 and pB-Ac102 (see Fig. 1).

Molecular Analyses. Southern hybridizations were done according to Maniatis *et al.* (35), and Northern hybridizations were done according to Kunze *et al.* (36); stringent conditions were used. Poly(A) RNA isolation was done with oligo(dT) Dynabeads (Dynal) by a modification of a method described by Jakobsen *et al.* (37).

RESULTS

Expression of the *Ac* Transposase Gene in *Arabidopsis*. Schematic diagram of the binary vectors pB-Ac101 and pB-Ac102 is shown (Fig. 1*A*); details of the vector constructions are described in *Materials and Methods*. A tandem CaMV 35S promoter was used to overexpress the *Ac* transposase gene, and a TMV leader sequence was inserted upstream of the coding sequence to enhance translation.

The mRNAs of transgenic *Arabidopsis* plants containing single-copy pB-Ac101 and pB-Ac102 T-DNA insertions were analyzed by Northern hybridizations (Fig. 2). Assuming that the *Ac* segments of the mRNAs are spliced as in maize and that a poly(A) tail of 200 nucleotides is added to the messengers, we expect the pB-Ac101 and pB-Ac102 transcripts to be 3.0 and 2.9 kb long, respectively (lanes 3 and 4); however, many other bands hybridized with the *Ac* probe. This finding indicates that only a fraction of the *Ac* message is correctly processed, whereas the majority of the transcripts is misprocessed.

High Rates of Somatic Transposition in the F_1 Generation. Genetic crosses were done between transgenic plants containing pB-Ac102 and pDsDHFR (Fig. 1); the plants were resistant to hygromycin and methotrexate, respectively. The F_1 progeny of these crosses, presumably heterozygous for *Ac* and *DsDHFR*, were selected on medium (32) containing hygromycin (20 μg/ml) and methotrexate (0.1 μg/ml) to verify the presence of *Ac* and *DsDHFR*, or with kanamycin (100 μg/ml) to select for early excision events (12). We examined 25 *Ac*/*DsDHFR* F_1 generation plants by Southern analysis in order to monitor *DsDHFR* transposition events. Representative examples are shown, including eight F_1 progeny derived from crosses between independent transgenic *Arabidopsis* plants containing single T-DNA copies of *Ac* (Ac102.1 and Ac102.2) and *Ds* (DsDHFR.252 and DsDHFR.310) (C.G. and R.M., unpublished data), and high rates of somatic transposition were observed (Fig. 3).

A prominent 7.1-kb *Eco*RI restriction fragment, indicative of an empty donor site, is visible in all eight F_1 plants hybridized with a T-DNA-specific probe (see Fig. 3 legend); an 11.5-kb *Eco*RI band represents full donor sites (Fig. 3*A*). Similar results were obtained with the other 17 *Ac*/*Ds* crosses including different single copy pDsDHFR T-DNA insertions

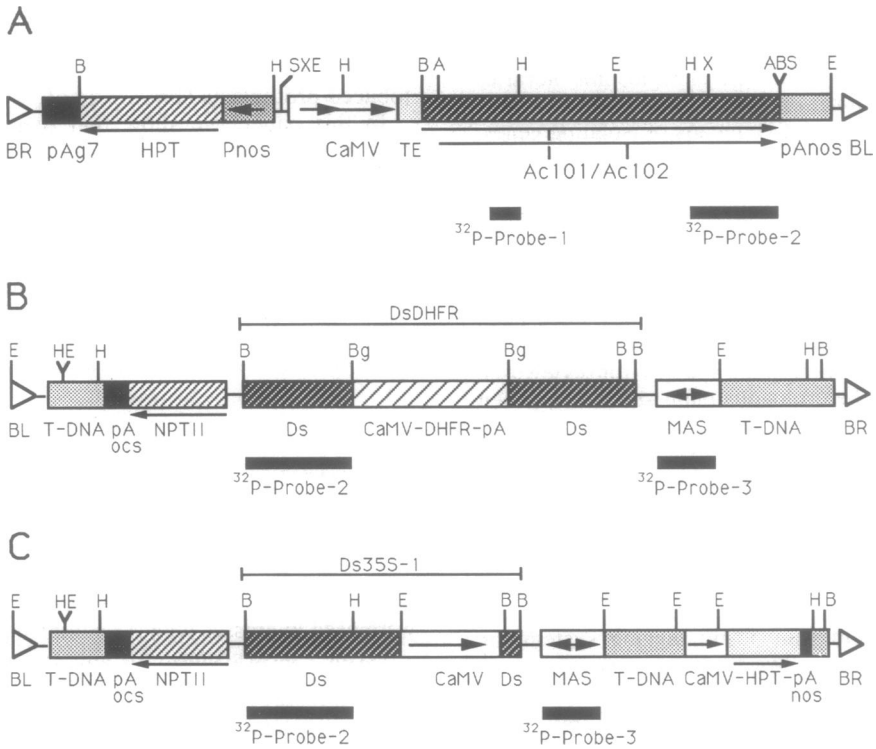

FIG. 1. Schematic diagram of the *Ac* and *Ds* constructs used in this study. (*A*) Binary vectors pB-Ac101 and pB-Ac102 (see *Materials and Methods* for details). HPT, hygromycin phosphotransferase; CaMV, tandem CaMV promoter; TE, part of a TMV translation enhancer sequence (33); Pnos, nopaline synthase promoter (*NOS*); pAnos, *NOS* poly(A) tail; pAg7, T-DNA gene 7 poly(A) tail. A, *Acc* I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal* I; X, *Xho* I; BR and BL, T-DNA borders. ^{32}P probes: *Pvu* II/*Hin*dIII fragment (probe 1) and *Hin*dIII/*Bam*HI fragment (probe 2). Arrows within boxed areas show direction of transcription; arrows below indicate orientation of coding regions. Not drawn to scale. (*B*) Cointegrate vector pGV3850 containing pDsDHFR (25); NPTII, neomycin phosphotransferase II; DHFR, dihydrofolate reductase; MAS, manopine synthase promoter; Bg, *Bgl* II. ^{32}P probes: *Hin*dIII/*Bam*HI fragment (probe 2; same as in *A*), *Eco*RI/*Bam*HI fragment (probe 3). (*C*) Cointegrate vector pGV3850HPT containing pDs35S-1; a CaMV 35S promoter is located near the 5′ end of the *Ds* element (see text). ^{32}P probes are the same as in *B*; other abbreviations are the same as in *A* and *B*. Not drawn to scale.

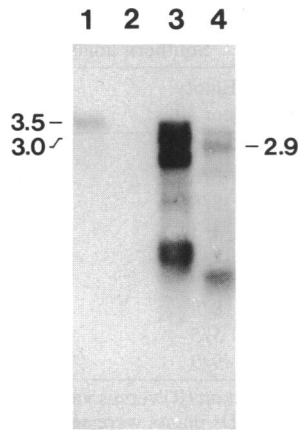

FIG. 2. Northern hybridization of Ac poly(A) mRNA. Lanes: 1, maize (2 μg); 2, *Arabidopsis* Columbia nontransformed control (1 μg); 3, transgenic *Arabidopsis* plant Ac101.1 (1 μg); 4, transgenic *Arabidopsis* plant Ac102.2 (1 μg). Hybridization was performed with a single-stranded *Ac*-specific DNA probe [*Pvu* II/*Hin*dIII fragment (probe 1); see Fig. 1*A*]. Sizes shown are kb.

(results not shown). Somatic excision of two elements occurred in F_1 progeny plants containing a tandem pDsDHFR T-DNA insertion (DsDHFR.250; Fig. 3*B*, lanes 7 and 8). Other hybridization signals are presumably due to aberrant excision events and have not been examined further.

A rehybridization of the same Southern blot with a probe specific to the *DsDHFR* element showed *DsDHFR* insertions in new locations; the same probe also hybridized with a 2.0-kb *Eco*RI fragment corresponding to *Ac* (see Fig. 1), indicating, as expected, that all eight plants contained pB-Ac102 (Fig. 3*B*). It is not clear whether a common, large molecular signal (>15 kb) observed in most lanes is due to reinserted *DsDHFR* elements because of the limited resolution of migrating fragments in this size range; no hybridization signals were observed with wild-type controls (data not shown). Insertions were also observed with the pB-Ac101 construct and two different *Ds* elements, *DsDHFR* and *Ds35S-1* (results not shown). The *Ds35S-1* element contains a CaMV 35S promoter near one end in order to generate

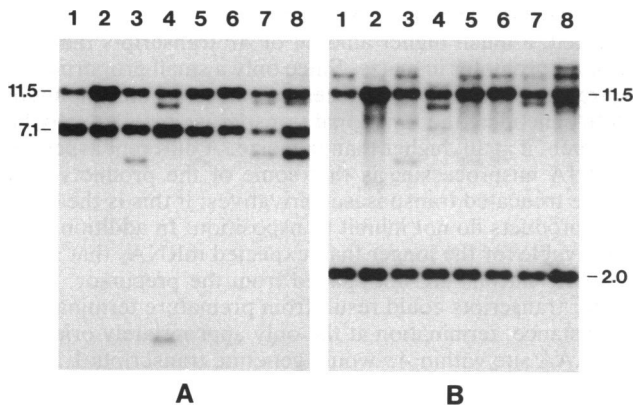

FIG. 3. Southern hybridization of F_1 generation *Ac/Ds* plants. (*A*) Lanes: 1, DsDHFR.126.4 × Ac102.1; 2, DsDHFR.252 × Ac102.1; 3, Ac102.1 × DsDHFR.252; 4, Ac102.1 × DsDHFR.252; 5, DsDHFR.252 × Ac102.2; 6, DsDHFR.252 × Ac102.2; 7, Ac102.2 × DsDHFR.250; 8, Ac102.2 × DsDHFR.250. Plant DNA (≈2 μg) was digested with *Eco*RI. ^{32}P probe was the *MAS* promoter (see Fig. 1*B*; ref. 25); sizes of full (11.5 kb) and empty (7.5 kb) donor sites are shown. (*B*) Southern blot shown in *A* was stripped and rehybridized with a ^{32}P-labeled 1.2-kb *Hin*dIII/*Bam*HI *Ac* fragment (probe 2) specific for both *Ac* and *DsDHFR* (Fig. 1 *A* and *B*); sizes shown are kb.

dominant mutations upon insertion (V.F., C.G. and R.M., unpublished data).

High Rates of Germinal Transposition in the F_2 Generation. An examination of 25 *Ac/DsDHFR* and 20 *Ac/Ds35S-1* plants indicated that a minimum of 27% of the F_1 generation *Ac/Ds* plants had independent *Ds* insertions, without *Ac*, in the F_2 generation. Representative examples of these germinal transposition events are shown (Fig. 4; Table 1).

A 5.5-kb *Eco*RI band, corresponding to a single *DsDHFR* insertion, was observed in an F_2 generation plant (without *Ac*) derived from a genetic cross between pB-Ac102 and pDsDHFR-310 (Fig. 4, lane 3; Table 1); genetic segregation resulted in two siblings that contained only *Ac* (lanes 1 and 2). Each parent contained single-copy T-DNA insertions (C.G. and R.M., unpublished data). In the F_2 progeny of a pAc-101/pDs35S-1 cross, a 2.2-kb *Eco*RI band indicative of a *Ds35S* insertion was observed in five progeny plants including three that did not contain *Ac* (lanes 4–8; Table 1); the *Ac* parent contained a single-copy T-DNA insert, and the larger hybridizing bands observed (lanes 4–8), including an 8.9-kb *Eco*RI band, represent multiple pDs35S-1 T-DNA insertions (V.F. and R.M., unpublished data).

A total of 44% of the families examined by Southern analysis contained both *Ac* and new *Ds* insertions; thus, significant rates of somatic or germinal transposition had occurred (see Table 1 for examples). Since *Ac* was present in some of these progeny plants, it was not always possible to conclude that a germinal transposition event had occurred.

The F_2 germinal revertants were resistant to kanamycin, and variable rates of excision were observed in the F_2 progeny (Table 1); kanamycin-sensitive progeny appeared as bleached white seedlings, variegated plants contained green and white sectors, and resistant plants were green. For example, the F_2 progeny of cross 1 (Ac102.1 × DsDHFR-310) contained germinal insertions (Fig. 4) and showed variable levels of kanamycin resistance. The ratio of resistant versus variegated and sensitive plants was 0.48:1 (Table 1, cross 1). The overall kanamycin-resistance levels varied from 0.03:1 to 1.52:1 in the examples shown (Table 1); kanamycin-resistance levels were as high as 7.7:1 in other F_2 progeny plants examined (data not shown). Germinal transposition events occurred in F_2 progeny that displayed different rates of kanamycin resistance (Table 1, crosses 3, 5, 7, and 8). Germinal excision events that did not result in reinsertions have also been observed (cross 9; see below), and new, presumably somatic, insertions were observed in some other F_2 families (cross 6).

Reciprocal *Ac/Ds* crosses resulted in similar transposition frequencies. Although the examples shown of reciprocal

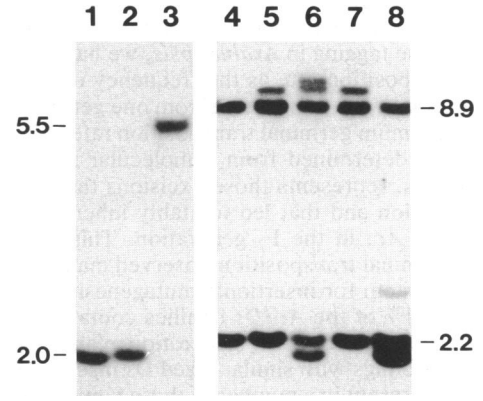

FIG. 4. Southern hybridization of F_2 generation *Ac/Ds* plants. Lanes: 1–3, Ac102.1 × DsDHFR.310 (siblings); 4–8, Ac101.1 × Ds35S-1.481 (siblings). ^{32}P probe is the same as in Fig. 3*B*; sizes shown are kb.

Table 1. Transposition activity of *Ac/Ds* in transgenic *Arabidopsis*

Ac/Ds cross	Kanamycin selection			Southern analysis			
	R/V+S	Total R+V+S	Ratio R:V+	Excision	Empty donor sites	*Ds* insertion	Germinal insertion
1. Ac102.1 × DsDHFR.310	24/50	(74)	0.48:1	+	14/14	5/14	+
2. Ac102.1 × DsDHFR.252	42/151	(193)	0.28:1	+	6/6	5/6	(+)
3. Ac102.1 × DsDHFR.252	120/79	(199)	1.52:1	+	8/8	3/8	+
4. Ac102.2 × DsDHFR.250	13/101	(114)	0.13:1	+	3/3	0/3	−
5. Ac102.2 × DsDHFR.250	74/110	(184)	0.67:1	+	12/12	9/12	+
6. DsDHFR.252 × Ac102.2	11/176	(187)	0.06:1	+	7/7	2/7	−
7. DsDHFR.252 × Ac102.2	5/188	(193)	0.03:1	+	8/8	3/8	+
8. Ac101.1 × Ds35S-1.481	6/180	(186)	0.03:1	+	5/5	5/5	+
9. Ac101.1 × Ds35S-1.481	23/177	(200)	0.13:1	+	9/9	0/9	−

F_2 generation seeds were selected with kanamycin sulfate (150 μg/ml); ratios are the number of resistant (R) versus variegated (V) and sensitive (S) seedlings; numbers of germinated seeds are shown in parentheses. Individual F_1 generation *Ac/Ds* plants were scored + or − for excision (determined by Southern hybridizations; see Fig. 3); the numbers of plants with empty donor sites in the F_2 generation and with *Ds* insertions are shown. Germinal insertions were scored as + or − determined by the presence of a *Ds* element in a new location and the absence of *Ac* in F_2 progeny. Cross 2 resulted in a putative germinal insertion (+) in that a similar-sized *Ds* insert segregated in the F_2 generation; however, *Ac* was still present.

crosses including DsDHFR.252 did display significant variation with respect to F_2 generation kanamycin resistance (Table 1, crosses 2, 3, 6, and 7), the general trend among the 45 families examined, including other Ac102 × DsDHFR.252 crosses, indicated that the use of *Ac* as a male or female donor did not appear to greatly affect the kanamycin-resistance ratios. In addition, molecular analysis showed that the frequency of germinal insertions among reciprocal crosses was approximately the same.

Germinal Excision Without Concomitant Ds Reinsertion. A number of F_2 families have been examined in which *Ds* excised in the F_1 generation and presumably failed to reinsert; an empty donor site was transmitted to the F_2 progeny. For example, a reinserted *Ds35S-1* element was not detected in nine F_2 progeny of an *Ac/Ds35S-1* cross; however, empty donor sites were observed, thus indicating that germinal excisions had occurred in the F_1 generation (Table 1, cross 9). In addition, Southern analysis of another *Ac/Ds35S-1* family, in which seven F_2 progeny were examined, indicated that germinal excision had occurred without an accompanying *Ds35S-1* insertion (V.F. and R.M., unpublished data). These results are consistent with our earlier finding that *DsDHFR* elements do not always reinsert after excision (25).

DISCUSSION

We have shown that overexpression of the *Ac* transposase gene, under the control of a tandem CaMV 35S promoter, results in a significant increase in the germinal transposition frequency of *Ds* elements in *Arabidopsis* in comparison to our previous results with an unmodified *Ac* element. For the purpose of gene tagging in *Arabidopsis*, we have defined the germinal transposition rate as the frequency of independent *Ds* insertions that are transmitted from one generation to the next. The minimum germinal transposition rate of 27% found in this study, determined from a molecular analysis of 45 *Ac/Ds* families, represents those excisions that occurred in the F_1 generation and that led to stably inherited *Ds* insertions, without *Ac*, in the F_2 generation. This minimal frequency of germinal transposition observed makes *Arabidopsis* an ideal system for insertional mutagenesis.

A total of 44% of the *Ac/Ds* families contained *Ds* insertions at new locations in F_2 generation plants, and some families had siblings with similar-sized *Ds* inserts. However, since *Ac* was present in a number of these families, it was not clear whether the *Ds* insertions were the result of germinal or early somatic transposition events. Therefore, we conclude that the actual germinal transposition frequency is most likely between 27 and 44%.

While the germinal transposition frequencies of the autonomous *Ac* element (26, 28) and the *Ac/Ds* system used in this study are not directly comparable, there is a clear indication that the two-element system is effective in producing significant rates of germinal insertions. The two-element system offers a number of advantages including the segregation of *Ac* and *Ds* in progeny generations, and the utilization of marked *Ds* elements, such as *DsDHFR*, which can be monitored by antibiotic selection. Furthermore, *Ac* and *Ds* parents can be screened for mutations before making *Ac/Ds* crosses.

Transgenic plants containing single and multiple T-DNA insertions, analyzed by Southern hybridizations (C.G., V.F. and R.M., unpublished data), were used to more precisely monitor excision and reinsertion events. The relationship between full and empty donor sites was determined by Southern hybridizations (Fig. 3*A*), and a kanamycin excision assay (12) allowed the selection of those *Arabidopsis* plants in which *Ds* elements had excised from the leader sequence of an NPTII gene (Table 1). While transposition patterns are more complex with multiple T-DNA insertions, somatic and germinal transposition events were nevertheless observed in plants containing a tandem pDsDHFR T-DNA construct and multiple pDs35S-1 T-DNA insertions (Figs. 3 and 4; Table 1).

The strong tandem CaMV promoter can generate, as expected, a much higher amount of *Ac* transcripts than the weak *Ac* promoter in maize. Since only a small proportion of the transcripts seem to be processed correctly, the amount of mRNA encoding the functional transposase may actually be not much, if at all, higher than in maize. A different aspect of Ac RNA misprocessing is that some of the products may encode truncated transposase derivatives; if this is the case, these products do not inhibit transposition. In addition, it is conceivable for the longer than expected mRNAs that some of the *Ac* introns are not spliced from the precursor; thus, shorter transcripts could result from premature termination. For instance, termination at the only appropriately oriented AATAAA site within *Ac* would generate transcripts 1.1 and 1.0 kb long from pB-Ac101 and pB-Ac102, respectively, which are about the sizes of the smallest transcripts on the Northern blot (Fig. 2).

Overexpression of the full-length (pB-Ac101) and truncated (pB-Ac102) CaMV–Ac fusions, using a tandem CaMV promoter previously shown to increase transcription in chimeric gene fusions (38), and a TMV sequence to enhance translation (33), resulted in similar high rates of *Ds* transposition, regardless of whether *Ac* was used as a male or female donor. While the same truncated *Ac* gene, under the control of the *MAS* 2′ promoter, produced significantly higher rates of *Ds* excision in petunia protoplasts compared to the full-

length *Ac* transposase gene (29), we did not notice a similar difference with the CaMV–Ac fusions in this study. In addition, both *Ac* constructs mobilized the two *Ds* elements used in this study, including *Ds35S-1*, which contains a CaMV 35S promoter located near one end of the element. The *Ds35S-1* element was designed to generate dominant mutations upon insertion (V.F., C.G. and R.M., unpublished data).

Molecular analysis of the F_1 generation, kanamycin-resistant *Ac/Ds* plants indicated high rates of somatic excision (Fig. 3*A*). The variation in kanamycin resistance observed in the progeny of these plants most likely indicates the differences in timing of *Ds* excisions that occurred during development of F_1 generation plants (Table 1). Dean *et al.* (28) have observed similar results with an autonomous *Ac* element in *Arabidopsis*.

The germinal inheritance of an empty donor site without a concomitant *Ds* insertion found in some of the transgenic *Arabidopsis* families examined in this study is consistent with our earlier finding that *DsDHFR* elements do not always reinsert, and thus are presumably lost in the transposition process (25). It is also possible that *Ds* insertions were not identified for other reasons, including insertions into sister chromatids after DNA replication (39). Nevertheless, the identification of germinal excisions and insertions is facilitated by selection with kanamycin and methotrexate, respectively; thus, fewer *Ac/Ds* progeny are needed for mutant screening.

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