Germinal and Somatic Activity of the Maize Element *Activator* (*Ac*) in Arabidopsis

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ABSTRACT

We have investigated the germinal and somatic activity of the maize *Activator* (*Ac*) element in Arabidopsis with the objective of developing an efficient transposon-based system for gene isolation in that plant. Transposition activity was assayed with a chimeric marker that consists of the cauliflower mosaic virus 35S promoter and a bacterial streptomycin phosphotransferase gene (SPT). Somatic activity was detected in seedlings germinated on plates containing streptomycin as green-resistant sectors against a background of white-sensitive cells. Germinal excisions resulted in fully green seedlings. The transposition frequency was extremely low when a single copy of the transposon was present, but appeared to increase with an increase in *Ac* copy number. Plants that were selected as variegated produced an increased number of green progeny. The methylation state of the *Ac* elements in lines with either low or high levels of excision was assessed by restriction analysis. No difference was found between these lines, indicating that the degree of methylation did not contribute to the level of *Ac* activity. Germinal excision events were analyzed molecularly and shown to carry reinserted transposons in about 50% of the cases. In several instances, streptomycin-resistant siblings carried the same transposed *Ac* element, indicating that excision had occurred prior to meiosis in the parent. We discuss parameters that need to be considered to optimize the use of *Ac* as a transposon tag in Arabidopsis.

DUE to its small genome size and rapid life cycle, Arabidopsis has been promoted as a model for the genetic and molecular analysis of plant processes (ESTELLE and SOMMERVILLE 1986; MEYEROWITZ 1989). Many mutants carrying lesions in metabolic or developmental pathways have been described. The next phase of the analysis will be to isolate the genes that have been mutated and to characterize them at the molecular level. However, for most genes defined mutationally, either no gene product has been characterized or the gene product is in very low abundance. In these cases, genetic strategies for gene isolation, such as insertional mutagenesis, appear promising.

Insertional mutagenesis using T-DNA from *Agrobacterium tumefaciens* as a tag has been used to isolate a number of genes from Arabidopsis (MARKS and FELDMAN 1989; KONCZ *et al.* 1990; and YANOFSKY *et al.* 1990). Transposon tagging, *i.e.*, insertional mutagenesis using transposons, has proved to be a powerful technique for the isolation of genes from maize and snapdragon where well characterized endogenous transposons exist (reviewed in CONE *et al.* 1988; SHEPHERD 1988; CARPENTER and COEN 1990). The maize *Activator* (*Ac*) element was one of the first transposons discovered (MCCLINTOCK 1951) and its activity has been studied extensively in its natural host. *Ac* has been shown to be capable of transposing in several heterologous plant species (for a review see HARING *et al.* 1991) including Arabidopsis (VAN SLUYS, TEMPE and FEDEROFF 1987; MASTERSON *et al.* 1989; SCHMIDT and WILLMITZER 1989) and therefore may serve as a useful alternative insertional "tag" in Arabidopsis.

A number of findings about *Ac* activity in maize are pertinent when designing gene tagging experiments. In general, it has been observed that a small percent of the progeny of plants which carry an active *Ac* element show evidence of germinal transposition and of these 50–70% contain the transposon at a new location in the genome. Reinsertions have been found to be nonrandom, with a preference for sites within 5 centimorgans from the donor site (GREENBLATT 1984; DOONER and BELACHEW 1989). Finally, *Ac* shows negative dosage effects in maize, *i.e.*, two copies of the element give rise to fewer germinal and later somatic transposition events than does one copy (MCCLINTOCK 1951; BRINK and NILAN 1952).

The activity of *Ac* has also been studied extensively in a heterologous system, tobacco. As in maize, reinserted *Ac* elements are detected in about 60% of progeny selected for germinal excisions and are linked to the donor site 70–80% of the time (JONES *et al.* 1990; DOONER *et al.* 1991). In a study of the sequences bordering transposed *Ac* elements, it was shown that, of the six reinsertion events that were analyzed, five were in low copy regions of the genome and at least

one was in a transcribed sequence (HEHL and BAKER 1990). Unlike the situation in maize, an increase in *Ac* copy number in tobacco leads to an increase in transposition frequency (JONES *et al.* 1989, 1991).

The finding that *Ac* acts in tobacco in a similar manner to maize indicates that the transposon may be very useful for tagging genes in heterologous plant species. The potential advantage of *Ac vs.* T-DNA as a mutational tag is the mobility of *Ac* within the genome from generation to generation. Consequently, fewer transformed plants need to be generated and characterized for a tagging experiment. Since *Ac* tends to transpose to linked sites in the genome it will be especially useful as a tag when linkage can be established between the transposable element and a gene of interest. A potential problem with using transposon tagging for gene isolation is the possible loss of the transposon by subsequent transposition before the desired gene can be isolated. However, infrequent reversions with restoration of function may be useful in establishing a cause and effect relationship between the transposed element and the mutant phenotype. The frequency of transposition can be modulated, if necessary, by using a two element system in which the transposase source and a nonautonomous tranposon segregate independently of each other.

SCHMIDT and WILLMITZER (1989) have found that the germinal excision frequency of *Ac* is very low in Arabidopsis, but that *Ac* can continue to transpose in the generations following transformation. In the present study we have used a streptomycin assay (JONES *et al.* 1989) to examine other features of *Ac* transposition that bear on the usefulness of *Ac* as a heterologous transposon in Arabidopsis. We have found that though the germinal excision frequency of *Ac* in Arabidopsis is low, in general, transformed lines with higher number of *Ac* elements give higher excision frequencies. Most importantly, we have established that about 50% of germinal excision products carry a transposed *Ac*, a critical parameter that validates *Ac*'s potential as a tag. We have used the somatic variegation conditioned by SPT::*Ac* to show that most of the transformed lines which gave no evidence of germinal excisions did have an *Ac* element that was active somatically. Peculiarly, in variegated seedlings the distribution of somatic excision events was not that expected of infrequent, random events. We have also analyzed the effects of the methylation status of the element on transposition frequency. Finally, we have obtained evidence that germinal transposition events can occur prior to meiosis, resulting in siblings carrying the same transposed *Ac* element.

MATERIALS AND METHODS

Constructions: The binary vectors used in our experiments were constructed by JONATHAN JONES and are shown in Figure 1. They are described in more detail elsewhere (J. KELLER, J. JONES, E. LIM, F. CARLAND, E. RALSTON and H. K. DOONER, submitted).

Plant transformation: *Arabidopsis thaliana* (ecotype Ws) seedlings were grown aseptically in magenta cups containing OMS agar (recipe given below) at 24°. Lighting was provided at 200 μE/m2/s for 12 hr followed by 12 hr of darkness. After 4–5 weeks bolted flowering stems were used as explants for transformation. Additional cuttings of flowering stems were obtained from the same plants over a period of about 1 month from the initial bolting. The stems were cut into 0.5–1-cm lengths and precultured on callus induction (I) medium (given below) for 5 days in dim light generated by placing the explants under several layers of cheesecloth in an incubator. On the morning of day 5 an overnight culture of Agrobacterium, strain LBA4404 (HOEKEMA *et al.* 1983), containing either pJJ4404 or pJJ4411 was diluted to an O.D. 595 of 0.2 and allowed to grow to an O.D. of 0.3 to 0.7. Just prior to starting the cocultivation, the culture was again diluted to an O.D. of 0.15 so as to contain approximately 1×10^8 bacteria per ml of culture solution. The pre-callused explants were dipped in the bacterial broth for 5 min and transferred onto a fresh plate of I medium containing a piece of sterile Whatman #1 filter paper. The explants were cocultivated for 2 days under dim light. At the end of the cocultivation, the explants were washed in OMS liquid medium containing 500 mg/liter cefotaxime for 3–5 hr. The explants were then plated on shooting medium (SM–given below) containing 15 mg/liter hygromycin and 500 mg/liter carbenicillin. Within 1 month green callus appeared at the ends of the explants, and was transferred to fresh SM medium. Calli were transferred to fresh SM medium every 3–4 weeks until shoots developed to the bolting stage. Bolting shoots were transferred into magenta cups containing OMS medium and allowed to set seed.

OMS agar consists of 1 × MS salts (MURASHIGE and SKOOG 1962), 3% sucrose, 1 × B_5 vitamins (1 mg/liter nicotinic acid, 10 mg/liter thiamine HCl, 1 mg/liter pyridoxine HCl, and 100 mg/liter myoinositol), 0.6 g/liter 2-(*N*-morpholino)ethanesulfonic acid, and 0.8% tissue culture grade agar. Induction (I) medium contains the same ingredients as OMS with the addition of 18.2 g/liter mannitol, 0.2 mg/liter 2,4-dichlorophenoxyacetic acid, 3.0 mg/liter kinetin and 100 mg/liter acetosyringone. Shooting medium (SM) is the same as OMS but with the addition of 1 mg/liter benzyladenine phosphate and 0.1 mg/liter naphthalene acetic acid.

Streptomycin seedling assay: Seedlings were analyzed for somatic and germinal *Ac* activity by germinating them on agar plates containing 1 × MS salts (MURASHIGE and SKOOG 1962), 3% sucrose, and 200 μg/ml streptomycin. This assay is based on the sensitivity of many plant species to the antibiotic streptomycin. In brief, the cotyledons of wild-type seeds germinated on agar containing streptomycin are white. However, transformed plants carrying a chimeric 35S-SPT gene will appear green in the presence of the antibiotic. If the SPT gene is interrupted by the *Ac* element, the seedlings will appear variegated, with *Ac* excision giving sectors of green streptomycin-resistant cells against a white streptomycin-sensitive background. If *Ac* excises germinally, the progeny will appear completely green on streptomycin.

DNA extraction and analysis: DNA was extracted from about 0.5 g of leaf tissue using the procedure described in DOONER *et al.* 1991. The DNA samples were analyzed by digesting with appropriate restriciton enzymes, size fractionating through agarose gels, and blotting to Duralon-UV (Stratagene). The blots were probed either with the *Ac* 5′

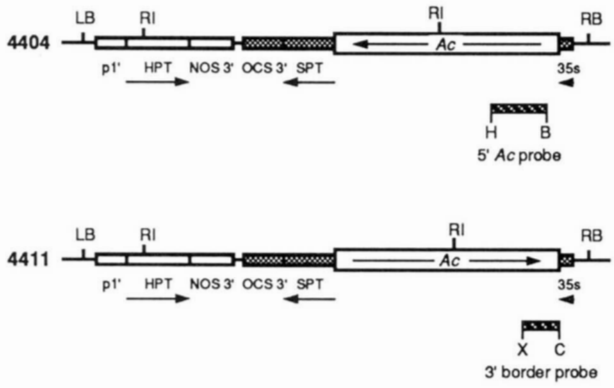


FIGURE 1.—The T-DNA regions of pJJ4404 and pJJ4411 are shown diagramatically. The positions of the 5′ probe bordered by *Bam*HI (B) and *Hin*dIII (H) sites and the 3′ probe terminating in *Xba*I (X) and *Cla*I (C) sites are also shown. p1′ is the T-DNA 1′ promoter, HPT is hygromycin phosphotransferase, and SPT is streptomycin phosphotransferase. NOS 3′ and OCS 3′ refer to the nopaline and octopine synthetase polyadenylation sequences, respectively. 35s indicates the CaMV 35S promoter. LB and RB are the left and right T-DNA border sequences, respectively.

and 3′ end probes shown in Figure 1 or with the 35S probe shown in Figure 5.

RESULTS

Generation of SPT::*Ac* transformants of Arabidopsis: The binary vectors used in the streptomycin excision assay, pJJ4404 and pJJ4411, are shown in Figure 1. The vectors contain a hygromycin resistance transformation marker and an SPT::*Ac* excision marker between the right and left T-DNA borders. The wild type *Ac7* element (MULLER-NEUMANN, YODER and STARLINGER 1984) interrupts the 35S-SPT gene in the 5′ untranslated leader sequence and is in either the same or opposite transcriptional orientation relative to the 35S-SPT gene in pJJ4404 and pJJ4411, respectively. The T-DNA was introduced into Arabidopsis, ecotype Ws, by cocultivating flowering stem sections with Agrobacterium strain LBA4404 (see MATERIALS AND METHODS). Primary transformants, which are heterozygous for the introduced DNA, are referred to as T1 plants to conform with the designations F1 and M1 commonly used for the heterozygotes produced by sexual crosses and mutagenesis, respectively. Subsequent generations are referred to as T2, T3, and so forth.

Twenty independent 4404 and eight independent 4411 transformants were generated and allowed to self-pollinate. Each transformant was analyzed for T-DNA content by germinating the seeds of the primary transformant on hygromycin to determine the segregation ratios and by DNA blot analysis of the T2 progeny. The DNA analysis was accomplished by digesting chromosomal DNA with *Eco*RI, which cleaves the *Ac* element once, and hybridizing with the 5′ border probe for 4404 transformants or the 3′ border probe for 4411 transformants (as shown in Figure 1). Each *Ac*-hybridizing band was taken to represent a T-DNA border, which allowed an independent determination of *Ac* copy number from that based on segregation ratios. Multiple progeny were sampled for their DNA content when uncertainty about the T-DNA copy number in a transformant arose. Tables 1 and 2 (second column) summarize the results of the Southern blot analysis. All of the transformants but one (C204) had a single T-DNA locus. Southern blot analysis showed that C204 had five T-DNA borders and at least three independently segregating loci (see discussion below). Of the single locus transformants, B120 and B246 had two T-DNA copies and C201 had three T DNA copies at the insertion site.

***Ac* activity in single insert transformants:** Somatic *Ac* activity was indicated by the presence of variegated seedlings on streptomycin. Examples of highly variegated seedlings are shown in Figure 2. Most variegated seedlings had only one to five sectors as will be discussed below. Table 1 shows the level of somatic activity (%*V*) detected in the T2 and T3 generations for the 24 transformants that contained single T-DNA inserts. In the T2 generation only 17 of the transformants had sufficient seeds (25 or more) to germinate directly on streptomycin. Of these, 13 gave some variegated seedlings, but none gave green seedlings, indicating a lack of germinal *Ac* excision. In the T3 generation the average level of somatic *Ac* activity remained the same as in the previous generation. However, in the T3 progeny of a few individual plants (*e.g.*, B104.3H, and B222.2H) greater than 25% of the seedlings showed variegation. Transformants B116, B214, B222, C231 and C245 gave some green progeny. Only four of the transformants, B106, B111, B211 and B229, gave no evidence of somatic *Ac* activity in either the T2 or T3 generation.

On average, only 7% of the T3 seedlings from the single insert transformants listed in Table 1 gave evidence of somatic *Ac* activity. This finding indicates that although the *Ac* elements were capable of excision, they remained largely inactive throughout cotyledon development. The question arose as to whether the seedling progeny of transformants containing detectable *Ac* activity could sporadically be released from some constraint on activity to give a large number of green sectors. To address this, counts were made of the number of sectors per seedling for several of the transformants containing single T-DNA inserts with *Ac* activity. Figure 3 shows the results of this analysis performed with seedlings from five homozygous T2 plants. The expected Poisson distribution is also shown in Figure 3 for comparison. The observed distribution has zero sectors per seedling as the most frequent class and one sector per seedling as the next most frequent class. However, the observed values do not fit the expected Poisson distribution ($\chi^2 = 216$, 2

TABLE 1

Analysis of *Ac* activity in progeny of the single insert transformants

T1 plant[a]	No. T-DNAs[b]	No. T2 seedlings[c]	%V[d]	%G[e]	T2 plant[f]	T2 Hyg genotype[g]	No. T3 seedlings[c]	%V[d]	%G[e]
B102	1	24	0	0	B102.1H	+/+	15	0	0
					B102.2H	+/+	29	4	0
B104	1	142	10	0	B104.1H	+/+	96	2	0
					B104.3H	+/+	68	35	0
B106	1	50	0	0	B106.1H	+/−	35	0	0
					B106.2H	+/+	35	0	0
B111	1	100	0	0	B111.2H	+/+	32	0	0
					B111.3H	+/+	48	0	0
B115	1	130	5	0	B115.3H	+/−	38	0	0
B116	1	75	41	0	B116.2H	+/+	71	13	0
					B116.3H	+/+	121	10	37
B117	1	164	6	0	B117.3H	+/−	177	0	0
B118	1	59	7	0	B118.2H	+/−	120	0	0
					B118.6V	+/+	28	0	0
B211	1				B211.1H	+/+	67	0	0
B214	1				B214.4H	+/−	64	0	0
					B214.5H	ND	93	11	1
					B214.16V	ND	32	25	0
B215	1				B215.1H	+/+	88	5	0
					B215.5H	ND	30	7	0
B217	1				B217.1H	+/−	87	6	0
B218	1				B218.1H	+/−	107	1	0
B222	1	26	8	0	B222.1H	ND	9	22	0
					B222.2H	ND	199	45	2
					B222.3H	+/+	58	7	0
B223	1	44	0	0	B223.1H	+/+	130	1	0
B228	1	75	8	0	B228.1H	+/+	44	11	0
B229	1	203	0	0	B229.2H	+/−	36	0	0
					B229.3H	+/−	57	0	0
B236	1	274	3	0	B236.1V	+/+	335	2	0
C203	1	94	3	0	C203.6H	+/−	75	21	0
					C203.7H	+/−	167	0	0
					C203.13H	+/+	30	13	0
C205	1	50	18	0	C205.1H	+/−	116	9	0
					C205.6V	+/−	158	3	0
					C205.7V	+/+	374	3	0
					C205.17H	+/−	126	5	0
					C205.16H	+/−	57	0	0
C224	1	27	4	0	C224.1H	ND	62	5	0
					C224.3H	+/+	70	0	0
C230	1	198	1	0	C230.1H	ND	148	2	0
					C230.2H	ND	56	7	0
C231	1	47	6	0	C231.1H	ND	192	17	1
					C231.2H	ND	50	2	0
					C231.4H	+/−	38	14	0
C245	1	2	0	0	C245.1H	+/−	103	8	2
					C245.3H	+/+	50	0	4
					C245.4H	ND	89	5	0
					C245.5H	+/−	66	13	2
					C245.6H	+/+	74	8	1
					C245.7H	ND	33	3	0
Overall averages			7%	0				7%	1%

[a] Transformants carrying the 4404 construct have a "B" before their numerical designation and those carrying 4411 are indicated with a "C."

[b] T-DNA insert number is based on Southern blot analysis.

[c] Number of seedlings germinated on streptomycin.

[d] %V = 100 (No. variegated/total) for seedlings on streptomycin.

[e] %G = 100 (No. green/total) for seedlings on streptomycin.

[f] V or H after the T2 plant name refers to the selection of that plant as variegated on streptomycin (V) or as resistant to hygromycin (H).

[g] Based on segregation of T3 seedlings on hygromycin (ND = not determined).

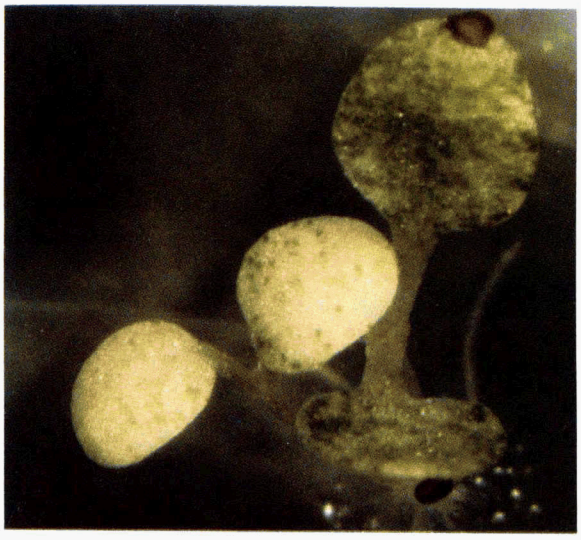
TABLE 2

Analysis of *Ac* activity in progeny of the multiple insert transformants

T1 plant	No. T-DNAs	No. T2 seedlings	%V	%G	T2 plant	T2 Hyg genotype	No. T-DNAs[a]	No. T3 seedlings	%V	%G
B120	2	30	3	0	B120.2H	+/−	2	236	10	0.5
					B120.3H	+/−	2	97	3	4
					B120.4H	+/−	2	54	0	0
					B120.5H	+/+	4	45	2	2
								Average	4%	1.6%
B246	2				B246.1H	+/−	2	57	14	0
					B246.2H	+/+	4	66	12	0
					B246.3H	+/+	4	102	38	2
					B246.4H	+/−	2	34	20	0
					B246.6H	+/−	4	39	24	4
								Average	21%	1.2%
C201	3	62	40	3	C201.5H	+/−	3	241	56	0
					C201.13H	+/−	3	60	53	2
					C201.16H	+/−	3	57	0	0
					C201.1H	+/+	6	421	92	2
					C201.2H	+/+	6	78	8	0
					C201.3H	+/+	6	238	50	0
					C201.4H	+/+	6	29	16	0
					C201.12H	+/+	6	152	4	0
					C201.15H	+/+	6	146	64	0
					C201.17H	+/+	6	346	58	0
								Average	42%	0.4%
					C201.19V	+/−	3	624	64	1
					C201.22V	+/−	3	100	31	1
					C201.29V	+/−	3	469	44	0.5
					C201.20V	+/+	6	658	49	0
					C201.31V	+/+	6	100	80	4
					C201.32V	+/+	6	182	36	0
					C201.33V	+/+	6	137	70	8
					C201.34V	+/+	6	143	55	2
					C201.36V	+/+	6	634	77	0
					C201.37V	+/+	6	989	97	1
					C201.38V	+/+	6	95	31	25
					C201.39V	+/+	6	240	ND	42
					C201.40V	+/+	6	445	74	2
					C201.41V	+/+	6	90	77	1
								Average	61%	6%
C204	5	50	38	4	C204.1H		3	101	0	0
					C204.4H		4	771	73	0
					C204.5H		3	560	73	0
					C204.6H		5	379	49	0
					C204.7H		5	469	97	2
								Average	58%	0.4%
					C204.10V		4	501	9	1
					C204.13V		2	260	2	0
					C204.14V		6	198	33	0
					C204.15V		5	150	17	0
					C204.17V		5	197	69	0.5
					C204.18V		5	144	25	1
					C204.20V		5	265	11	0
					C204.21V		5	525	ND	5
					C204.22V		4	146	30	1
								Average	25%	1%

For an explanation of the column headings see the footnotes for Table 1.

[a] T-DNA insert number is based on Southern blot analysis of the T2 plants and hygromycin segregation in their T3 offspring. For the T2 progeny of C204, the minimum number of *Ac* copies was estimated by counting border fragments in the DNA blots of the T2 parents. If one or more band within the lane had a two-fold increase in intensity then that insert(s) was taken to be homozygous and given a weight of 2. These estimates were corroborated in a few cases by Southern blot analysis of T3 progeny.



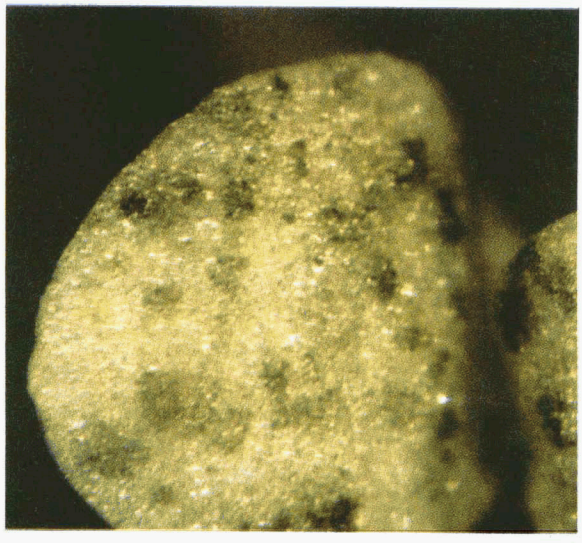


FIGURE 2.—The variegated phenotype of Arabidopsis seedlings on streptomycin.

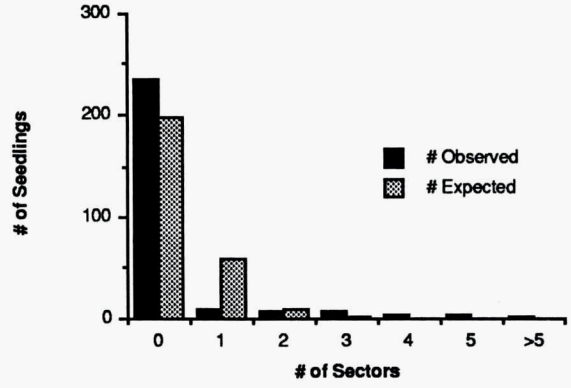


FIGURE 3.—Analysis of number of green sectors per seedling. T3 self progeny of B102.1, B102.2, B104.1, B104.3H and B222.3H were germinated in the presence of streptomycin and green sectors were counted at 3 weeks. The data from the five families were pooled (black bars). The expected Poisson distribution for a random, infrequent event is also shown (stipled bars) for comparison.

d.f.) suggesting that *Ac* excision is not random among Arabidopsis seedlings that carry a single T-DNA insert. Possibly, factors are operating in the Arabidopsis genome which negatively regulate *Ac* transposition activity, but in a few seedlings the *Ac* element can escape this constraint.

Activity in transformants containing multiple *Ac* elements: Four transformants containing more than one copy of the T-DNA insert were generated and are listed in Table 2. Transformants B120 and B246 had two T-DNA inserts at one locus, C201 had three T-DNA inserts at one locus and C204 had five inserts at three or more loci. It was of interest to compare the levels of somatic and germinal *Ac* activity in these multicopy transformants to those in transformants with single copies of *Ac* to determine if there is a positive dosage affect for the transposon in Arabidopsis, as there is in tobacco (JONES *et al.* 1989). In tobacco, all individuals carrying an SPT::*Ac* allele show variegation and, furthermore, there is a positive dosage effect of the transposon on the level of variegation within a seedling. In Arabidopsis, only a fraction of individuals carrying SPT::*Ac* show variegation. Therefore, we decided to address the issue of *Ac* dosage effect in Arabidopsis on a population, rather than on an individual, basis. We asked if the percentage of variegated seedlings seen in the T2 and T3 progeny of transformants carrying multiple copies of *Ac* increased relative to that seen with single copy transformants.

B120 and B246 on average gave twice as many variegated seedlings in the T3 generation as the single insert transformants and both gave green streptomycin resistant progeny. However, a few of the single insert transformants (*e.g.*, B222 and C245) have comparable somatic and germinal *Ac* activity to B120 and B246. Consequently, the apparent increase in the *Ac* activity in B120 and B246 relative to all of the single insert transformants could be due either to a chromosomal location that promotes *Ac* transposition or to a positive gene dosage effect in a less active region of the genome. Transformants C201 and C204, which had 3 and 5 T-DNA inserts, respectively, gave about 40% variegated seedlings and several fully green seedlings in both the T2 and T3 generations (see Table 2). Comparing the results from the transformants with one or two T-DNA inserts with those from C201 and C204, it appears that there may be a positive effect of increasing *Ac* copy number on the frequency of somatic and germinal excisions. However, a linear increase in transposon activity with number of T-DNA alleles is not seen and position effects probably contribute significantly to the activity of *Ac* in individual transformants.

It was of interest to determine if selection of varie-

gated seedlings would increase the number of green progeny obtained in the next generation. Only transformants C201 and C204 produced enough variegated T2 seedlings with which to do this analysis. Both transformants showed an increase of germinal excision frequency when the selection of the parents was based on *Ac* activity rather than on hygromycin resistance(see Table 2). This is observed, for example, by comparing the number of families having green progeny from hygromycin-selected T2 plants (C201.1H-C201.13H) with those from T2 plants selected for variegation on streptomycin (C201.19V-C201.39V). The former group gave green progeny in 2 out of 9 families whereas the latter gave green progeny in 11 of 14 families tested (see the last column of Table 1). Progeny of C204 gave similar results, with 5 out of 9 variegated selections giving green progeny in the T3 generation but only 1 out of 5 hygromycin selected plants giving green progeny.

Test for correlation between the activity and methylation state of *Ac*: In maize it has been shown that loss of *Ac* activity is correlated with C-methylation at a number of restriction enzyme recognition sites including, *Pvu*II, *Sac*II and *Hpa*II (SCHWARTZ and DENNIS 1986; CHOMET, WESSLER and DELLAPORTA 1987; KUNZE, STARLINGER and SCHWARTZ 1988). A sample of the Arabidopsis transformants was tested to determine if methylation of the *Ac* elements might explain their low activity level. DNA was isolated from hygromycin resistant or variegated T2 progeny of transformants with no *Ac* activity (B220, B223 and B229), low *Ac* activity (B236 and C230) or higher *Ac* activity (C201, C204, C205 and B228), as determined by the frequency of variegated seedlings in the streptomycin assay. Figure 4 shows that the 2.6-kb band generated by cleavage at both *Pvu*II sites within *Ac* was the major species in all of the transformants tested, indicating little or no methylation at these sites. Transformant B220 has been shown to carry an *Ac* element truncated at the 5′ end (data not shown), and even this inactive transposon was not methylated. Higher molecular weight fragments were detectable only in the lanes containing DNA from progeny of C201 and C205 (Figure 4, lanes 1 and 3), which had active *Ac* elements. These larger bands are most likely due to partial digestion, although the possibility that *Ac* is methylated in a subset of cells cannot be eliminated. The DNA from the T2 progeny was further tested for *C*-methylation using *Sac*II, but no correlation between loss of cleavage and *Ac* activity level was found (data not shown). Thus, the level of *Ac* activity in Arabidopsis is not correlated with *C*-methylation at the sites analyzed.

Molecular analysis of green selections: To confirm that the green phenotype on streptomycin occurred as a result of excision of the *Ac* element from

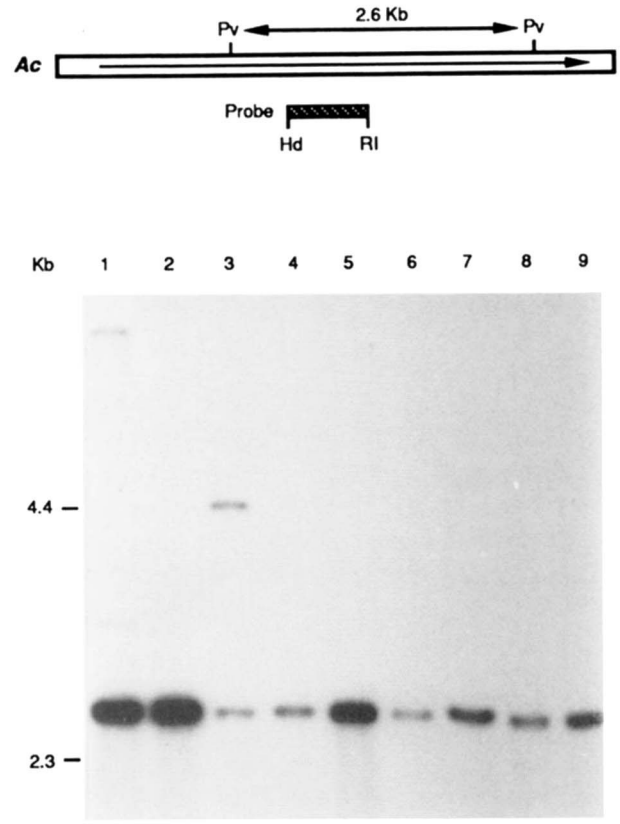


FIGURE 4.—Analysis of methylation of *Ac* in Arabidopsis. DNA was extracted and digested with *Pvu*II which should give a 2.6-kb band in the absence of methylation. The lanes contain DNA from the following F_2 progeny: (1) C201.32G, (2) C204.14V, (3) C205.1H, (4) B236.1V, (5) B228.1H, (6) B220.1H, (7) B223.1H, (8) B229.1H and (9) C230.1H.

the 35S-SPT gene, Southern blot analyses were performed, using *Ssp*I which restricts the T-DNA on either side of and within the *Ac* element, as shown in Figure 5. If a probe for the 35S promoter sequence is used, the unexcised T-DNA gives a characteristic 2-kb resident site (R.S.) band. When *Ac* excises, a 1.8-kb excision product or "empty" site (E.S.) is generated. Lane 7 of Figure 5 contains plasmid DNA for the 35S-SPT gene and shows the approximate size of the excision product. It is slightly smaller than the product of transposon excision in plants because the *Ac* element in 4404 and 4411 is flanked by about 80 bp of maize DNA which remains after excision. In all of the green progeny tested, an excision product was present (*e.g.*, lanes 1, 2, 3, 5 and 6). The DNA analyzed in lanes 1 to 3 was from green T2 progeny of transformants C201 and C204 which had multiple inserts of the T-DNA. Consequently, a resident band was detected from intact copies of the SPT::*Ac* gene, together with the excision product. However, green progeny of single insert transformant B222 did not show a resident site band (see Figure 5, lanes 5 and 6). A few of the variegated individuals that were tested had weakly detectable excision products (data not shown). A hy-

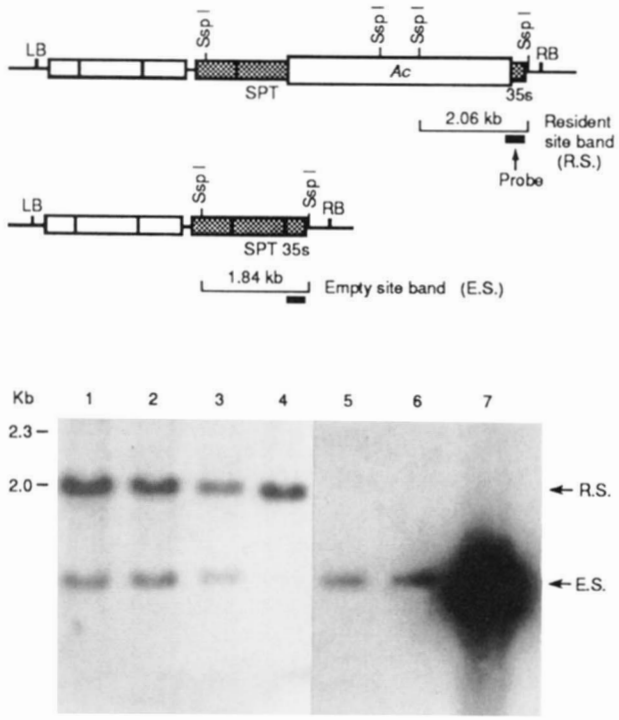


FIGURE 5.—Empty site analysis of germinal revertants. Genomic DNA was isolated from T2 progeny of C201 and C204 and T3 progeny of B222, digested with *Ssp*I and subjected to Southern blot analysis using the 35S promoter as a probe. The probe hybridizes to a 2.06-kb fragment prior to *Ac* excision (R.S.) and to a 1.84-kb band after *Ac* excision (E.S.) as is shown diagramatically at the top of the figure. Lanes 1, 2, 3, 5 and 6 contain DNA from streptomycin-resistant plants: (1) C204.26G, (2) C20412G, (3) C201.23G, (5) C222.2.1G and (6) C222.2.3G. DNA from a hygromycin resistant plant, C201.5H, is in lane 4. Lane 7 contains plasmid pJJ3995 DNA with an uninterrupted 35S-streptomycin gene.

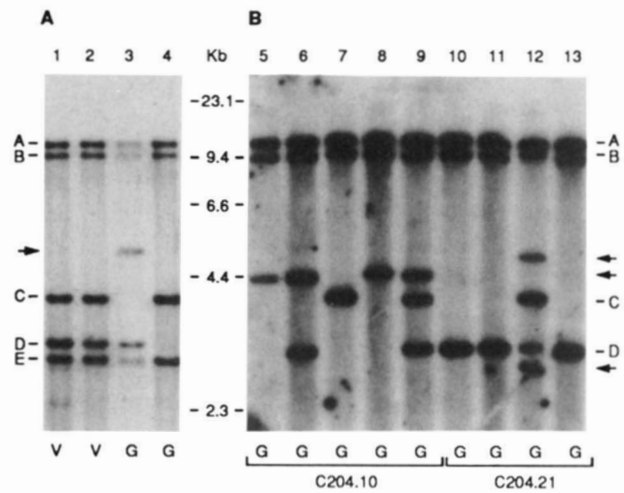


FIGURE 6.—Analysis of green progeny for transposed *Ac* elements. *Eco*RI-digested DNA from T2 (lanes 1–4) and T3 (lanes 5–13) progeny of C204 was analyzed by Southern blot. The positions of the five parental T-DNA border fragments A to E are indicated to the left and right of the figure. Reinserted *Ac* elements are indicated with arrows. Lanes 1 and 2 contain variegated progeny C204.14V and C204.15V. Lanes 3 and 4 contain green progeny C204.12G and C204.26G. Lanes 5–9 contain green progeny of C204.10V and lanes 10–13 contain green progeny of C204.21V.

gromycin-selected individual (Figure 5, lane 4) did not have an excision product.

The green phenotype was shown to be due principally to germinal excision of the *Ac* element from the SPT gene rather than to early somatic excision by testing progeny of the green selections for heritability of the streptomycin resistant trait. In 95% of the green selections that were progeny tested (174 out of 184), streptomycin resistance was inherited in a Mendelian fashion.

To tag genes with *Ac*, it is necessary for excised elements to reinsert into the genome. Reinsertion in our system was detected by Southern blot analysis of DNA from green streptomycin-resistant progeny. *Eco*RI-digested DNA was blotted and analyzed with an *Ac* 3′ probe (as shown in Figure 1). Reinserted *Ac*s were detected as new bands in the DNA from green progeny that were not observed in parental or variegated sibling plants. Examples of this type of analysis are shown in Figure 6. Lanes 1 and 2 of Figure 6A show the DNA banding pattern for two variegated T2 progeny of C204, C204-14 and C204-15. The five parental T-DNA borders are present in both individuals. DNA from green T2 progeny, C204-12 and C204-26, are shown in lanes 3 and 4. C204-12 (lane 3) had lost parental band C, either due to segregation or excision, and had a new *Ac* border of 4.5 kb. C204.26 (lane 4), which had lost parental band D, had no reinsertion band. Border fragment analysis of several green T3 progeny from C204.10V (lanes 5 to 9) and C204.21V (lanes 10 to 13) is shown in Figure 6B. Reinsertion bands of 4.4 kb and 4.8 kb (indicated by arrows) were detected in the progeny of C204.10 and C204.21, respectively. The green progeny of C204.21 shown in lane 12 may have a second reinsertion at 3.0 kb (bottom arrow, right) since DNA blot analysis showed that the parental E fragment (also 3.0 kb) was not present in C204.21 (data not shown). The *Ac* elements which had excised to give rise to the green revertants shown in lanes 7, 10, 11 and 13 had either not reinserted into the genome or had segregated away. Empty site border analysis (data not shown) indicated that in all green progeny of C204.10V and C204.21V, transposition was from either the C or D locus. Other analyses showed that the A locus also carries a transpositionally active *Ac* element.

Table 3 summarizes the results of the reinsertion analyses of 49 green progeny. Twenty-six out of 49 green progeny (53%) had reinsertion bands. This level of reinsertion is in the same range as that observed with *Ac* in maize (McCLINTOCK 1956; Greenblatt 1984; DOONER and BELACHEW 1989) and tobacco (JONES *et al.* 1990; DOONER *et al.* 1991). As can be seen in Table 3, the reinsertion bands in the progeny from any one parent are frequently the same. For example, only 1% of the self progeny of C204.10 were fully streptomycin resistant, but four of the five

TABLE 3

Summary of *Ac* reinsertion data

Name	Reinsertion (band size in kb)	Name	Reinsertion (band size in kb)
C201.23	+ (3.4)	C204.12	+ (4.7)
C201.42	+ (6.0)	C204.26	–
C201.29-15	–	C204.10-001	+ (4.4)
C201.29-03	–	C204.10-158	+ (4.4)
C201.29-04	+ (9.0)	C204.10-238	+ (4.4)
C201.35-10	+ (2.3)	C204.19-243	+ (4.4)
C201.35-05	–	C204.10-245	–
C201.35-06	–	C204.16-05	+ (4.6)
C201.37-01	–	C204.16-12	–
C201.37-02	–	C204.16-13	–
C201.38-03	+ (3.6)	C204.16-26	–
C201.38-07	+ (3.6)	C204.21-63	–
C201.38-13	–	C204.21-64	–
C201.38-15	+ (3.6)	C204.21-74	+ (4.8)
C201.38-19	–	C204.21-94	–
C201.38-23	–	C204.07-17	–
C201.39-07	+ (2.5)	C204.07-18	–
C201.39-10	+ (2.5)	C204.07-19	+ (8.8)
C201.39-17	+ (2.5)	C204.07-20	+ (3.5)
C201.39-18	+ (2.5)	C204.07-21	+ (15)
C201.39-21	+ (2.5)	C204.07-22	–
C222.02-01	+ (2.4)	C204.07-25	+ (25)
C222.02-03	+ (19)	C204.07-27	–
C222.02-04	–	C231.01-01	+ (2.4)
		C231.01-02	–

Total green progeny analyzed = 49; reinsertion events = 26.

progeny analyzed had the same reinsertion band (see also Figure 6B, lanes 5 to 9). The *Ac* 5′ end probe (shown in Figure 1) was also used to analyze DNA blots of the green revertants to determine if the progeny of a given parent had common 5′ reinsertion borders. This analysis confirmed that the similarity in 3′ border fragment size was not merely due to coincidental comigration (data not shown). These data are highly suggestive of premeiotic events giving rise to the green seedlings. For most of the T3 progeny from C201 and C204 T2 plants, the timing of premeiotic events during the development of the T2 plant cannot be determined because the seeds from several siliques were mixed and sterilized before plating. However, the green progeny of plant C204.07 were selected by plating individual siliques aseptically and only one green seedling from each silique was analyzed for reinsertion events. The data in Table 3 indicate that each transposed *Ac* detected using this approach was unique. More analysis will be necessary to determine if germinal transposition events can occur prior to the formation of the flowers and siliques.

DISCUSSION

The goal of this study was to better understand the activity of the maize transposable element *Ac* in Arabidopsis so that we can design experiments to tag genes of interest in this model plant species. Previous work has shown that the frequency of germinal *Ac* excision is generally low in Arabidopsis and the transposition activity of the element, as defined by germinal excision, remains low throughout three consecutive generations (SCHMIDT and WILLMITZER 1989). The SPT::*Ac* assay described in this study allowed detection, not only of germinal *Ac* transpositional activity, but also of somatic activity in generations subsequent to transformation. Twenty-eight independent transformants carrying one or more copy of *Ac* were analyzed and 19 of these showed no evidence of germinal transposition. However, all but four transformants (B106, B111, B211 and B229) had *Ac* elements which were active somatically, as determined by variegation in the presence of streptomycin. Somatic activity was not detectable in Mendelian ratios in Arabidopsis as it is in maize (MCCLINTOCK 1951) and tobacco (JONES *et al.* 1989, 1990) and was extremely low in most of the transformants containing one T-DNA insert.

To use transposons efficiently for insertional mutagenesis (*i.e.*, to avoid having to screen very large populations), germinal transposition activity should occur in 1% or more of the progeny. Germinal excisions were rarely detected in transformants carrying one copy of the *Ac* element. By comparing the results in Tables 1 and 2 corresponding to transformants with one and more than one *Ac* element, respectively, we have observed that germinal excision frequency was generally higher in plants containing more copies of the *Ac* elements. C201, which has three copies of *Ac* at one locus and thus six copies when homozygous, gave the highest frequency of green germinal revertants (Table 2). Germinal reversion frequencies are indicators of transposon activity in that they represent measurements of transposon excision. However, the critical parameter if a transposon is to serve as a tag in a heterologous system is the frequency with which the transposon reinserts in the new host genome. We have found that about 50% of the germinal excisions that were analyzed molecularly carried detectable reinsertions of the *Ac* element into the Arabidopsis genome. This fraction of reinsertions is similar to that detected in maize (GREENBLATT 1984) and tobacco (JONES *et al.* 1990; DOONER *et al.* 1991) and validates the use of *Ac* as a transposon tag in Arabidopsis.

The relative inactivity of the *Ac* element in Arabidopsis could be caused by a number of factors, including: (1) methylation of the element; (2) a lack of the host cell support functions required for transposition of the element in Arabidopsis; (3) incomplete or inaccurate splicing of the primary transcription product of the element; (4) inefficient translation of the mRNA; (5) weak *Ac* promoter activity in this heterologous system and (6) factors which modulate the

activity of the element. We will discuss these possibilities in light of our present results.

No evidence of methylation of *Ac* was detected in the transformants of Arabidopsis which were analyzed. Although the *Tam3* element from Antirrhinum has been shown to be inactive and methylated in tobacco (MARTIN *et al.* 1989), *Ac* remains hypomethylated in this plant species (NELSEN-SALZ and DORING 1990). It appears that *Ac* also remains unmethylated at the sites tested (*Pvu*II and *Sac*II) in Arabidopsis. Consequently, the low level of activity is probably not due to methylation.

The DNA-modifying enzymes, such as ligases, endonucleases and polymerases, which are required for transposition of the *Ac* element are undoubtedly present in Arabidopsis since some transposon activity is detectable in callus (VAN SLUYS, TEMPE and FEDEROFF 1987), in sporogenic tissue (SCHMIDT and WILLMITZER 1989; and this study), and in somatic tissue (this study). Our finding that the transformants containing more than one copy of *Ac* tend to have an increased number of transposition events suggests that the lack of activity is not due to a limiting amount of the host cell support functions required during excision of the transposon.

The low *Ac* activity in Arabidopsis could possibly be due to weakness of the transposase promoter, incomplete processing of the primary transcript or inefficient translation. Although, inaccurate splicing of some monocots genes has been shown to occur in dicotyledonous plant species (KEITH and CHUA 1986), the *Ac* element appears to be accurately spliced in tobacco (J. KELLER, unpublished results). Neither transcription levels of the *Ac* gene nor processing of the *Ac* transcript have been studied yet in Arabidopsis. However, the finding that transposition frequency, can be increased in plants carrying multiple copies of the SPT::*Ac* gene may indicate that the level of translatable mRNA generated by one or two copies of the gene is too low in most cells of the developing plant to allow detectable transposition to occur, but that greater gene dosage helps to overcome this limitation. The position of the T-DNA within the Arabidopsis genome may also affect the *Ac* promoter since there was considerable variability in transposon activity among transformants carrying the same number of *Ac* copies.

We have observed that the number of sectors per seedling does not fit the expected Poisson distribution based on the random occurrence of an infrequent event. This finding suggests that there may be regulatory factors in the Arabidopsis genome which limit transposition activity of *Ac*. Further studies will be necessary to determine what these factors are and how they function.

The results of this study indicate that *Ac* should work as an insertional mutagen in Arabidopsis. However, it may be desirable to increase the activity of the element by elevating the number of *Ac* copies in the genome or, alternatively, by using a two-element system in which the transposase is transcribed from a stronger promoter. *Ac* cDNA might be used to eliminate any inefficiencies in the processing of this monocot gene in Arabidopsis. To increase the chances of tagging genes of interest in a mutational screen, a large number of progeny carrying independent germinal transpositions must be generated. Since our studies indicate that germinal events frequently occur premeiotically, such that sibling plants carry the same transposed *Ac*, care must be taken to germinate seeds from individual siliques separately and use revertant progeny which appear from their segregation ratios to be independent. If the above considerations are kept in mind while planning gene tagging experiments, *Ac* should constitute a powerful gene isolation tool in Arabidopsis.

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