Mol Gen Genet (1989) 220:17–24

MGG

The maize autonomous element *Activator* (*Ac*) shows a minimal germinal excision frequency of 0.2%–0.5% in transgenic *Arabidopsis thaliana* plants

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Summary. The autonomous mobile element *Activator* from *Zea mays* was introduced into *Arabidopsis thaliana* via *Agrobacterium*-mediated gene transfer. The use of a chimaeric construct, where the *Ac* element is located in the leader of the neomycin phosphotransferase (NPT II) gene, enabled the excision of *Ac* to be monitored by assaying for the reconstitution of NPT II gene activity. Using this approach, the transpositional activity of *AC* was initially studied in primary transformants. About 50% of the regenerating *Ac* transformants showed evidence for excision of the element. Reintegration of *Ac* was confirmed by Southern blot analysis. Transposition events are transmitted to the F1 generation with a minimal frequency of 0.3%. In a few exceptional cases they are detected in a high proportion of the F1 generation. Seedlings from the F2 and F3 generations were assayed for the rate of germinal excisions by scoring for kanamycin resistance. The minimal frequency of germinal excision events amounts to 0.2%–0.5% and hence allows the use of the *Ac* element for gene tagging purposes in *A. thaliana*.

Key words: *Ac* transposition – Gene tagging – Germinal excision frequency – Transgenic *Arabidopsis thaliana*

Introduction

Arabidopsis thaliana is receiving increasing attention as a model system for studies of plant molecular biology. The main advantages of this small cruciferous plant are its short generation cycle, its high seed production, which coupled to self-fertilization, results in rapid recognition of recessive mutations, and its small genome size (for review see Meyerowitz and Pruitt 1985; Pang and Meyerowitz 1987). In addition, numerous mutants induced by chemicals or X-ray treatment have been identified in this plant species (for review see Estelle and Somerville 1986).

Molecular identification of genes via insertion mutagenesis is generally regarded to be a powerful tool in various eucaryotic systems. In plants both the T-DNA of *Agrobacterium tumefaciens* (Feldmann et al. 1989) and mobile elements have been used for this purpose. The use of mobile elements for the isolation of genes has up to now been restricted to *Antirrhinum majus* (e.g., see Martin et al. 1985) and *Zea mays* (e.g., see Fedoroff et al. 1984; O'Reilly et al. 1985; Theres et al. 1987). There are two main reasons for this: firstly various endogenous mobile elements such as the *Tam* elements, *Ac/Ds*, *En/Spm* or *Mutator* are well characterized for both species, and secondly the genetics of both species is highly developed. We decided to use the maize element *Activator* (*Ac*) in *Arabidopsis thaliana*. The reason for this was that it has already been shown that *Ac* can transpose in various heterologous plant species (Baker et al. 1986; Yoder et al. 1988; Knapp et al. 1988), including *A. thaliana* (Van Sluys et al. 1987). Also, the use of an alien element allows the introduction of low or single copies of this element into the plant, which obviously facilitates the subsequent genetic analysis.

Although *Ac* has been shown to transpose in a variety of heterologous plant species, no detailed analysis of the transpositional activity of *Ac* in subsequent generations has been performed. On the contrary, most studies were exclusively devoted to the analysis of transposition in the primary transformants. The introduction of mobile elements in most cases is performed using the *Agrobacterium* system or direct gene transfer, both of which involve extensive periods of tissue culture including dedifferentiation and redifferentiation steps. Under these conditions, however, independent mutations arise with high frequencies. It has been suggested that such mutations (under the synonym "somaclonal variations") might even be used as a tool for increasing variability of relevance to plant breeding (Larkin and Scowcroft 1981). Mutations that occur very early after transformation and therefore that are manifested in the F1 progeny could either be due to a mutation induced by the period of tissue culture, i.e., somaclonal variation, or to the transposition of the mobile element.

A study aimed at estimating the feasibility of a transposon-tagging approach should include analysis of the transposition frequency during vegetative growth of the regenerant and during subsequent generations. More importantly, it is not the general transposition rate that is of prime interest but rather the number of excisions that are transmitted to the progeny. Here we describe analysis of the transpositional activity of *Ac* in transgenic *Arabidopsis thaliana* plants. Both the rates of early transposition and the rate of transposition occurring later during development or in subsequent generations are estimated with respect to their transmission to the progeny. The data obtained show that transpositions occurring late in development in the original transformant or in subsequent generations of the original

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transformant are transmitted to the progeny at a minimal rate of about 0.2%–0.5%.

Materials and methods

Plant materials. A. thaliana genotype C24 was used for all transformation experiments, and was kindly provided by J.-P. Hernalsteens (Vrije Universiteit Brussels). The culture conditions for the plants and the media used were described previously (Schmidt and Willmitzer 1988).

Leaf disc transformation. Agrobacterium-mediated transformation of *A. thaliana* leaf or cotyledon explants was performed as described by Schmidt and Willmitzer (1988) using the *Agrobacterium* strain GV3850Hygro::pKU3 (Baker et al. 1987). *Ac* transformants were selected on 20 µg/ml hygromycin B.

Tissue culture selection for transposition. To select for transposition events in the progeny of *Ac* transformants, seedlings were germinated on kanamycin-containing (50 µg/ml) MS medium (Murashige and Skoog 1962) with glucose (1.6%) as carbon source; seedlings were scored for a resistant phenotype. Resistant seedlings developed a normal root system and the leaves showed no bleaching under the selection conditions.

Isolation and analysis of DNA. Genomic DNA was isolated according to Murray and Thompson (1980). After digestion with restriction enzymes the DNA was separated on agarose gels and blotted to Biodyne B membranes using alkaline transfer conditions. Hybridizations with radioactively labelled DNA fragments (Feinberg and Vogelstein 1983) were performed following the protocol of Amasino (1986).

Neomycin phosphotransferase activity test. The enzyme activity of chimaeric NPT II genes in transformed plant material was analysed according to Reiss et al. (1984) with the modifications of Schreier et al. (1985).

Results

General strategy

Baker et al. (1987) have developed a phenotypic assay for monitoring the transposition of *Ac* in transgenic tobacco plants, based on the use of a chimaeric neomycin phosphotransferase gene containing the *Ac* element inserted in the 5′-untranslated leader. This insertion inactivates the NPT II gene in tobacco cells (Baker et al. 1987) as well as in *Arabidopsis* cells (cf. Fig. 1). Transposition of *Ac* leads to the reconstitution of an active NPT II gene. The latter can be detected either biochemically by its enzymatic activity (Reiss et al. 1984), or through the fact that it confers resistance to kanamycin to the transgenic plantlets; the resistance can easily be scored in tissue culture. This construction was used throughout as expression of the neomycin phosphotransferase gene is highly correlated with transposition of *Ac* (see below). This allowed us not only to monitor biochemically the activity of *Ac*, but also to determine the frequency of germinal excision events by selecting for kanamycin-resistant seedlings.

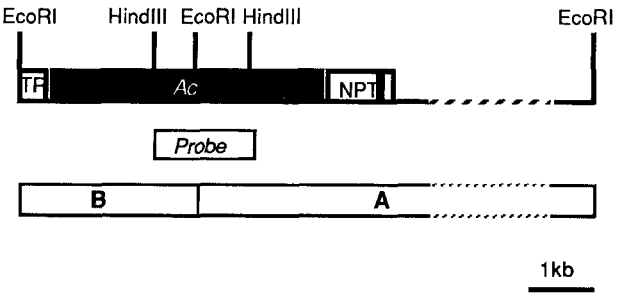

Fig. 1. Location of *Eco*RI cleavage sites in the T-DNA region of the *Agrobacterium* strain GV3850Hygro::pKU3 (Baker et al. 1987) encoding the neomycin phosphotransferase (NPT II) gene. The *Ac* element is located downstream from the TR promoter in the leader of the NPT II gene. The 3′ untranslated region of the octopine synthase gene and T-DNA sequences from pGV3850Hygro are situated downstream of the NPT II gene. The two *Eco*RI/*Eco*RI fragments (designated A and B) give a signal in genomic southern blots of *Ac* transformants probed with the *Hin*dIII/*Hin*dIII *Ac* sequences shown, if the element is still retained in its original position within the T-DNA

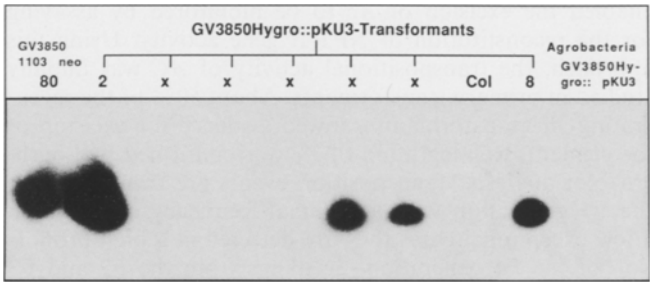

Fig. 2. Appearance of NPT II activity in primary transformed tissue. Regenerating calli from 7 independent *Ac* transformants were tested for enzyme activity. Untransformed *Arabidopsis* tissue (Col) and the *Agrobacterium* strain used for transformation (GV3850Hygro::pKU3) showed no NPT II activity whereas 4 *Ac* transformants (for example 2 and 8) displayed NPT II activity. Lane 1 (GV3850 1103 neo-80) serves as a positive control and contains extracts from a transgenic *Arabidopsis* plant transformed by the *Agrobacterium* strain GV3850 1103 neo (Hain et al. 1985) containing a chimaeric NPT II gene under the control of the nopaline synthase promoter

Transposition of Ac *as indicated by NPT II activity can be detected in 50% of the primary transformants*

In order to estimate the transpositional activity of *Ac* in primary *A. thaliana* transformants, independently regenerating calli derived from a transformation with *Agrobacterium* strain GV3850Hygro::pKU3 were analysed. This strain contains a chimaeric hygromycin gene as a selectable marker, in addition to the chimaeric neomycin phosphotransferase gene carrying the *Ac* element inserted in its 5′-untranslated leader (Baker et al. 1987). Of 37 hygromycin resistant calli analysed, neomycin phosphotransferase activity was detectable in 19 cases (cf. Fig. 2 for a representative result). As no NPT II activity is detected in untransformed *Arabidopsis* leaves or in the *Agrobacterium* strain used (Fig. 2) this result strongly indicates that the NPT II activity is due to the expression of a reconstituted NPT II gene in the transformed *Arabidopsis* cells, which in turn is a strong indication for the transposition of *Ac* (see also below).

Southern blot analysis of Ac *transformants*

The assay for NPT II activity allows biochemical testing for the reconstitution of the NPT II gene most probably arising due to excision of the *Ac* element; however no conclusion can be drawn as to whether or not the *Ac* element has reinserted into the genome. Therefore, genomic DNA of several independent primary *A. thaliana* GV3850Hygro::pKU3 transformants was analysed on Southern blots. There is one internal *Eco*RI site in the *Ac* element, as well as two *Eco*RI sites, one located upstream from the TR promoter, and one within the T-DNA downstream from the 3′ untranslated region of the octopine synthase gene (Fig. 1). Two fragments with sizes of 11.5 kb (A in Fig. 1) and 3.0 kb (B in Fig. 1) hybridize to a *Hin*dIII/*Hin*dIII fragment spanning the internal *Eco*RI site of the *Ac* element on condition that *Ac* is still present in its original location. As a result of a transposition, two new bands of unpredictable size would be expected, because the flanking *Eco*RI sites have to be provided by the plant DNA into which *Ac* has transposed.

Genomic DNA was isolated from regenerated *Ac* transformants and restricted with *Eco*RI. Using the *Ac Hin*dIII/*Hin*dIII fragment as a probe, strong hybridizations to the *Eco*RI fragments A and B were detectable (Figs. 3 and 4), showing that the *Ac* element is still in its original location in the leader of the NPT II gene. In a number of cases, however, additional bands indicative of transposition events were detectable (Fig. 3, transformants 64, 75, 77 and 81 and Fig. 4, transformants 74 and 89). The additional hybridizing fragment with a size of about 4.5 kb in transformant 75 gives a strong signal (Fig. 3). This is most probably due to aberrant integration of the T-DNA, as a TR promoter probe detects an *Eco*RI/*Eco*RI fragment of the same size (data not shown).

The intensity of the signals for additional *Ac* fragments were in most cases weaker than those corresponding to fragments A and B. This most probably reflects integration of more than one T-DNA copy into the *Arabidopsis* genomic DNA, as seen by segregation data for hygromycin resistance of the *Ac* transformants and hybridizations of genomic blots with a border probe (data not shown). On the other hand, depending on the timing of excision and the regeneration capacity of the cells that contain a transposed copy of the element, the regenerating callus might represent a chimaeric tissue with respect to the transposed *Ac* copy.

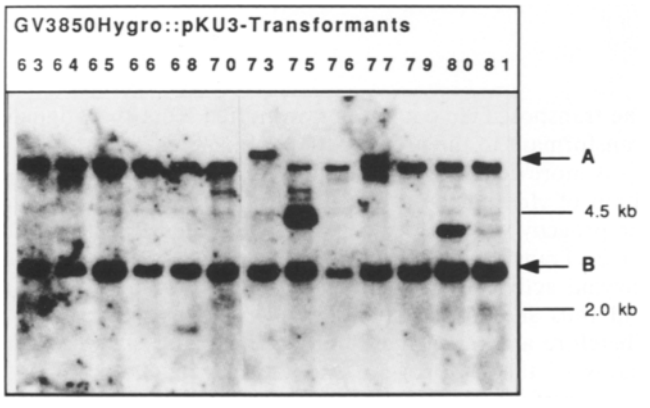

Fig. 3. Southern blot analysis of independent *Ac* transformants. The *numbers* along the top identify different independent primary *Arabidopsis thaliana* transformants harbouring the chimaeric NPT II/*Ac* construct. *Eco*RI-restricted genomic DNA was hybridized to the *Hin*dIII/*Hin*dIII fragment containing an internal part of the *Ac* element (see Fig. 1). The bands marked by *arrows* correspond to fragments A and B of Fig. 1. Some of the transformants show additional hybridizing bands of weaker intensity, indicative of transposition events that occurred during regeneration of the transformants (e.g. transformants 64, 75, 77 and 81)

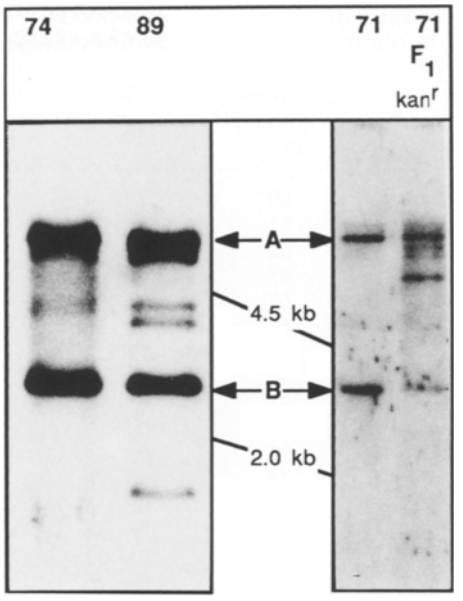

Fig. 4. Southern blot analysis of original *Ac* transformants which in their F1 or F2 progeny gave rise to kanamycin resistant seedlings. Genomic DNA restricted by *Eco*RI was hybridized with the internal *Ac* probe (see Fig. 1). The *numbers* 74, 89 and 71 identify primary transformants of *Arabidopsis thaliana*. The lane 71 F1 kan^r shows hybridization of the genomic blot of the one descendant of transformant no. 71 in the F1 progeny which was resistant to kanamycin (cf. Table 1). In addition to fragments A and B it contains other *Ac* hybridizing bands indicative of transposition

Transmission of excision events to the F1 generation of Ac *transformants*

During the regeneration regime of the *Ac* transformants no selection was applied for cells containing the reconstituted NPT II gene which is indicative of the excision of an *Ac* copy. An important consideration is the frequency by which transposition events occurring during regeneration of transformants are transmitted to the progeny. Therefore the F1 progenies of 30 independent original *A. thaliana* GV3850Hygro::pKU3 transformants were analysed for *Ac* excision by plating seeds on kanamycin-containing (50 μg/ml) medium. The numbers of resistant and sensitive plantlets obtained are summarized in Table 1. While a number of transformants did not give rise to any kanamycin-resistant plantlets (Table 1, for example transformants 64 and 75) seven transformants gave rise to one or more resistant seedlings in the F1 progeny. All resistant seedlings showed clear NPT II activity (data not shown). Furthermore, the progeny analysis of these plantlets for kanamycin resistance revealed segregations typical for a dominant Mendelian trait (Table 2). In order to confirm that kanamycin resistance is due to transposition of an *Ac* element, genomic DNA was isolated from some kanamycin-resistant plants, restricted with *Eco*RI and probed with the

Table 1. Appearance of kanamycin-resistant plants in F1, F2 and F3 generations of transgenic *Arabidopsis thaliana* plants containing the chimaeric NPT II/*Ac* construct

Transformant[a] no.	F1 generation[b]	Transformant[a] no	F2 generation[c]	Transformant[a] no.	F2 generation[c]	Transformant[a] no.	F3 generation[d]
60	3: 46	60	2: 4	103	0: 61	62	0: 30
61	0: 22	61	0: 21	106	3: 26		0: 13
62	0: 16	62	0: 6	107	122: 0	65	0: 29
63	0: 194	65	0: 3	110	1: 32		0: 56
64	0: 127	67	0: 13	111	0: 14		0: 39
65	0: 16	68	0: 55	115	0: 30		0: 68
66	0: 155	69	1: 122	118	0: 99	69	1: 67
67	1: 184	70	1: 8	121	0: 121		0: 23
68	0: 104	71	0: 10	126	0: 9		0: 123
69	0: 188	72	0: 3	129	2: 75		0: 142
70	0: 132	73	0: 24	133	0: 186		0: 120
71	1: 79	74	29: 8	134	0: 18	77	0: 24
72	0: 28	75	0: 6	135	0: 12		5: 43
73	0: 7	77	0: 54	136	0: 16		0: 39
74	15: 166	79	0: 32	140	0: 165		0: 105
75	0: 71	81	0: 30	142	15: 8	81	1: 82
76	0: 103	84	0: 55	145	6: 0		0: 48
77	1: 111	87	0: 21			118	0: 10
79	0: 58	89	52: 0				0: 70
80	0: 69	90	0: 7				0: 10
82	3: 111	91	0: 84				0: 78
83	1: 23	92	0: 16			133	0: 118
84	0: 51	93	70: 0				0: 134
94	0: 37	94	1: 94			136	0: 28
96	0: 41	95	4: 5				0: 59
100	0: 21	96	0: 105				0: 85
113	0: 32	98	24: 0				0: 34
116	0: 25	99	0: 25				1: 112
140	0: 60	101	0: 18				
141	0: 25	102	28: 0				

[a] The numbers identify independent primary transformants of the *Agrobacterium* strain harbouring the chimaeric NPT II/*Ac* construct
[b] From 1 to 4 shoots derived from independently regenerating calli were used for seed production. Seeds from individual transformants were harvested separately and germinated on kanamycin-containing (50 µg/ml) medium in order to select for germinal excision events. Scoring was performed 4–5 weeks after plating. The segregation data show kanamycin-resistant versus kanamycin-sensitive plants
[c] Seeds were harvested from 1 hygromycin-resistant F1 plant of each individual transformant and tested for kanamycin-resistance (see the Materials and methods)
[d] Seeds derived from 2 to 5 F2 plants of 8 independent transformants were harvested and tested for kanamycin resistance. Each line represents segregation of seeds from an individual F2 plant

*Hin*dIII/*Hin*dIII *Ac* fragment. An example is shown in Fig. 4. Whereas no additional *Ac* fragments are detected in the original transformant no. 71, the kanamycin-resistant F1 plant reveals *Eco*RI fragments indicating transposition of *Ac*. This result supports the interpretation that kanamycin resistance is caused by *Ac* transposition and that NPT II activity can be used as a reliable marker for the excision of the element.

Comparison of the Southern blot analysis and the segregation data reveals that not all transposition events occurring in the original transformants were transmitted to the progeny. Transformants 64 and 75 for example (Fig. 3) show *Eco*RI fragments indicative of *Ac* transposition, but no kanamycin-resistant plant was obtained in the progeny of these transformants (Table 1). Thus those cells containing the transposed copy of the element did not give rise to a fertile shoot during regeneration of the original transformant. Transformant 74, in contrast, shows kanamycin-resistant progeny and additional *Ac* hybridizing fragments (Table 1, Fig. 4). It could be demonstrated that at least one transposed *Ac* copy was transmitted from the original transformant to the progeny (data not shown).

A more detailed analysis aimed at studying the transposition of *Ac* and the transmission of transposed copies to the progeny was performed with the original transformant no. 2. Transformant 2 gave a clear signal in the NPT II enzyme activity assay early after transformation (Fig. 2) and thus should contain cells where *Ac* has transposed. Therefore a comparative Southern blot analysis of eleven plants of the F1 progeny and the original transformant 2 was performed. The result of a hybridization of *Eco*RI-digested DNA to the *Hin*dIII/*Hin*dIII *Ac* probe is shown in Fig. 5. The presence of the 11.5 and 3.0 kb band in the original transformant indicate that an *Ac* element is located at its original position in the T-DNA. Two additional bands are visible, indicative of a transposition of *Ac*. This is in accordance with the NPT II activity detected in the original transformant (Fig. 2).

Concerning the F1 plants a complex picture emerges, because in the original transformant at least four copies

Table 2. Progeny analysis of plants displaying germinal excision of *Ac*

Transformant[a] no.	F1 generation[b]	F2 generation[c]
60	3: 46	10: 4
		3: 0
67	1: 184	31: 9
74	15: 165	109: 8
		129: 40
		45: 0
		103: 0
		14: 2
		111: 0
		17: 9
		58: 16
		30: 0
		91: 0
83	1: 22	55: 13

[a] The numbers identify independent primary *A. thaliana* transformants harbouring the chimaeric NPT II/*Ac* construct

[b] Seeds from regenerated shoots were harvested separately from individual transformants. To select for germinal excision events, seeds were plated on kanamycin-containing (50 µg/ml) medium. The plates were scored after 4–5 weeks. Only fully resistant plantlets (see the Materials and methods; cf. Fig. 7) were considered resistant to kanamycin. The segregation data show kanamycin-resistant versus kanamycin-sensitive plantlets

[c] Seed from kanamycin-resistant F1 plants were harvested and scored for kanamycin resistance (see above). Each line represents segregation of seeds from an individual F1 plant

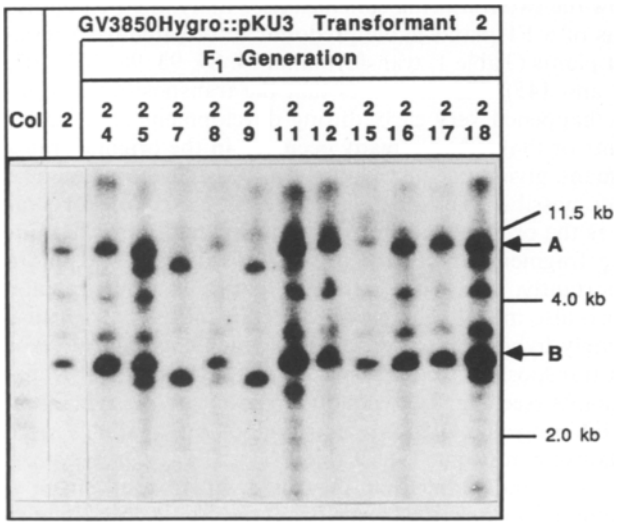

Fig. 5. Southern blot analysis of F1 progeny of plants of transformant nos. 2 and 11. Genomic DNA was restricted by *Eco*RI and hybridized to the internal *Hin*dIII/*Hin*dIII fragment of the *Ac* element (see Fig. 1). A and B represent the *Ac* fragments obtained when the element is located in its original position in the leader of the NPT II gene. Additional hybridizing fragments indicate transposition events. No hybridization is detected when DNA from a non-transformed control (Col) is used

of the *Ac* element were integrated via the T-DNA at a minimum of two independent loci in the *Arabidopsis* genomic DNA (data not shown). Five out of the eleven plants analysed (plants 2/4, 2/12, 2/15, 2/16 and 2/17) show the same hybridization pattern as that observed in the original transformant. In four of the F1 progeny (plants 2/5, 2/8, 2/11 and 2/18) two additional bands with molecular weights of about 8.0 and 2.7 kb appear, this being indicative of a second independent transposition event which was not detected in the original transformant. Two F1 plants contain only these two bands (plants 2/7 and 2/9).

These data demonstrate that a transposition event occurring early after transformation, and which therefore is detectable on a genomic blot in the original transformant, can be fixed at a high frequency in the subsequent F1 generation, i.e. in 9 out of 11 cases analysed. Furthermore, in this transformant at least one additional independent transposition event has occurred, giving rise to the *Ac* hybridizing bands with molecular weights of approximately 8.0 and 2.7 kb. This transposition was fixed in 6 out of 11 F1 plants,

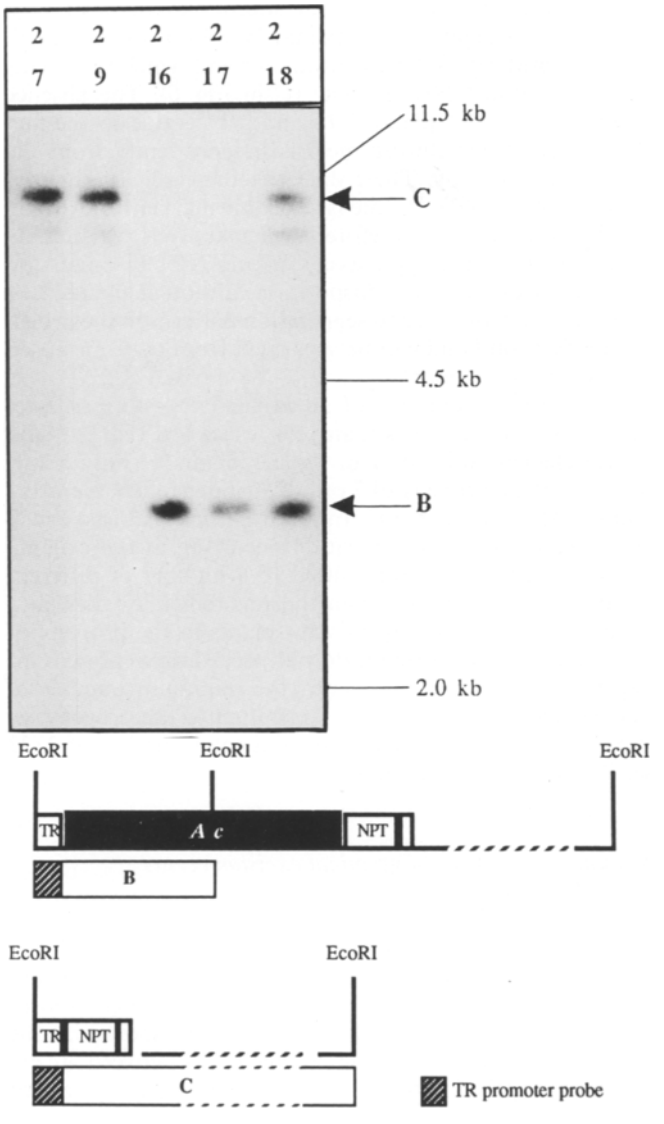

Fig. 6. Southern blot analysis of F1 progeny of the original transformant no. 2. Genomic DNA was hybridized, after *Eco*RI restriction, to the TR promotor fragment. Fragment B represents the *Eco*RI/*Eco*RI fragment when *Ac* is located in its original position in the leader of the NPT II gene, whereas fragment C represents the reconstituted NPT II gene after excision of the element (see *lower* panel). The bands marked by *arrows* correspond to fragments B and C, respectively

and therefore is at a lower frequency than that observed for the first transposition event. Together with the failure to detect these bands in the original transformant, this might indicate that the second transposition event occurred later during plant development.

All 11 F1 plants were analysed for NPT II activity. With the exception of the two plantlets, 2/16 and 2/17, all displayed clear enzymatic activity (data not shown). Genomic blots were performed to confirm the reconstitution of the NPT II gene at the DNA level. The TR promoter fragment was used as a probe for *Eco*RI-cut genomic DNA of the F1 plants of transformant 2 (Fig. 6). As expected, the promoter probe hybridized to a band with a molecular weight of 3.0 kb in the genomic DNA of plants 2/16, 2/17 and 2/18 (B in Fig. 6) but not in that of plants 2/7 and 2/9, as the latter do not contain an *Ac* element located in the leader of the NPT II gene (see above). In all transformants tested except 2/16 and 2/17 an additional fragment with a molecular weight of approximately 9.9 kb was observed, which hybridizes to the TR promoter and which represents the reconstituted NPT II gene (C in Fig. 6). The absence of this band in F1 plants 2/16 and 2/17 is due to the fact that it segregated during meiosis independently from the transposed *Ac* copy. These data together indicate that expression of the NPT II gene is a reliable marker for excision of the *Ac* element. Therefore further analysis was mainly based on this phenotypic assay, taking NPT II expression as an indicator of *Ac* transposition, although plants 2/16 and 2/17 show that due to segregation patterns in the progeny the excision band can be separated from the transposed copy of the *Ac* element.

The progeny analysis of 30 original transformants revealed, on the basis of kanamycin resistance, that excision of the element was fixed in the F1 plants at only a low rate, i.e. in the progeny of 7 out of 30 independent transformants tested (see Table 1). In a few exceptional cases such as transformant 2, transposition occurring in the original transformant can be transmitted to a number of different F1 plants (see Fig. 5). When the possibility is taken into account that kanamycin-resistant plants in the progeny of an individual transformant do not necessarily represent independent transposition events, the minimum number of independent excision events transmitted to the progeny, as shown in Table 1, equals 7 out of a total number of 2327 seedlings, resulting in a frequency of 0.3%.

Minimal frequencies of germinal excision events are as low as 0.2%–0.5%

Analysis of the F1 progeny revealed that the vast majority of the seedlings were kanamycin sensitive, indicating that the integrated *Ac* elements were still in their original location in the leader of the NPT II gene. As shown by the data described above a close linkage exists between *Ac* excision and NPT II activity, although the possibility cannot be excluded that a low level of NPT II activity could also be due to a rearrangement of the gene not connected to excision of *Ac*. These plants represent ideal stock for looking for *Ac* transposition in subsequent generations, because the progeny simply needs to be selected for reconstitution of the NPT II gene. Therefore F2 seed from 47 randomly chosen hygromycin-resistant F1 plants, each derived from an independent transformant, was germinated on kanamycin-containing medium. The data summarized in Table 1 allow the two following conclusions. Firstly, the F2 populations of 8 F1 plants show predominantly kanamycin-resistant plants (Table 1, transformants 74, 89, 93, 98, 102, 107, 142 and 145). This indicates that the transposition had either happened very early during development of these F1 plants or that it had already occurred in the original transformant, giving rise to a kanamycin-resistant F1 plant. The latter possibility is most probably true for transformant 74, as the original transformant showed additional hybridizing fragments indicative of *Ac* transposition and, more importantly, gave rise to kanamycin-resistant F1 plants. This is also most likely the case for transformant 89 because strongly hybridizing additional *Ac* fragments indicative of two transpositions could be detected in the original transformants (see Fig. 4). Secondly, in 8 out of the remaining 39 F2 progenies a low proportion of F2 seedlings were kanamycin resistant (see Table 1 and Fig. 7 for a typical selection), indicating that they most likely result from a germinal transposition event. From these data an estimated minimal frequency of germinal excisions is 0.5% (see Table 1).

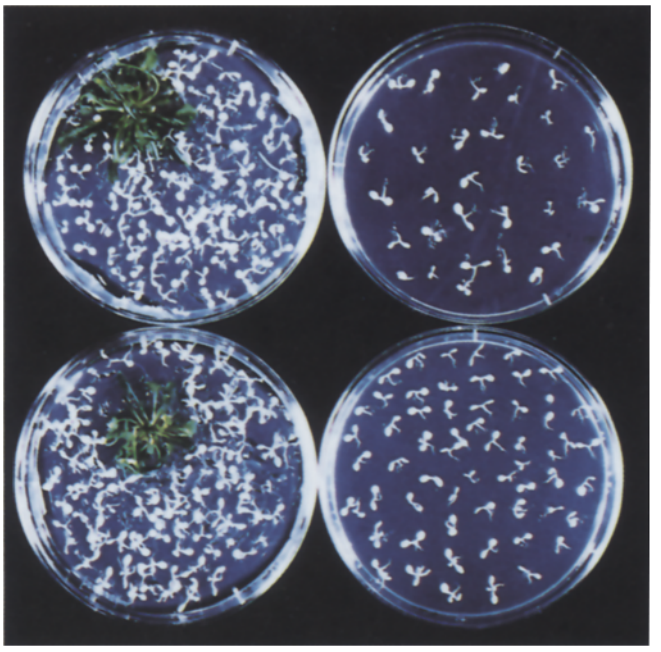

Fig. 7. Selection for germinal excisions in tissue culture. F2 seedlings were selected on kanamycin-containing medium (50 µg/ml). F2 seeds were derived from transformants no. 69 (*upper left*), 94 (*lower left*), 79 (*upper right*) and 103 (*lower right*) (cf. Table 1)

To verify the occurrence of *Ac* excision in subsequent generations the progeny of 28 F2 plantlets originating from 8 independent transformants were tested for kanamycin resistance. Of the 1797 F3 seedlings plated (see Table 1), 8 were resistant to the antibiotic, which is equivalent to a minimal excision frequency of 0.4%. However, if as described above several kanamycin-resistant plants are derived from one transposition event, the resulting minimal excision frequency is 0.2%. This is in agreement with the frequencies obtained from the F1 and F2 plants and suggests that the *Ac* element remains active throughout several generations in the heterologous host *A. thaliana*.

Discussion

Isolation of plant genes by transposon tagging is potentially a powerful technique. In maize several genes have been identified in this way. Particularly in cases where the biochemical product was previously unknown, as in the case of a controlling gene such as the *c* locus (Paz-Ares et al. 1986), it has proven to be a useful tool. The transposon tagging strategy might be even more powerful when applied to a smaller sized target. Thus applying this concept to *A. thaliana* which has a genome size of 7×10^7 base pairs (Leutwiler et al. 1984), should, under the assumption of completely random insertions, result in a higher frequency of insertional mutations compared to maize.

In this study we analysed transpositional activity of the maize-derived element *Activator* (*Ac*) in transgenic *Arabidopsis* plants. The reason we chose *Ac* was that this element has been demonstrated to transpose in a variety of heterologous plant species such as tobacco (Baker et al. 1986), tomato (Yoder et al. 1988), potato (Knapp et al. 1988) carrot and also *Arabidopsis* (Van Sluys et al. 1987). Most of these studies have been mainly concerned with analysis of the transpositional activity of *Ac* in primary transformants. As a general observation, in about 25%–75% of the primary transformants *Ac* had transposed. This finding is in agreement with the data described here showing that in about 50% of the transformed primary regenerants *Ac* had already excised.

Interesting as these data are for an understanding of the mechanism of *Ac* transposition, they provide no answer to the question whether the *Ac* element can be used in *A. thaliana* in order to identify genes by insertional mutagenesis. As already outlined in the Introduction, one major problem in applying insertion mutagenesis for identifying plant genes has been the fact that independent mutations are induced at a high rate. This is probably related to the tissue culture conditions needed for introducing genes into plants. Whatever the cause, the result is a significant reduction in the probability of a mutant phenotype being created by the insertion of DNA. This problem can be circumvented by the use of a transposable element, but only when the excision events occurring in subsequent generations and not the ones appearing in the primary transformants are used for screening of mutants. The reason for this assumption is that mutant phenotypes identified as coinciding with the transposition of the mobile element in later generations are very probably caused by the transposon, as after regeneration of the primary transformants no further tissue culture steps are needed.

All current assays that allow screening and/or selection for the transposition of a mobile element do not actively select for the transposed element, but rather for the reconstitution of an empty donor site. Therefore to identify transposition events occurring in subsequent generations a screening is needing for regenerants and/or F1 plants where *Ac* has not yet transposed. The descendants of these regenerants are then followed through the F1 and subsequent generations in order to select and/or screen for transpositions. Obviously, for this approach the transposition rate observed early after transformation is irrelevant. It is rather the transposition rate observed later on which is of primary importance. Furthermore, not the transposition rate per se, but the number of transposition events transmitted to the next generation is the major determinant which decides upon the feasibility of the whole transposon-tagging approach.

In order to get an idea of the frequency at which *Ac* excisions are transmitted to the progeny, seed of 30 primary transformants was germinated in the presence of kanamycin to select for resistant F1 plants. In total, 25 kanamycin-resistant seedlings could be detected in the progeny of 7 primary transformants. In the case of transformants 2 and 74, it was shown that excision events happening early after transformation can be transmitted to several independent F1 plants. Therefore a minimal estimate of the frequency of excision events transmitted to the F1 generation has to account for this fact, and this minimal excision frequency amounts to 7 out of 2327 seeds tested or 0.3%. However, due to the fact that an excision event happening in somatic cells can be cloned via the regeneration of shoots and give rise to kanamycin-resistant progeny, the data do not reflect the situation in a normal growing *A. thaliana* plant. More accurate estimates for germinal excision frequencies are therefore obtained from the F2 or F3 generations. The minimal rates obtained are 0.5% for the F2 progeny and 0.2% for the F3 progeny and do not deviate from the data of the F1 generation. Thus analysis of the progeny reveals that *Ac* transposes throughout several generations in *A. thaliana* – a prerequisite that has to be fulfilled for transposon tagging. An important question is the general validity of the estimated frequency of germinal excisions. For 21 out of the 58 transformants tested we have evidence for germinal excisions. Furthermore for 19 transformants that gave no kanamycin-resistant seedlings in the F1, F2 or F3 generations, somatic excisions were seen when plants were put back into tissue culture thus showing that the *Ac* element is able to excise in all transformants tested (R. Schmidt, unpublished observations). Hence all *Ac* transformants are to be considered in the estimation of the frequency of germinal excisions.

Analysis of the transposition of *Ac* in transgenic *A. thaliana* has revealed a frequency that makes the application of the strategy of insertional mutagenesis a realistic possibility. Thus screening of about 1 000 000 *Arabidopsis* seeds derived from 1500 transgenic F2 plants should result in 4000 to 9000 progeny plants where *Ac* has excised. This would already present a valuable stock for screening and/or selecting for mutant plants. Even if it is taken into account that the actual number of independent excision events might be lower due to the possible cloned nature of some of these plants (see above), and might be reduced further still due to the fact that not all excision events will lead to integration (Jones et al. 1989), this number is still high enough to allow a mutant screening program.

In maize the germinal excision rates of *Ac* were determined for two mutable alleles (Greenblatt 1968; Brink and Williams 1973), and are as high as 0.4%–17%. Jones et al. (1989) studied the manifestation of *Ac* transposition in tobacco using a chimaeric *Ac*/streptomycin phosphotransferase construct. Here between 1% and 9% of the F1 progeny was observed to inherit the transposition event as assayed by phenotypic expression of the empty donor site. Lassner et al. (1989) also report for transgenic tomato plants a high frequency of germinal transmission in the progeny of primary *Ac* transformants. As no subsequent generations were included in these studies, no conclusion can yet be drawn concerning the question of whether or not this rate was also observed during normal growth of the plant, i.e. in

the absence of any tissue culture. Thus it is not possible to conclude whether or not a seemingly low rate of germinal excisions is specific for *A. thaliana* or rather is a general phenomenon.

Acknowledgements. We thank G. Coupland and P. Starlinger for providing *Agrobacterium* strain GV3850Hygro::pKU3. The photographic expertise of J. Bergstein is also gratefully acknowledged. This work was supported in part by a grant from the Bundesministerium für Forschung und Technologie (BCT0389 „Molekular- und Zellbiologie von höheren Pilzen und Pflanzen").

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Communicated by H. Saedler

Received August 28, 1989