Theor Appl Genet (1993) 86:919–926

Selection for enhanced germinal excision of Ac in transgenic *Arabidopsis thaliana*

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Received: 4 January 1993 / Accepted: 28 January 1993

Abstract. Gene tagging in *Arabidopsis thaliana* using the autonomous Ac (Activator) transposable element has so far been hampered by low frequencies of germinal transposition events. Here we describe a procedure by which the frequency of independent germinal reinsertions has been much improved by a process of long-term selection on kanamycin for the continued growth of tissues in which somatic excisions have occurred. Growth on artificial media increased the somatic excision frequency, and the long-term selection procedure channelled somatic transposition events into the germline. This resulted in an overall germinal excision frequency in the progeny of long-term selected plants of 15%, as confirmed by Southern blotting, with 63% of the plants bearing excision events having detectable reinsertions of the Ac element. This compares with a germinal excision frequency of approximately 1% when no long-term selection is employed. However, offspring from individual plants tended to have identical germinal Ac reinsertion patterns, thus the critical parameter for evaluating the system for tagging purposes is the frequency of individual plants yielding offspring with reinsertions, which was 64%. This high frequency, when coupled to the enhanced germinal transposition rate overall, easily allows the generation of a large population of plants with independent reinsertions.

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

Introduction

There have been considerable efforts in the past few years aimed at transposon tagging in the cruciferous plant *Arabidopsis thaliana* (reviewed Balcells et al. 1991; Haring et al. 1991; Van Lijsebettens et al. 1991) with the emphasis being on the introduction of the well-characterised heterologous transposon Activator (Ac) derived from maize (McClintock 1951; Federoff et al. 1983). Molecular identification of genes via insertion mutagenesis using mobile elements has proven a successful strategy in both prokaryotic and eukaryotic molecular biology (e.g., Bingham et al. 1981; Berg et al. 1989) and, in particular, much has been learned about insertion mutants caused by endogenous transposons in the higher plants *Antirrhinum majus* and *Zea mays* (e.g., Federoff et al. 1984; Martin et al. 1985).

Arabidopsis with its small genome of between 70 and 145 Mbp (Leutwiler et al. 1984; Arumuganathan and Earle 1991), and other attendant advantages of scale (Meyerowitz 1989), is an ideal plant species in which to carry out insertion mutagenesis, as demonstrated by the high frequency of tagged mutants obtained by T-DNA transformation (Feldmann 1991). However the establishment of self-generating mutants, arising from excision and reintegration of mobile elements, should in principle greatly reduce the effort involved in generating many independent insertions as compared to T-DNA transformation. In such a trans-

Communicated by G. Wenzel

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poson-based system, the use of an heterologous element, for example the maize Ac element inserted into *Arabidopsis*, should also reduce the labour involved in the isolation of a tagged gene since, on the level of Southern analysis and identification of cloned sequences, there should be no detectable endogenous transpositional background additional to that of Ac.

Although the Ac transposon has been successfully introduced into *Arabidopsis* and indeed demonstrated to be mobile (Van Sluys et al. 1987; Masterson et al. 1989), its use in practise has been hampered by a relatively low sexual transmission frequency of excision and reintegration events into progeny plants (germinal excisions) (Schmidt and Willmitzer 1989). Germinal excisions are essential to obtain plants which are non-chimaeric and homozygous for reinsertions, so that mutant phenotypes can be screened for. The reasons for the low rate of transmission are not understood but are probably connected with a low somatic excision rate of Ac in *Arabidopsis* (Dean et al. 1992) coupled with a relatively small proportion of cells which will give rise to gametes (Li and Rédei 1969; Shevchenko and Grinikh 1990). However there is a possibility to enhance the transmission frequency, based on the flexible nature of the plant germ-line, which is not fixed as in animal systems (Walbot 1985). Most forms of plant somatic tissue can be induced under suitable conditions to give rise to reproductive structures. Here we have taken advantage of this capability to achieve an enhanced frequency of inheritance of somatic Ac excision events.

The use of a chimaeric construct where Ac is located in the leader of the neomycin phosphotransferase (NPTII) gene enables excision events to be selected for on the basis of kanamycin resistance (Baker et al. 1987). Kanamycin resistance due to NPTII is not cell autonomous so that no direct evaluation of the number of independent somatic events is possible as is the case when streptomycin phosphotransferase is used as the excision marker (Jones et al. 1989; Dean et al. 1992) However, somatic excision allows the continued growth and development of a sector of predominantly kanamycin-resistant tissue, whilst tissues in which an excision has not occurred are inhibited from growth. The continued selection for resistant tissues channels transposon excisions, and any accompanying reintegration events, into germ-line cells with a high frequency. Hence, they are transmitted to the next generation, where they are observed as germinal excision events. Together with the observation that kanamycin-sensitive plants are not killed by the antibiotic, but are simply bleached and inhibited from further growth, this permits the approach employed here to fix high numbers of independent transposition events in the germ-line.

Materials and methods

Plant materials

Transgenic *A. thaliana* genotype C24 plants harbouring the construct pKU3, introduced by *Agrobacterium*-mediated transformation of cotyledons with the strain GV3850Hygro::pKU3, were as described in Schmidt and Willmitzer (1989).

Tissue culture selection for excision

In order to select for excision on the basis of a visible phenotype, F_3 seeds derived from single soil-grown F_2 plants were sown on kanamycin-containing (50 mg l^{-1}) MS medium (Murashige and Skoog 1962) with glucose at 1.6% as the carbon source. Fully resistant seedlings were removed after three weeks and kanamycin-sensitive seedlings cultured further. Green shoots which arose from the sensitive seedlings were excised and cultured further in tubes on MS medium to allow for seed-set. The F_4 progeny was analysed by segregation analysis on kanamycin-containing medium and by Southern-blot analysis.

Isolation and analysis of DNA

Genomic DNA was isolated as described by Murray and Thompson (1980). After digestion with restriction enzymes, the DNA was separated by electrophoresis in agarose gels and blotted onto Hybond N nylon membranes. Hybridisation with radioactively-labelled DNA fragments (Feinberg and Vogelstein 1983) was performed as described in Sambrook et al. (1989).

Results

Long-term selection on kanamycin results in the selection of kanamycin-resistant tissues

F_3-generation seeds from individual soil-grown F_2 plants of five independently transformed lines harbouring a chimaeric gene, composed of Ac located in the 5′ untranslated leader between the TR1′ promoter and the coding sequence of NPTII (Baker et al. 1987), were sown on hygromycin-bearing media (20 mg l^{-1}), and the number of resistant and sensitive seedlings assessed after 21 days in order to obtain segregation data for the insertion loci of the T-DNA (Table 1). Analysis of F_1 segregation data already indicated that the primary transformants of these lines contained insertions at one or two loci only (data not shown). Lines 1A and 1B were derived from two separate plants of the F_1 generation and thus may be isogenic. The F_3 generation of line 5 segregated for hygromycin resistance in a 3:1 ratio indicating that the plant chosen was heterozygous for one locus with introduced hygromycin resistance genes, whereas all other lines did not segregate, indicating that F_2 parent plants were homozygous for at least one locus.

In parallel, F_3 seed were sown on 50 mg l^{-1} of kanamycin-containing media to score for germinal excision and somatic excision events. These lines had previously all been shown to exhibit a low frequency of

Table 1. Segregation of the F_3 of pKU3-transformed *Arabidopsis* lines on hygromycin- and kanamycin-containing media, and the production of kanamycin-resistant shoots after long-term kanamycin selection

pKU3[a] line	Hygromycin resistant	Hygromycin sensitive	Kanamycin resistant	Kanamycin intermediate	Kanamycin sensitive	Shoots[b] harvested	Yield[c] (%)
1A	287	0	0	53	114	105	63
1B	140	0	0	25	138	100	61
2	168	0	0	3	129	18	14
3	292	0	0	3	144	23	16
4	509	0	0	12	166	17	10
5	56	20	2	26	32	41	68

[a] F_3 populations from single F_2 plants of five independently transformed lines were analysed
[b] The number of kanamycin-resistant shoots produced by the previously kanamycin-sensitive seedlings over a period of 112 days of continued selection on kanamycin is shown
[c] The yield of kanamycin-resistant shoots as a percentage of the kanamycin-intermediate and sensitive seedlings is given

excision and reintegration of the Ac transposon (Schmidt and Willmitzer 1989). Fully-green seedlings with well developed roots (fully kanamycin-resistant, Altmann et al. 1992) were counted and removed after 21 days (Table 1). Only for one line (5) was a small number of fully resistant progeny seen. The remaining seedlings were partially or fully sensitive to kanamycin. Those fully sensitive were blocked in growth at the cotyledon stage, showed very little root growth, and were completely bleached. The partially sensitive seedlings showed a variegated appearance or had sectors of green tissue. These variegated plants did not develop further than the six or eight leaf stage. Seedlings with well developed roots but bleached or variegated leaves were also scored as sensitive.

However, after 4 weeks of selection on kanamycin-containing medium, a proportion of both fully bleached and variegated seedlings started to produce fully green leaves and flowering shoots, which were removed and cultured further in tubes in which they flowered and set seed. Green shoots from sensitive seedlings continued to be produced and harvested over a 4 month period during which the still kanamycin-sensitive seedlings were transferred to fresh kanamycin plates every 4 weeks. The majority of green shoots arose within the first 2 months. The different lines produced different numbers of green shoots (Table 1). For example, 68% of the plants from line 5 produced green shoots whereas only 10% of the plants from line 4 did so. Lines 1A and 1B produced equal numbers of shoots (63%) indicating that this property may be a function of the transformant.

Kanamycin resistance is inherited by a high proportion of progeny plants

F_4 seed from 30 rescued green F_3 shoots, five from each line, was sown on kanamycin-bearing media and the segregation of fully green seedlings and fully white or variegated seedlings was determined after 3 weeks (Table 2). Twenty-four of the shoots (80%) yielded a proportion of fully resistant offspring. However, for only four shoots was a three to one ratio of resstant to sensitive seedlings observed (shoots 1A-3, 2-2, 4-2, and 5-3) arguing that, in the majority of instances, the selected green parent shoot tissue was chimaeric for an Ac excision event, since the fully-resistant offspring were under-represented. From a total of 1661 seeds sown (from all lines), 295 (18%) were judged to be fully kanamycin-resistant. The proportion of intermediate phenotypes was increased compared to the number seen prior to long-term selection, whereas the number of fully sensitive seedlings was much reduced.

Molecular analysis of F_4 progeny derived from kanamycin-resistant shoots

The fully green F_4 seedlings from up to five F_3 shoots from each line were transferred to soil and Southern analysis carried out on DNA extracted from up to four fully-resistant F_4 plants from each shoot. In a few instances, F_4 plants which showed a partially variegated phenotype were also transferred to soil and subsequently a Southern analysis was carried out. The DNA was digested with *Eco*RI and *Hin*dIII, the blotted gels were hybridised against the TR1′ promoter to check for Ac excision events and with the *Bam*HI fragment of Ac to check for reintegration of the transposon. When probing DNA from plants in which no excision had taken place, the expected band with the TR1′ promoter was 2.3 kb, whereas in the case of a excision event, a band of 2.9 kb was expected. DNA from plants in which Ac remained in the donor site should, when probed with Ac, yield bands of 3.6, 2.3, 0.9 and 0.7 kb whereas if a reintegration of Ac had occurred, the bands corresponding to the internal Ac fragments of 0.9 and 0.7 kb should be present as for unexcised Ac, but the two other bands would be un-

Table 2. Segregation on kanamycin of the F_4 progeny from long-term selected F_3 plants

pKU3 line	F_3[a] shoot	Kanamycin resistant	Kanamycin intermediate	Kanamycin sensitive
1A	1	2	69	2
	2	8	90	0
	3	43	19	0
	4	8	53	0
	5	2	59	0
1B	1	2	87	0
	2	2	101	0
	3	1	100	0
	4	1	76	3
	5	21	38	0
2	1	24	22	14
	2	60	16	1
	3	0	29	20
	4	0	30	15
	5	0	0	4
3	1	4	26	9
	2	2	51	2
	3	2	26	0
	4	2	18	0
	5	0	21	7
4	1	1	38	8
	2	54	22	2
	3	5	74	7
	4	0	34	4
	5	0	54	0
5	1	4	18	1
	2	5	43	1
	3	34	9	0
	4	1	37	1
	5	7	5	0
$\sum$		295	1265	101

[a] The F_4 progeny from up to five kanamycin-resistant shoots (1–5), derived from long-term selection on kanamycin of the F_3 line, were analysed

predictable in size since the *Eco*RI restriction sites would be located within the plant DNA (Fig. 1).

In total 57 plants were analysed (Table 3). Of the 53 F_4 plants judged as being fully resistant on kanamycin, 45 (85%) showed an excision band. Of the four plants with a variegated phenotype, three did not show a TR1′ excision band and one showed a weak excision band in addition to the donor band. In all instances, excepting four plants, the original Ac bands were still present. The four exceptions also did not show a TR1′ donor band; thus in these cases the F_4 generation had not inherited an untransposed element.

Twenty-nine plants had additional Ac bands, indicative of a reinsertion of the transposon. In all of these cases except one (line 4, shoot 3, plant a), a TR1′ excision band was also present. Thus 63% of those plants with excision bands showed reinsertions. F_4 siblings from a single F_3 shoot generally showed the same Ac reintegration pattern, with two instances of one plant with different bands to its siblings (line 2, shoot 2, plants a and c; line 4, shoot 3, plants a and c). For all lines analysed, F_4 plants from independent F_3 shoots with reinsertions always had the new Ac bands in different positions, indicative of independent excision and reinsertion events occurring in the F_3 plants. Had the events occurred earlier (e.g., F_2), the same reinsertion pattern would have been observed in F_4 progeny from independent F_3 shoots of the same line. Had the events occurred in the F_4, the patterns would have consistently varied within the progeny of each

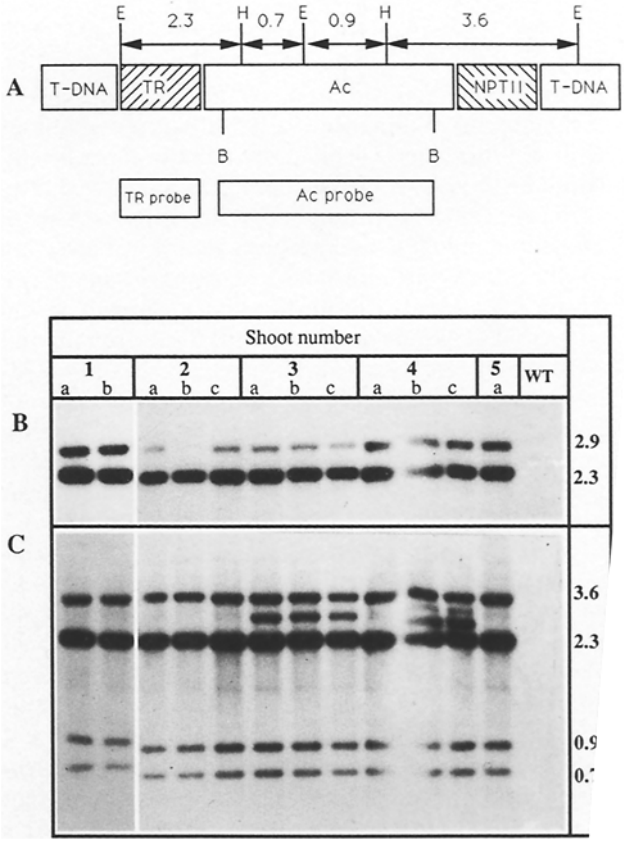

Fig. 1. Southern-blot analysis of kanamycin-resistant progeny from five long-term kanamycin selected shoots of line 1A. Genomic DNA was digested with *Hin*dIII and *Eco*R1. **A** Structure of the pKU3 T-DNA indicating the length of fragments expected after a *Hin*dIII-*Eco*R1 digestion and probing of the blot with either the major *Bam*H1 fragment of Ac or the TR1′ promoter. *B* = *Bam*HI, *E* = *Eco*RI, *H* = *Hin*dIII. **B** Hybridization to the TR1′ promoter. The fragment hybridizing at 2.3 kb indicates that Ac is in its original position whereas the fragment at 2.9 kb indicates an excision event. No hybridization is detected when DNA from a non-transformed control is used. **C** Hybridization to the major *Bam*H1 Ac fragment. Bands at 3.6 and 2.3 kb represent Ac fragments seen when the element is located in its original position in the leader of the *NPTII* gene, bands at 0.9 and 0.7 kb represent internal Ac fragments. Additional hybridizing fragments indicate reintegration events

Table 3. Southern analysis of kanamycin-resistant progeny from long-term selected pKU3 *Arabidopsis* shoots

pKU3 line	F_3 shoot	F_4[a] plant	Tr donor[b]	Tr exci[c]	Ac orig[d]	Ac new[e]	Comment
1A	1	a, b	+	+	+	−	
	2	a, c	+	+	+	−	
		b	+	−	+	−	
	3	a, b, c	+	+	+	+	New Ac bands the same in a, b, c
	4	a	+	+	+	−	New Ac bands the same in b, c
		b, c	+	+	+	+	
	5	a	+	+	+	−	
1B	1	a	+	+	+	+	
	2	a	+	+	+	−	
		b	+	+	+	+	
	3	a	+	+	+	−	
	4	a	+	+	+	+	
	5	a, b	+	+	+	−	
		c	+	+	+	+	
2	1	a, b	−	+	−	+	New Ac bands the same in a, b, c, d
		c	−	+	−	+	
		d	+	+	+	+	
	2	a, b	+	+	+	+	New Ac bands the same in a, b
		c	−	+	−	+	New Ac bands differ from those in a, b
	3	a	+	−	+	−	Variegated phenotype
3	1	a, c	+	−	+	−	
		b	+	+	+	+	
		d	+	−	+	−	Variegated phenotype
		e	+	Weak	+	Weak	Variegated phenotype
	2	a, b	+	+	+	−	
	3	a, b	+	+	+	−	
	4	a	+	+	+	+	
		b	+	−	+	−	
4	1	a	+	−	+	−	
		b	+	−	+	−	Variegated phenotype
	2	a, b	+	+	+	+	New Ac bands the same in a, b, c
		c	−	+	−	+	
	3	a	+	−	+	+	New Ac bands differ in a and c
		b	+	+	+	−	
		c	+	+	+	+	
5	1	a	+	+	+	+	
		b	+	−	+	−	
	2	a	+	+	+	+	
		b	+	+	+	−	
		c	+	−	+	−	
	3	a, b, c	+	+	+	+	New Ac bands the same in a, b, c
	4	a	+	+	+	+	
	5	a	+	−	+	−	

[a] Up to five kanamycin-resistant F_4 plants (a–e) from each shoot were analysed

[b,c] "TR donor" and "TR exci" refer to the bands expected when probing with the TR1′ promoter if an Ac element is still in situ or has excised, respectively. The symbols + and − indicate the presence and absence respectively of a band

[d,e] "Ac orig" and "Ac new" refer to the bands expected when probing with the Ac fragment if an Ac element is still in situ or has reintegrated respectively. The symbols + and − indicate the presence and absence respectively of a band

shoot. In total, 17 different Ac reinsertion pattens were seen in the progeny of the 30 shoots. The Southern blots for all of the F_4 plants analysed in line 1A are shown in Fig. 1. It can be seen for shoot 3 that only one new Ac band is visible whereas two are to be expected from the digest carried out. It is not known whether this represents a partial reintegration of Ac or whether the second new Ac band is masked by the original bands.

Inheritance of kanamycin resistance of the F_5 generation agrees with the molecular analysis of excision in the F_4

The segregation on kanamycin of F_5 progeny from the same F_4 plants from the line 1A plants as analysed in the Southern of Fig. 1 is shown in Table 4. Segregation of the F_5 was in accordance with the Southern analysis of the F_4 in that those plants that show an excision band also produce progeny that segregate 3:1 for kanamycin resistance, whereas the progeny of plant 2b which did not show an excision band contain no fully resistant plants. In one instance (1a) a 15:1 segregation was observed, arguing for two independent excision events. No fully kanamycin-sensitive seedlings were found; correspondingly, the number of variegated phenotypes was much increased as compared to the number seen prior to long-term selection (Table 1).

Selection on hygromycin does not lead to enhanced germinal excision frequencies

The mechanism by which greater numbers of germinal excisions are produced after prolonged selection on kanamycin could be via enhanced transpositional activity of Ac due to physiological stresses induced by selection pressure and tissue culture (e.g., Dennis and Brettell 1990; Brettell and Dennis 1991). In order to test this hypothesis, F_3 seed from each line was sown on hygromycin-containing medium. Hygromycin-resistant seedlings were chosen after 4 weeks and subsequently grown in tubes in the same way as the long-term selected kanamycin-resistant shoots. F_4 seed derived from these plants were sown on kanamycin and the segregation analysed (Table 5). Five of the twenty-eight plants yielded a proportion of fully kanamycin-resistant offspring. Of 722 seeds sown in total, 12 (1.7 %) were judged fully kanamycin-resistant, as compared with 18 % after long-term selection, thus it does not seem that the culture conditions in the absence of long-term selection on kanamycin are inducing enhanced numbers of germinal excisions in the progeny. However, the proportion of intermediate phenotypes was much increased compared to the number seen when hygromycin-resistant plants were soil grown (compare Tables 1 and 5), indicative of enhanced somatic activity.

Table 4. Segregation on kanamycin of progeny from plants of the line 1A which have undergone Southern analysis (Table 3, Fig. 1)

F_3[a] shoot	F_4[b] plant	Kanamycin resistant	Kanamycin intermediate	Kanamycin sensitive
1	a	101	6	0
	b	88	24	
2	a	52	27	0
	b	0	94	0
	c	93	21	0
3	a	29	12	0
	b	76	30	0
	c	137	42	0
4	a	130	29	0
	b	142	45	0
	c	87	31	0
5	a	155	47	0

[a] Five kanamycin-resistant F_3 shoots resulting from long-term selection were chosen

[b] Up to three kanamycin-resistant F_4 progeny from each shoot were analysed by Southern blotting, and the F_5 generation seed was collected

Discussion

We have extended our studies on transposon tagging in transgenic *Arabidopsis* (Schmidt and Willmitzer 1989) using the autonomous Ac element. Initial studies on *Arabidopsis* transformed with pKU 3 showed that the transposon was mobile in up to 50 % of the primary transformants, that it was active also in subsequent

Table 5. Segregation on kanamycin of F_4 progeny from hygromycin-selected pKU3 *Arabidopsis*

pKU3 line	F_4[a] plant	Kanamycin resistant	Kanamycin intermediate	Kanamycin sensitive
1A	1	0	22	0
	2	0	38	0
	3	0	10	0
	4	0	12	0
	5	2	16	0
1B	1	0	83	0
	2	0	32	0
	3	0	27	0
	4	0	8	0
	5	0	41	0
2	1	0	13	10
	2	0	8	0
	3	1	13	3
	4	0	4	0
	5	0	15	0
3	1	0	27	0
	2	1	12	0
	3	0	27	0
	4	0	28	0
	5	0	6	0
4	1	0	32	2
	2	0	13	0
	3	0	62	0
	4	0	41	0
	5	0	65	1
5	1	1	23	0
	2	0	14	0
	3	7	2	0
$\sum$722		12	694	16

[a] F_3 seed was sown on hygromycin-containing media (20 mg l^{-1}) and grown for 28 days. Five hygromycin-resistant plants from each line were further cultured to maturity in glass tubes, the seed collected and the segregation of the F_4 on kanamycin analysed

generations but that excision and reintegration events were passed on to the progeny only at a very low frequency. Thus of 30 primary transformants, only seven had a limited number of kanamycin-resistant progeny, and of 2327 F1 seedlings sown, only 25 (1%) were fully kanamycin-resistant (Schmidt and Willmitzer 1989). Similarly, when an autonomous Ac element was cloned into the 5′ leader of the streptomycin phosphotransferase (STP) gene, and resistance to streptomycin used as an excision marker, an overall average of 1.4% germinal excisions was seen (Dean et al. 1992).

This rate imposes limits on the use of such a system for transposon tagging since very large numbers of plants have to be produced and screened in order to detect independent tagged mutants. One possible reason for the low incidence of germinal Ac excisions in *Arabidopsis* as compared to maize (1–10%, Brink and Nilan 1952), tobacco (1–10%, Jones et al. 1990), or tomato (30%, Belzile et al. 1989), may be connected to the frequency and/or timing of Ac transposition in somatic tissues, which in maize is not a stochastic event but is developmentally and environmentally regulated (Levy and Walbot 1990). For example, if Ac transposition were to occur primarily in the maturing seed or germinating seedling of *Arabidopsis*, the frequency of transmission to the gametes would be relatively low, since it has been estimated by sector analysis that of the 7000 cells comprising the mature seed of *Arabidopsis*, only two meristematic cells will ultimately give rise to gametes (Li and Rédei 1969; Rédei and Koncz 1992). Transpositions occurring later in the life cycle of the plant are also statistically unlikely to be passed on to the progeny, as the vegetative and floral meristems are very small, and the number of cells in these organs are much less compared to the rest of the vegetative tissue. Thus Shevchenko and Grinikh (1990) found the genetically-effective cell number in rosette stage *Arabidopsis* to be about five. Therefore enhancing the incidence of somatic excisions and the transmission of these events into germ-line cells should result in increased numbers of germinal excisions.

In the pKU3 construct employed here, somatic excision of the transposon results in activation of the *NPTII* gene, leading to kanamycin resistance. Although the exact number of individual somatic excisions per plant cannot be monitored because the *NPTII* gene product does not appear to be cell autonomous, somatic activity overall can be recognised since the seedlings have a variegated phenotype on kanamycin. Only when the vast majority, or every, cell in the plant contains an active *NPTII* gene is full kanamycin resistance observed (Altmann et al. 1992). What is apparent after long-term selection on kanamycin and further growth on artificial media is that the proportion of somatic excisions is much enhanced as judged by the number of variegated plants seen in the next generation. This effect is also seen when plants were selected for on hygromycin rather than kanamycin (Table 5). It is known that tissue culture of maize will lead to activation of otherwise inactive Ac elements, and that this is associated with reduced cytosine methylation in the 5′ region of the gene (Peschke et al. 1987; Dennis and Brettell 1990). Furthermore, the methylation status, and the activity of the element, can be transmitted through at least two sexual generations (Brettell and Dennis 1991). It is of interest that the F_5 generation of plants (derived from soil-grown F_4 plants which in turn were germinal excision-containing progeny of long-term selected F_3 plants) also contained a very high proportion of plants which showed somatic Ac excision, in contrast to the progeny of soil-grown F_2 plants (Tables 1 and 4). Thus it seems that the somatic activity of Ac in *Arabidopsis* is enhanced by culture of the plant under "stressful" or sub-optimal conditions, and that this active status can be inherited by the progeny even when the plants are grown under optimal conditions. This may indicate that Ac transposon activity in *Arabidopsis* is also mediated by the methylation status of the gene.

Selection for continued growth of tissues in which an excision has taken place allows us to obtain an average overall germinal excision frequency in the next generation, as based upon the visible phenotype, of 18% which, with an 85% probability of the phenotype corresponding to a true germinal excision (Tables 2, 3, 4, Fig. 1), equals an average germinal excision frequency of 15%. Of the offspring bearing germinal excisions, 63% also contained reinsertions of the Ac element (Table 3, Fig. 1). This reinsertion frequency compares well with those found by other workers (e.g., 50–83% in tobacco, Hehl and Baker 1990; 50% in *Arabidopsis*, Altmann et al. 1992). Thus 9.6% overall of the total progeny after long-term selection contain new Ac insertions (18% phenotypically fully resistant × 85% corresponding to a true excision × 63% reinsertions). This figure is an underestimate since for this analysis those plants in which the reintegration of Ac was genetically unlinked to the excision (kanamycin resistance) would tend to be excluded.

Of more relevance for the use of Ac as a means of producing mutants is the number of independent Ac insertions seen rather than the total number of insertions overall. Progeny from the same plant tend to contain the same insertion event (Table 3, Fig. 1) and thus the frequency of individual plants yielding progeny with new insertions after long-term selection is the critical parameter for evaluating the potential of this method for transposon tagging. In this study, 16 out of 25 (64%) long-term selected plants produced offspring with new, independent Ac insertions (Table 3, Fig. 1), as compared to 7 out of 30 (23%) (Schmidt and

Willmitzer 1989) or 5 out of 25 (18%, Table 5) when no long-term kanamycin selection was employed. Coupled with the increased number of germinal excisions obtained from each plant (15% overall versus 1% without long-term selection) these independent Ac insertions are easily generated. Thus the advantages of the long-term selection scheme are that a high number of independent Ac reinsertions at a high frequency can be produced.

The detailed analysis described above was carried out on plants from five lines only, but less extensive studies on 30 other lines (analysis of kanamycin resistance and *NPTII* activity in shoots and progeny after long-term selection on kanamycin, Schmidt and Willmitzer unpublished results) indicate that the same outcome is obtainable with all lines tested, and thus this methodology is a viable option for the production of many independent Ac transpositions.

Acknowledgements. We thank Gisela Felix and Salomé Prat for critical reading of the manuscript.

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