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Establishment of a gene tagging system in *Arabidopsis thaliana* based on the maize transposable element *Ac*

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Summary. An *Ac*-derived, two-component transposable element system has been developed and analyzed with respect to its use in *Arabidopsis thaliana*. This system consists of an immobilized *Ac* element ("Ac clipped wing", Ac_{cl}) as the source of transactivating transposase and a nonautonomous "*Ds*" element, Ds_A, which is inserted into a chimaeric neomycinphosphotransferase gene used as excision marker. After separate introduction of Ac_{cl} and Ds_A into *Arabidopsis thaliana*, progeny analysis of crosses between five different Ac_{cl} lines and seven different Ds_A lines shows that: (1) different Ac_{cl} lines differ greatly in their capacity to transactivate Ds_A; (2) different Ds_A lines do not differ significantly with respect to Ds_A transactivation by one Ac_{cl} line; (3) reintegration of excised Ds_A elements, both at (genetically) linked and unlinked sites, occurs in about 50% of the excision events; and (4) plants with a high rate of somatic excisions can be used as source of new Ds_A transpositions, allowing the creation of a large number of independent Ds_A insertions.

Key words: *Arabidopsis thaliana* – Ds transposition – Transposon tagging – Insertion mutagenesis

Introduction

Gene tagging is a strategy that allows a rapid molecular identification and isolation of genes only defined by a mutant phenotype. It is based on the dual property of a known DNA fragment of inactivating a gene upon insertion and simultaneously marking its site of integration. The main bottleneck for using this method is the creation of high numbers of independent insertions by a DNA element of known sequence in the desired host organism. Due to the presence of well-characterized endogeneous transposable elements in *Drosophila*, maize and *Antirrhinum*, these were among the first eukaryotic organisms in which this tool was used for gene isolation (Bingham et al. 1981; Fedoroff et al. 1984; Martin et al. 1985).

Arabidopsis thaliana, a member of the *Brassicaceae* family, has almost all of the properties to serve as an ideal genetic model system for higher plants. Among these are the small size of the mature plant, a short generation time, the numerous seeds produced from a single plant by self-fertilization, the small and simple organization of its genome and its susceptibility to transformation. Intensive classical genetic studies have been carried out, and molecular genetic studies have also been initiated, including the isolation of a variety of genes (for reviews see Rédei 1975; Bowman et al. 1988; Meyerowitz 1989).

The small genome size in particular should be an advantage for setting up an efficient gene tagging system in *Arabidopsis*. This strategy has been successfully applied using the T-DNA of *Agrobacterium tumefaciens*: insertion mutants have been obtained (Feldman et al. 1989, 1990; Koncz et al. 1989), and the corresponding genes have been isolated (Marks and Feldman 1989; Yanofsky et al. 1990; Koncz et al. 1990). The most efficient way to induce mutations by T-DNA insertion has turned out to be by seed transformation (Feldman and Marks 1987), which omits the dedifferentiation/redifferentiation step involved in other transformation protocols that is known to induce independent mutations (Larkin and Scowcroft 1981) at a high frequency. An alternative way to circumvent this problems of "somaclonal variation"

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would be to use a transposable element that could cause many independent insertion mutations in the sexual progeny of a plant. In addition, as recent data seem to indicate that the T-DNA is not integrated into the plant genome completely randomly with respect to the target sequence or the state of the chromatin (Koncz et al. 1989), certain areas of the genome might be under-represented in a stock of T-DNA transformants and a variety of genes might be left untagged. In that respect a transposon displaying probably a different insertion site preference could complement a set of T-DNA insertion mutants.

It has only been very recently that indications for the presence of an active endogeneous transposon have been obtained in *Arabidopsis thaliana* (Peleman et al. 1991). The maize *Activator* (*Ac*) element (McClintock 1951; Fedoroff et al. 1983; Behrens et al. 1984) and its related *Ds* elements (McClintock 1952; Döring and Starlinger 1984), one of the best characterized family of plant transposable elements (for reviews see Nevers et al. 1986; Döring and Starlinger 1986) have been used to isolate genes from maize (e.g. Fedoroff et al. 1984; Theres et al. 1987; Motto et al. 1988; Hake et al. 1989). *Ac* was shown to transpose in transgenic *Arabidopsis* plants (Van Sluys et al. 1987; Schmidt and Willmitzer 1989) as well as in other heterologous hosts like tobacco, potato, tomato and carrot (Baker et al. 1986; Knapp et al. 1988; Yoder et al. 1988; Van Sluys et al. 1987). Remarkably however, *Ac* was shown to exhibit a lower germinal excision frequency in *Arabidopsis* than in tobacco (Schmidt and Willmitzer 1989; Hehl and Baker 1990; Jones et al. 1990).

A two-component system such as the maize *Ac/Ds* system (McClintock 1952) provides striking advantages over the use of an autonomous element with respect to its application for gene tagging: Firstly, the separation of *cis*- und *trans*-acting functions provides the possibility to control transposition and thus to obtain stabilized insertions. Secondly, stable lines containing a strong activating element can be produced and continuously used, whereas in the case of autonomous elements, very active *Ac* copies probably transpose early during the regeneration process of transgenic plants (Schmidt and Willmitzer 1990). These plants would be of no further use as a phenotypic excision assay that allows the easy detection of excisions (Baker et al. 1987) can no longer be applied. In order to establish an efficient two-component transposon tagging system, a deletion derivative of *Ac* lacking 2.8 kb of internal sequences and an "Ac clipped wing" (Ac_{cl}) generated by deleting the 3′ outermost 11 bp comprising one of the inverted repeats of *Ac* were constructed. The first element, Ds_A, should serve as a nonautonomous element just like a maize *Ds* element; the second element, Ac_{cl}, was designed to be an immobile source of transactivating function(s). These constructs were subsequently introduced separately into *Arabidopsis thaliana* via *Agrobacterium* transformation.

To test the applicability of this Ac_{cl}/Ds_A system for gene tagging purposes a series of experiments was performed aimed at answering the following questions:

1) Is the frequency of transactivation of the nonautonomous Ds_A element high enough to allow the creation of large numbers of germinal transpositions?
2) Are different Ds_A elements activated at the same frequency irrespective of their chromosomal location?
3) What proportion of excised Ds_A elements reintegrate and therefore represent true transpositions?
4) Does Ds_A transpose only to genetically linked sites or do transpositions also occur to unlinked sites?

The work presented here demonstrates that Ac_{cl} is able to induce Ds_A transposition and that independent Ac_{cl} lines differ greatly in their potential to transactivate Ds_A, whereas different Ds_A lines do not differ significantly with respect to Ds_A transactivation by one Ac_{cl} line. We show that in about 50% of the cases where Ds_A excision has been observed a Ds_A reintegration can be monitored and that the level of Ds_A transactivation obtained in the progeny of crossings between a particular Ac_{cl} line and different Ds_A lines is high enough to allow the creation of large numbers of independent germinal transpositions. Finally, preliminary evidence for Ds_A transposition to genetically nonlinked sites is presented.

In conclusion, the system described here fulfills all of the criteria necessary for gene tagging purposes in *Arabidopsis thaliana.*

Materials and methods

Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (Maniatis et al. 1982).

Construction of E. coli plasmids

To obtain pTA1 the *Xba*I-*Pst*I fragment of pKU3 (Baker et al. 1987) was subcloned into M13 mp18, and oligonucleotide-directed mutagenesis was performed in vitro according to Taylor et al. (1985) and Nakamaye and Eckstein (1986) using an 40-mer oligonucleotide spanning 20 nucleotides on each side of the outermost 11 bp of *Ac* from position 1 to 11 according to Müller-Neumann et al. (1984). Position 1 is here referred to as 3′ end of *Ac* with respect to the direction of transcription. After deletion of this 11-bp fragment the *Xba*I-*Sal*I fragment of the mutated M13 was used to replace the 1.1-kb *Xba*I-*Sal*I fragment of the partially digested pKU3, resulting in pTA1. In order to construct pTA4, pTA1 was digested with *Sal*I and religated, and transformed *E. coli* were selected for Carbenicillin resistance (Cb^r) and Kanamycin resistance (Km^r) to delete only the *Sal*I fragment containing the neomycin phosphotransferase II (*NPT* II)-coding region together with the octopine synthase (*ocs*) polyadenylation signal, but not the fragment carrying the bacterial Km^r gene.

pTA3 was generated from pKU35, a deletion derivative of pKU3 in which the fragment from about position 370 to about 1580 of *Ac* was replaced by a duplicated *Bgl*II linker (G. Coupland and P. Starlinger, personal communication). After cutting

with *Bgl*II and religation (removal of the second *Bgl*II linker fragment) the internal *Hin*dIII fragment of the *Ac* derivative in pKU35 was removed by complete digestion with *Hin*dIII, religation and selection of transformed *E. coli* for Cb^r/Km^r. The resulting plasmid was called pKU35ΔH. A *bar* gene driven by the CaMV 35S promoter and provided with the *ocs* polyadenylation signal was inserted into the *Sca*I site of pKU35ΔH (located in the Cb^r gene) to produce pTA3.

Agrobacterium tumefaciens strains

In the *A. tumefaciens* strain C58C1 Rif the pGV3850HPT Ti plasmid (Baker et al. 1987) was separately recombined with plasmids pKU3 (Baker et al. 1987), pTA1, pTA3 and pTA4 after conjugative transfer from *E. coli* (Van Haute et al. 1983). The resulting *A. tumefaciens* strains named GV3850HPT::pKU3, GV3850HPT::pTA1, GV3850HPT::pTA3 and GV3850HPT::pTA4 were tested for the correct structure of the T-DNA by Southern blot analysis.

Transformation of Arabidopsis thaliana and growth in aseptic culture

The *Arabidopsis thaliana* ecotype C24 was used for *Agrobacterium tumefaciens*-mediated gene transfer. Cotyledon infections, the regeneration of transformed plants and the growth of plants to maturity in aseptic culture were performed as described by Schmidt and Willmitzer (1988).

Selection for Kanamycin resistance

To select progeny plants of crossings between Ac_{cl} and Ds_A lines for Kanamycin resistance (indicating Ds_A excision events) seeds were surface sterilized (Schmidt and Willmitzer 1988) and seedlings were germinated on MS medium (Murashige and Skoog 1962) containing 1.6% glucose and 50 mg/l Kanamycin according to Schmidt and Willmitzer (1989). Plants were scored for the development of roots as well as for cotyledon and leaf coloration after 4 weeks of growth on the same medium.

Nopaline synthase assay

The test of transformed plant material for nopaline synthase enzyme activity was performed as described (Otten and Schilperoort 1978).

Neomycin phosphotransferase II assay

Analysis of transformed plants for NPT II enzyme activity was performed according to Reiss et al. (1984).

Isolation and analysis of plant DNA

Genomic DNA was isolated as described by Dellaporta et al. (1983) with the addition of a CTAB (Cetyl trimethylammonium bromide) precipitation, and digested with restriction enzymes. After electrophoretic separation on 1% agarose gels, depurination and denaturation (in 0.5 *M* NaOH, 1.5 *M* NaCl) the DNA was blotted onto BiodyneB membranes (Pall) using capillary transfer with 20 × SSC (Maniatis et al. 1982). Hybridization with radioactively labelled DNA fragments (Feinberg and Vogelstein 1983) was performed at 65 °C in 5 × Denhardt's Solution, 5 × SSC, 0.1% (w/v) SDS and 100 µg/ml salmon sperm DNA. Purified DNA fragments used as labelled probes were the pTR *Eco*RI-*Bam*HI fragment of pKU2 (Baker et al. 1987), the *Sal*I-*Sph*I fragment of the *NPT II* gene (from pKU2), the 5′*Bam*HI-*Hin*dIII fragment of *Ac* (position 4379 to 2777) and the *Hin*dIII (*Ac*-position 1172)-*Bam*HI fragment of pKU3 covering the 3′ end of *Ac*.

Crossing of Arabidopsis thaliana plants

Arabidopsis plants were grown in soil under a 16-h day (3000 lux fluorescent light, 20 °C): 8-h night (17 °C) regime at a relative humidity of 70%. For crossings flowers of the female parent were emasculated about 2 days prior to anthesis and cross-pollinated 2–3 days later.

Results

Construction of Ac derivatives serving as immobile transposase donor ("Ac clipped wing", Ac_{cl}) and as nonautonomous element (Ds_A)

To obtain a nonmobile element able to express *Ac*-encoded function(s) (Ac_{cl}), the 3′ (with respect to the direction of transcription) outermost 11 bp of *Ac* that comprise one of the inverted repeats (Pohlmann et al. 1984; Müller-Neumann et al. 1984) were removed by oligonucleotide-directed *in vitro*-mutagenesis. *Ac* sequences inserted between the TR 1′ promoter and the *NPT II* coding region in pKU3 (Baker et al. 1987) were replaced by Ac_{cl} to obtain pTA1. The deletion of the 11 bp was expected to result in an immobile *Ac* element, as all active members of the maize *Ac/Ds* family that have been sequenced contain the terminal 11-bp repetition (reviewed in Döring and Starlinger 1984). Deletion of the *NPT II* coding region and the ocs polyadenylation signal lead to pTA4, which is shown in Fig. 1 A.

Ds_A, as shown in Fig. 1 B, was constructed from an *Ac* deletion derivative that lacks internal sequences from about position 370 to about 1580, and where a *Bgl*II linker was inserted instead. It is located in pKU2 (Baker et al. 1987) in the same orientation as *Ac* in pKU3 (called pKU35, G. Coupland and P. Starlinger, personal communication). Deletion of the internal *Hin*dIII fragment of this *Ac* derivative in pKU35 resulted in the 1.8-kb Ds_A in pKU35ΔH. In order to link a dominant marker to Ds_A to facilitate crossing experiments of transgenic plants, a *bar* gene (Thompson et al. 1987) driven by the CaMV 35S promoter and provided with the polyadenylation signal of the *ocs* gene was inserted into pKU35ΔH near to Ds_A (pTA3). Expression of the *bar* gene in plants confers resistance to the herbicide BASTA® that can be applied by spraying (DeBlock et al. 1987).

The plasmids pTA1, pTA4 (Ac_{cl}) and pTA3 (Ds_A) were independently integrated into the T-DNA of the *Agrobacterium tumefaciens* strain GV3850HPT by recombination. The T-DNA of this Ti plasmid contains a chimaeric hygromycin phosphotransferase gene conferring Hygromycin B resistance in plants and a nopaline synthase (*nos*) gene that is also active in plants (Baker et al. 1987).

Ac_{cl} is nonmobile and Ds_A cannot transpose autonomously

To prove that Ac_{cl} is stable and Ds_A cannot transpose autonomously, primary *Arabidopsis thaliana* transfor-

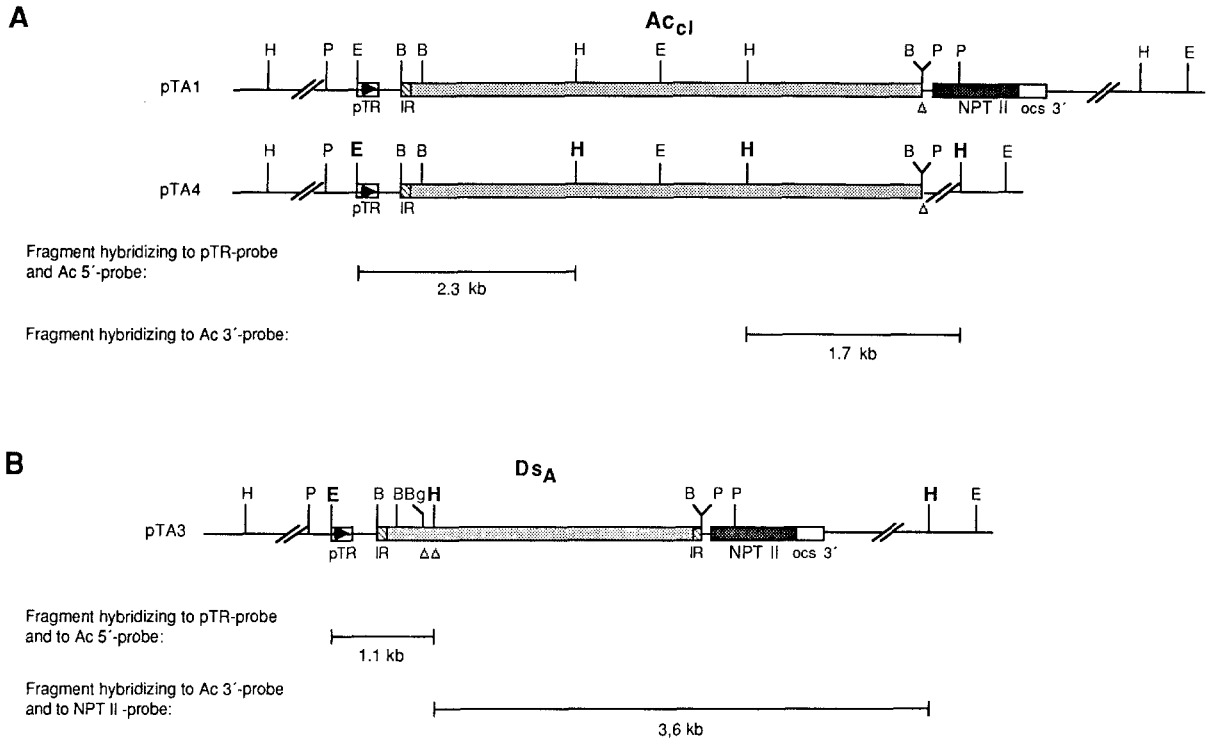

Fig. 1 A, B. Schematic representation and partial restriction map of the Ac_{cl} and the Ds_A element. The *boxes* indicate the transposable elements and their flanking sequences comprising the 1'-promoter of the Ti plasmid TR-DNA (*pTR*) and the *NPT II* gene coding region (*NPT II*) fused to the polyadenylation signal of the octopine synthase gene (*ocs 3'*). The *lines* drawn below the Ac_{cl} and the Ds_A element mark the fragments (created by using the restriction sites typed in *bold*) that hybridize to the specific probes used in the Southern blot analysis. **A** Ac_{cl}. The element is shown as located in front of the *NPT II* gene coding region (*pTA1*) as well as after removal of the excision marker gene (*pTA4*). The site of deletion of the 3' outermost 11 bp of *Ac* is indicated by *Δ*. **B** Ds_A. The location of the element in the 5'-untranslated leader region of the chimaeric *NPT II* gene (*pTA3*) is indicated. The *Δ*'s mark the sites of the internal deletions. *B BamHI, Bg BglII, E EcoRI, H HindIII, P PstI*. The *boxes* marked by *IR* represent the 11-bp terminal sequence repetition of *Ac*

mants obtained by *Agrobacterium tumefaciens*-mediated cotyledon transformation were tested for NPT II activity as shown in Fig. 2. For comparison primary transformants containing the intact *Ac* on the 3850HPT::pKU3 T-DNA (Baker et al. 1987) were analyzed in parallel. Whereas NPT II activity most probably due to *Ac* excision was detectable in 15 out of 18 *Ac* transformants (Fig. 2A), this was not the case in any of the 18 Ac_{cl} (3850 HPT::pTA1) transformants (Fig. 2B) or the 18 Ds_A (3850HPT::pTA3) transformants (Fig. 2C) tested. These results strongly indicate that the latter two elements are not able to excise. The immobility of Ac_{cl} was furthermore confirmed by Southern blot analysis performed on 5 F_1 plants derived from each of five independent Ac_{cl} transformants (data not shown). In agreement with these data, no Km^r plants were detected in the progenies of five different Ac_{cl} transformants (3850HPT::pTA1) and seven different Ds_A transformants (3850HPT::pTA3) (Table 1 and data not shown).

In crossings Ds_A can be activated by Ac_{cl} at different efficiencies depending on the particular Ac_{cl} line used

To show that Ds_A is a nonautonomous element that can be transactivated by the stabilized Ac_{cl} element crosses were performed between seven different Ds_A lines and five different Ac_{cl} lines. Each line descended from an independent transformant and thus represents a different chromosomal location of the respective element. F_2 progenies from the different crosses were analyzed for the presence of Km^r plants, which is indicative of Ds_A excision events. The data summarized in Table 1 clearly show that the five Ac_{cl} lines tested, all containing intact Ac_{cl} elements as shown by Southern blot analysis (data not shown), strongly differ in the number of Km^r plants in the F_2 progeny of crossings between Ac_{cl} and Ds_A lines. In this line of experiments plants growing in the presence of Kanamycin were scored as Km^r if they developed roots and completely green leaves or green leaves with small

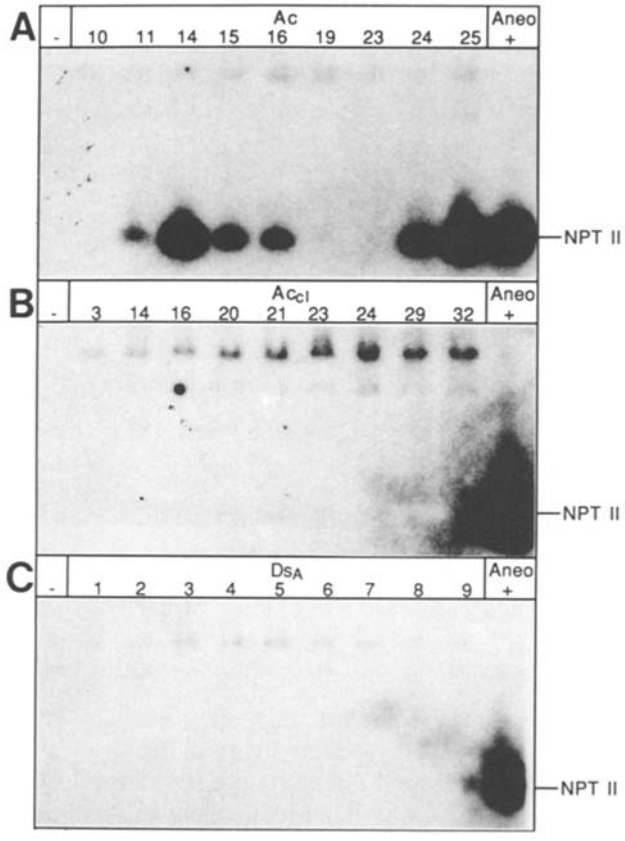

Fig. 2 A–C. *NPT II* enzyme activity is detectable in primary *Arabidopsis* transformants containing constructs of the autonomous *Ac* element but not in transformants containing constructs of either Ac_{cl} or Ds_A. **A** Transformants containing the intact *Ac* (3850HPT::pKU3); **B** Ac_{cl} transformants (3850HPT::pTA1); **C** Ds_A transformants (3850HPT::pTA3). *Numbered lanes* refer to independent regenerants transformed with the respective construct. In the *lanes* marked by − extracts of untransformed plants were applied. The lanes marked by *Aneo*+ show the *NPT II* activity in extracts of plants stably transformed with a chimaeric *NPT II* gene driven by the CaMV 35S promoter (von Schaewen 1989), which were used as positive controls. The extracts tested were prepared from Hygromycin-resistant and *nos*-(nopaline synthase)-positive calli with regenerating shoots. The position of the Kanamycin phosphate produced by the *NPT II* enzyme is indicated by *NPT II*

Table 1. Number of Kanamycin-resistant F_2 plants[a] derived from crosses of five Ac_{cl} lines with seven Ds_A lines and the respective selfings

Ac_{cl}	TA1/16/A	TA1/18/C	TA1/21/B	TA1/24/D	TA4/1/C
Ds_A	0% (0/640)	0% (0/250)	0% (0/250)	0% (0/250)	0% (0/250)
TA3/6/A	0% (0/100)	0% (0/100)	0% (0/160)	2.4% (4/170)	16.7% (40/240)	23.9% (43/180) 19.1%[b] (42/220)[b]
TA3/7/C	0% (0/880)	0% (0/400)	0% (0/240)	2.2% (2/90)	n.d.[c]	n.d.[c]
TA3/9/C	0% (0/915)	0.25% (1/400)	0.6% (1/180)	n.d.[c]	14.2% (37/260)	n.d.[c]
TA3/10/A	0% (0/630)	3% (6/200)	0% (0/170)	17.1% (12/70)	1.9% (2/105)	23.9% (43/180) 31%[b] (93/300)[b]
TA3/11/C	0% (0/740)	0% (0/200)	0% (0/300)	0.6% (1/170)	0% (0/80)	12.5% (25/200)
TA3/12/B	0% (0/525)	1.25% (5/400) 0%[b] (0/55)[b]	0% (0/300)	n.d.[c]	n.d[c]	11.1% (5/45)
TA3/15/A	0% (0/815)	0.45% (1/220)	0% (0/170)	n.d.[c]	11.8% (26/220)	27.7% (72/260) 28.5%[b] (84/295)[b]

The numbers given in brackets represent resistant plants versus the total number of individuals of the respective F_2 population.
[a] F_2 seeds bulk harvested from the different F_1 populations were germinated on MS medium containing 1.6% glucose and 50 mg/l Kanamycin. Resistant plants were defined here as those that developed roots and had green leaves, sometimes with small patches of white tissue, when scored 4 weeks after sowing
[b] Repeated selection of the same F_2 population
[c] Not determined

patches of white tissue. As seen in Fig. 3 this phenotype was never observed in progenies of selfed Ds_A or Ac_{cl} plants, thus it is strongly indicative of Ds_A excisions that either happened in the somatic tissues of the F_2 plants or had already happened in the tissue of F_1 plants and were germinally transmitted to the F_2 plants. Irrespective of the exact timing of the excision events, the number of Km^r plants in a particular F_2 population gives an indication of the frequency of Ds_A excisions induced by Ac_{cl}. As shown in Table 1, the Ac_{cl} line TA4/1/C had the highest activating potential of the lines tested, followed by the lines TA1/24/D and TA1/21/B with lower activity and the lines TA1/18/C and TA1/16/A with almost no detectable activity. The data given in Table 1 also indicate that the chromosomal location of a Ds_A element has little effect on its excision rate, as different Ds_A lines derived from independent transformants displayed only small differences with respect to the frequency of the appearance of Km^r F_2 plants when crossed with one particular Ac_{cl} line (e.g. TA4/1/C).

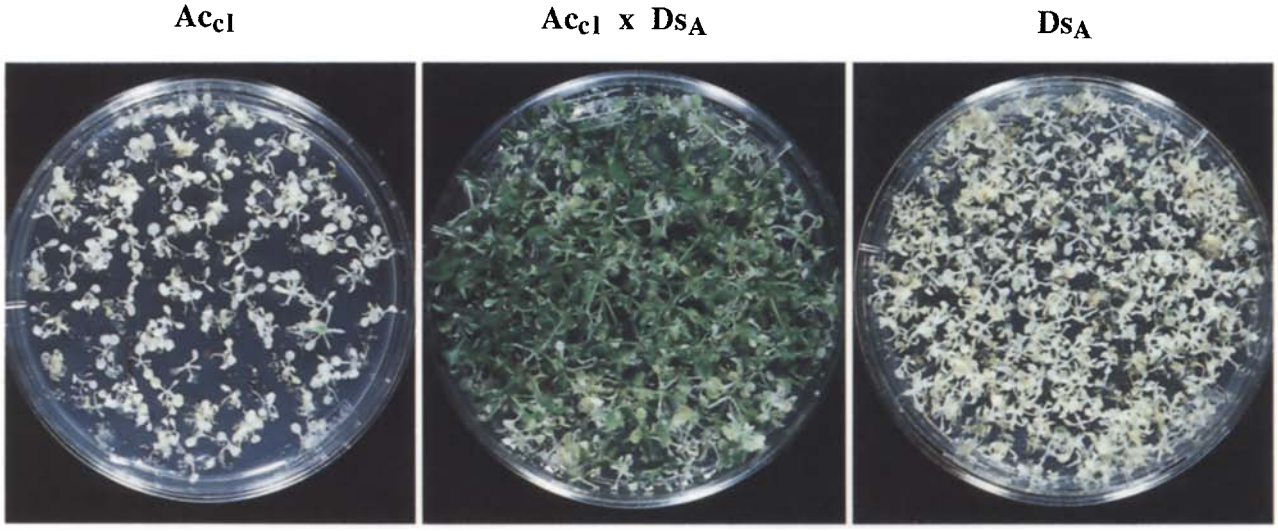

Fig. 3. Kanamycin-resistant plants arise only in the progeny of a crossing between Ac_{cl}- and Ds_A-containing lines. Progeny of the selfing of the Ac_{cl} parent TA4/1/C (*left*), the selfing of the Ds_A parent TA3/15/A (*right*) and the $Ac_{cl} \times Ds_A$ crossing TA4/1/C × TA3/15/A (*middle*) were germinated and grown for 4 weeks on MS medium containig 1.6% glucose and 50 mg/l Kanamycin

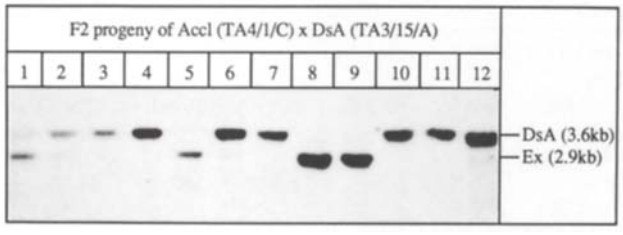

Fig. 4. DNA fragments indicative of Ds_A excision are present in several Kanamycin-resistant F_2 plants. Genomic DNA of Kanamycin-resistant F_2 plants (as defined in Table 1) from the cross of the Ac_{cl} line TA4/1/C with the Ds_A line TA3/15/A was digested with *Eco*RI/*Hin*dIII and hybridized with a fragment of the *NPT II* gene coding region. The bands corresponding to the Ds_A fragment in the original position (*Ds_A*, 3.6 kb) and the excision fragment (*Ex*, 2.9 kb) are indicated; compare with Fig. 1. In the exposure shown here, only those excision bands characterized as + + (strong signal) or + (medium strong signal) in Table 2 were visible

Excision bands of varying intensities are detected in the F_2 generation of crosses between one Ac_{cl} line and three different Ds_A lines

In order to test whether or not the appearance of Km^r F_2 plants in different crossings was indeed due to excision events, a series of Southern blot hybridization experiments was performed with *Eco*RI/*Hin*dIII-digested DNA of 32 Km^r F_2 plants derived from three $Ac_{cl} \times Ds_A$ crossings (TA4/1/C × TA3/10/A, TA4/1/C × TA3/11/C and TA4/1/C × TA3/15/A). The *Eco*RI and *Hin*dIII restriction sites and the expected sizes of the fragments hybridizing to the various labelled probes used in the analysis are indicated in Fig. 1. Upon hybridization with the *NPT II* gene (examples are shown in Fig. 4) a readily detectable signal for a 2.9-kb fragment, as expected for the excision band, was obtained in 9 samples. A signal at the same position was also observed after hybridization with the TR 1′ promoter (data not shown). In 16 other samples a very weak signal was present, and in the 7 remaining samples no excision fragment was detected.

These data clearly show that Ds_A excisions can be detected in Km^r plants. They also indicate that the *NPT II* gene serves as a very sensitive excision marker as plants that show only very weak excision bands, thus containing excision fragments underrepresented in their genomic DNA, were picked up as Km^r. This genomic situation is expected for plants in which somatic excisions happen, thus leading only to a subset of cells containing excision fragments.

F_2 plants exhibiting strong excision bands in the DNA blot analysis inherit germinal excision events

Detection of an excision band in the DNA blot analysis is not sufficient evidence to prove the germinal (i.e. premeiotic) nature of the Ds_A excision event that results in the presence of an excision fragment in every cell of the F_2 plant. Even the appearance of strong excision bands could be due to early somatic events (producing large sectors of tissue with cells containing excision fragments) or highly frequent late somatic events (resulting in many small sectors).

To test how many germinal Ds_A excision events were present in the 32 F_2 plants tested by Southern blot analysis, their F_3 progenies were germinated in the presence

Table 2. Intensities of the excision bands of F_2 plants[a] from three different $Ac_{cl} \times Ds_A$ crosses and segregation of their respective F_3 progeny under Kanamycin selection[b]

n	TA4/1/C × TA3/10/A		*n*	TA4/1/C × TA3/11/C		*n*	TA4/1/C × TA3/15/A	
	F_2 Excision band[a]	F_3 Km selection[b] r:hv:v:s:w		F_2 Excision band[a]	F_3 Km selection[b] r:hv:v:s:w		F_2 Excision band[a]	F_3 Km selection[b] r:hv:v:s:w
1	++	21:0:0:8:0	1	−	2:1:2:47:0	1	++	19:0:0:4:7
2	+/−	5:10:4:4:1	2	−	3:2:19:69:27	2	+	3:16:4:9:0
3	+/−	4:10:7:5:0	3	−	2:2:11:22:0	3	−	0:15:6:10:10
4	−	5:7:7:2:0	4	+/−	1:0:4:35:11	4	+/−	4:8:11:1:0
5	−	1:11:6:6:0	5	+/−	1(?):0:2:55:0	5	++	21:0:0:1:5
6	+/−	2:5:8:6:0	6	+/−	3:1:0:62:0	6	+	3:10:5:0:12
7	+/−	5:10:5:9:0	7	+/−	4:0:16:40:23	7	+/−	1:13:8:5:7
8	+/−	0:8:7:16:5	8	−	1:0:9:23:8	8	++	24:0:0:8:12
9	+/−	3:10:5:12:0	9	++	34:3:0:13:0	9	++	20:0:0:5:0
10	+/−	2:13:9:2:0	10	+/−	3:0:13:25:13	10	+/−	4(?):32(hv+v):8:0
						11	+/−	1:19(hv+v):5:0
						12	+	2:13:6:6:8

[a] The intensity of the excision band detected in Southern blot analysis was determined by the eye (compare Fig. 4): ++, strong signal; +, medium strong signal; +/−, weak signal; −, not detectable

[b] Seeds harvested from individual F_2 plants were germinated on MS medium containing 1.6% glucose and 50 mg/l Kanamycin and scored after 4 weeks of growth on this medium: r, resistant; hv, highly variegated; v, variegated; s, sensitive; w, white (recessive albino mutation present in Ac_{cl} line TA4/1/C). For definitions see results section. Numbers marked with (?) refer to plants that fitted into a particular class but showed abnormalities in growth

of Kanamycin. In the scoring of these F_3 populations three different groups of plants were distinguished: plants with properly developed roots and completely green leaves (termed resistant), plants with developed roots and leaves with patches of white tissue (termed highly variegated or variegated if the roots developed poorly and the leaves had large white patches) and sensitive plants that showed no root formation and did not develop true leaves, with the cotyledons finally bleaching. In these F_3 populations another group of plants with an albino phenotype (termed white) appeared. As these plants did not fully expand their cotyledons and appeared to be completely white, they were distinguishable from Kanamycin-sensitive plants and did not interfere with the segregation analysis. As shown in Table 2, a Mendelian segregation into resistant plants and nonresistant plants (highly variegated, variegated and sensitive) expected for at least one active locus ($\geq$3:1) was found in the F_3 progenies of 6 F_2 plants (Table 2), thus proving the germinal nature of the Ds_A excision events inherited in these F_2 plants. Notably, all these 6 F_2 plants displayed a strong excision band in the DNA blot analysis (c.f. Fig. 4), and their leaves did not show any white patches.

Reintegration of Ds_A is monitored in 3 out of 6 germinal excision events

An important characteristic with respect the applicability of the Ac_{cl}/Ds_A system for insertion mutagenesis purposes is the frequency of Ds_A reintegration after excision. In order to address this point, DNAs of all 6 F_2 plants that had been seen to inherit germinal Ds_A excision events were analyzed for the presence of new Ds_A bands indicative of reintegration and thus of a transposition event. Transposed Ds_A elements were expected to generate two new *Eco*RI/*Hin*dIII fragments unpredictable in size but different from the ones known for its position in the T-DNA, as Ds_A has one internal *Hin*dIII and no *Eco*RI site. In 3 of the 6 F_2 plants analyzed, new fragments hybridizing to end-specific probes of *Ac* were visible (Fig. 5A II, III) that were not present in the plant DNA of either the Ac_{cl} or the Ds_A lines (Fig. 5B, II, III). As these fragments did not hybridize to the *NPT II* gene (Fig. 5A I), or the TR 1′ promotor, or an Ac_{cl}-specific fragment (part of the sequence deleted in Ds_A) (data not shown) they most probably represent transposed Ds_A elements. Although the appearance of these new Ds_A bands proves the occurrence of transposition events, they do not yet prove their germinal nature. With respect to the use of this system for insertion mutagenesis purposes, however, this is an absolutely essential question. To show germinal transmission of the new Ds_A bands, DNAs of F_3 progeny plants of the 3 F_2 plants were analyzed by blot hybridization: strong excision bands as well as the same new Ds_A bands as those seen in their respective F_2 plants were present (Fig. 6). These F_2 plants, which transmitted both the excision fragment and the transposed Ds_A element to their progenies that segregated in a Mendelian fashion for the presence of the excision

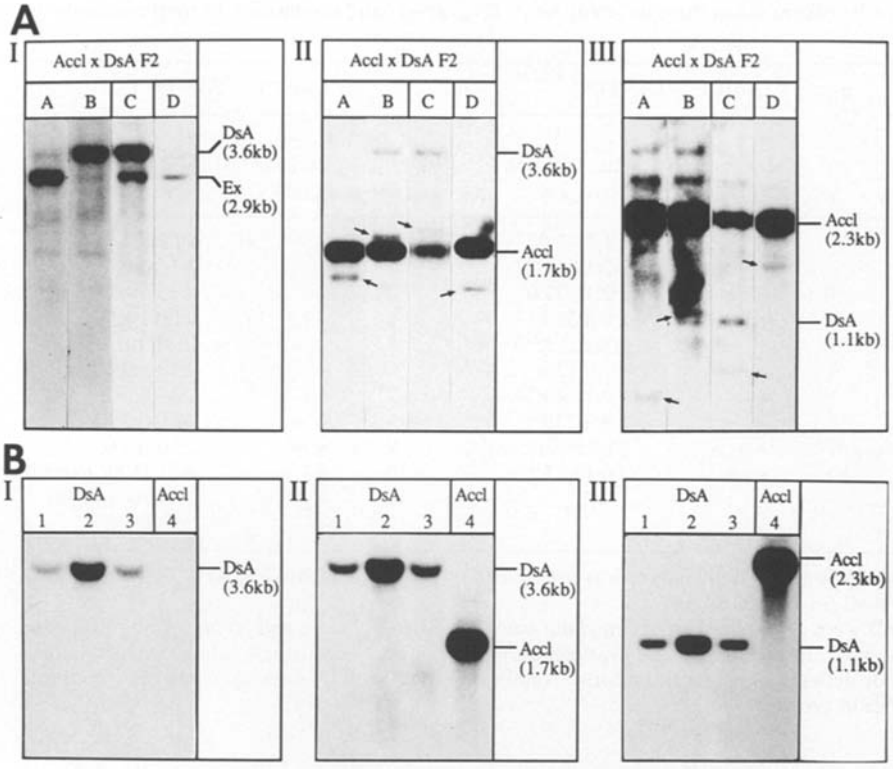

Fig. 5 A, B. A subpopulation of plants containing excision bands also contain new Ds_A bands indicative of transposition events. **A** Southern blot analysis of F_2 plants of crosses of Ac_{cl} line TA4/1/C with Ds_A lines TA3/10/A (*lanes A* and *B* refer to F_2 plants numbers, 1 and 3, respectively), TA3/11/C (*lane C*, F_2 plant number 9) and TA3/15/A (*lane D*, F_2 plant number 1), **B** Analysis of F_2 progeny plants derived from selfings of the respective Ac_{cl} and Ds_A parents. *Lane 1* TA3/10/A, *lane 2* TA3/11/C, *lane 3* TA3/15/A, *lane 4* TA4/1/C. Plant DNA was digested with *Eco*RI/*Hin*dIII and hybridized with the *NPT II* gene coding region (*I*), the 3′ *Hin*dIII/*Bam*HI fragment of *Ac* (*II*) and the 5′ *Bam*HI/*Hin*dIII fragment of *Ac* (*III*). The positions of bands corresponding to fragments of Ac_{cl} (*AccI*, 1.7 kb/2.3 kb) and Ds_A (*Ds*A, 1.1 kb/3.6 kb) in their original positions and the excision fragment (*Ex*, 2.9 kb) are indicated; compare with Fig. 1. Bands indicative of transposed Ds_A elements are marked by *arrows*

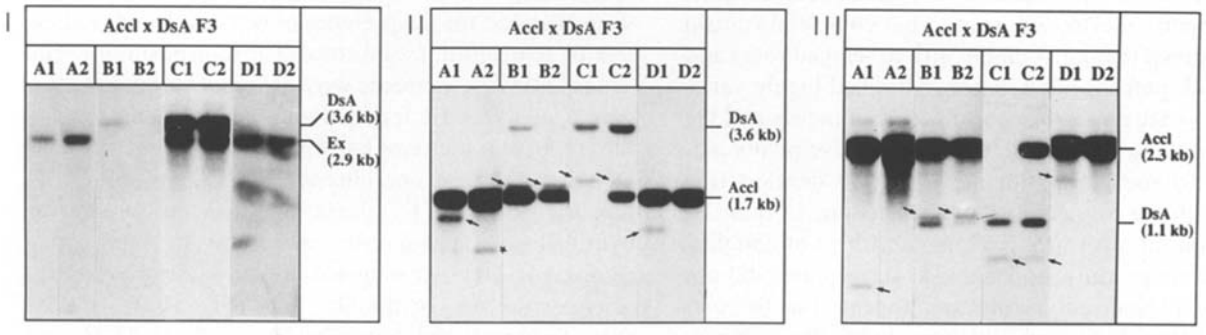

Fig. 6. Transposed Ds_A elements detected in 4 F_2 plants appear also in their respective F_3 progeny plants. Southern blot analysis of F_3 progeny plants of F_2 plants TA4/1/C × TA3/10/A number 1 (*lanes A1, A2*) and number 3 (*lanes B1, B2*), TA4/1/C × TA3/11/C number 9 (*lanes C1, C2*) and TA4/1/C × TA3/15/A number 1 (*lanes D1, D2*). Compare with Fig. 5. Genomic DNA digested with *Eco*RI/*Hin*dIII was hybridized with the *NPT II* gene coding region (*I*), the 3′*Hin*dIII/*Bam*HI fragment of *Ac* (*II*) and the 5′ *Bam*HI/*Hin*dIII fragment of *Ac* (*III*). The bands corresponding to Ac_{cl} (*AccI*, 1.7 kb/2.3 kb) and Ds_A (Ds_A, 1.1 kb/3.6 kb) in their original positions and the excision fragment (*Ex*, 2.9 kb) are indicated. Bands indicative for the transposed Ds_A elements seen in the respective F_2 parent plants are marked by *arrows*. The band marked by an *asterix* in *lane A2* part II most probably does not correspond to a transposed Ds_A element but has rather to be attributed to star activity of the enzyme as bands at the same position were detected with various intensities in DNAs of several other F_2 and F_3 plants

marker, thus fulfill the criteria for a germinal transposition event.

Ds_A can transpose to genetically unliked sites

One further critical feature of a transposon system for its efficient use in insertion mutagenesis is the distance the mobile element can move, as this would principally limit the number of loci accessible to a limited number of insertion sites of the transposon in the genome.

Positive evidence for Ds_A transposition to a genetically nonlinked site was found in F_2 plant number 3 of the cross TA4/1/C × TA3/10/A. This plant showed a very

weak signal for the excision fragment but contained new Ds_A fragments (Fig. 5 A I, II, III lane B). Km^r plants comprised consistently less than 15% in its F_3 generation (Table 2), whereas the same new Ds_A fragments as present in the F_2 plant were found in the F_3 progeny plants (examples are shown in Fig. 6 I, II, lanes B1, B2), thus proving that the transposed Ds_A is germinally transmitted. This strongly indicates that Ds_A had transposed to a position genetically not linked to its excision site and that either mitotic segregation, in the case of transposition to the sister-chromatid, or meiotic segregation led to the presence of the transposed Ds_A but the absence of the corresponding excision fragment in the F_2 plant. Two further examples for the separation of the excision fragment and the transposed Ds_A were found in the F_3 progenies of the F_2 plants TA4/1/C × TA3/10/A number 1 (Fig. 6 I, II, III, lanes A1, A2) and TA4/1/C × TA3/15/A number 1 (Fig. 6 I, II, III, lanes D1, D2). Here, in contrast to the example mentioned above, the excision band but not the bands of the respective transposed Ds_A elements were present in a subset of F_3 plants (Fig. 6 I, II, III, lanes A2, D2). This could either be explained by genetic segregation of the loci of excision and the loci of reinsertion, by further excision of the Ds_A from its new position or by the creation of new excision fragments due to independent Ds_A excisions from the other allele. As in general relatively low frequencies of Ds_A excisions readily detectable in the Southern blot analysis were observed and in this case no selection for such events was applied, the latter two explanations seem to be less likely than the first one.

Variegated plants represent a pool for the identification of new and independent germinal transposition events in their progeny

Out of the 32 F_2 plants analyzed, 6 plants were shown to be due to germinal excisions/transpositions of Ds_A. The detection of only weak excision bands in the remaining 26 F_2 plants (examples are shown in Fig. 4) in combination with at least partial Km resistance can be explained by assuming that somatic excision/transposition events occurred in these F_2 plants. This assumption is in agreement with the fact that all F_3 populations of these 26 plants contained less than 75% completely Km^r plants (Table 2).

With respect to the development of this system for transposon tagging purposes the appearance of fully Km^r plants in these F_3 populations is important. These fully Km^r plants could be due to germinal excision/transposition events of Ds_A. Most importantly, however, these events must have occurred during the growth of the F_2 plants and thus have to be independent and new with respect to the germinal excision/transposition events detected in the 6 F_2 plants described above, as the latter events are due to excisions/transpositions that happened during development of the F_1 plants. Therefore, by applying a scheme of collecting fully resistant plants (representing a source for independent insertion mutants) in each progeny generation of crossings between Ac_{cl} and Ds_A lines and continuously propagating the variegated population as a source for new and independent germinal transpositions in the next generation we should be able to

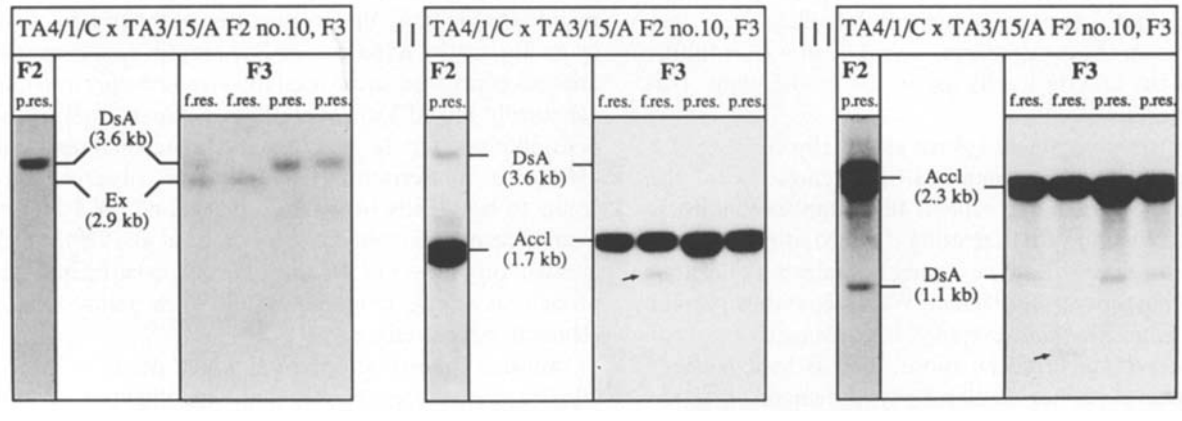

Fig. 7. Fully Kanamycin-resistant F_3 progeny of partially Kanamycin-resistant F_2 plants contain germinal excisions/transpositions of Ds_A. Southern blot analysis of the partially Kanamycin-resistant (*p. res.*) F_2 plant number contain 10 of the cross TA4/1/C (Ac_{cl}) × TA3/15/A (Ds_A) and partially as well as fully Kanamycin-resistant (*f. res.*) F_3 progeny plants. After digestion with *Eco*RI/*Hin*dIII genomic DNA was hybridized with the *NPT II* gene coding region (*I*), the 3′ *Hin*dIII/*Bam*HI fragment of *Ac* (*II*) and the 5′ *Bam*HI/*Hin*dIII fragment of *Ac* (III). The positions of the original 3.6-kb and 1.1-kb Ds_A fragments (Ds_A), the 2.9-kb excision fragment (*Ex*) and the 1.7 kb and 2.3 kb Ac_{cl} fragments (Ac_{cl}) are indicated. Bands indicative for a transposed Ds_A element are marked with *arrows*.

select for an essentially unlimited number of independent insertions.

In order to test whether or not this scheme would hold true, fully Km^r F_3 plants derived from variegated F_2 plants were tested for the presence of excision fragments and transposed Ds_A elements. After transfer to soil or to nonselective medium about one-half to two-thirds of the F_3 plants scored as Km^r after 4 weeks of growth in the presence of Kanamycin (Table 2) developed small white lesions on their youngest leaves. Only those plants showing no white patches of tissue through development until maturity were termed fully Km^r. This phenomenon was not observed in the Km^r F_3 plants inheriting the germinal excision events detected in their respective F_2 plants (data not shown).

Out of 18 fully Km^r F_3 plants picked out in 11 different F_3 populations of the two crossings TA4/1/C × TA3/10/A and TA4/1/C × TA3/15/A, 12 (66%) were positive for a strong excision band indicative of a germinal excision event. For example, see Fig. 7I. In 6 of these 12 plants, new Ds_A bands were also identified that were all different from each other (for example, see Fig. 7 II, III) and different from the ones observed in the F_2 plants described earlier. Thus, new and independent germinal transpositions were detected in the progeny of variegated plants. About 30% of the plants identified as fully Km^r were positive for new Ds_A fragments.

Discussion

The molecular cloning of genes via insertional mutagenesis is an attractive method for the isolation of genes only identified by a mutant phenotype. The rate-limiting step for the application of this method is the need for a very large number of independent and randomly scattered insertions in the chromosomes. In this article we present evidence that a two-component mobile element system derived from the maize transposon *Activator* (*Ac*) fulfills most of the criteria for its use in the model plant *Arabidopsis thaliana*.

This two-component system essentially consists of a stabilized *Ac* element obtained by deleting one of the terminal 11-bp inverted repeats that supplies the necessary transfunction for activating the second component, a nonautonomous *Ds*-type element obtained by deleting internal parts of an *Ac* element. When separately present in transgenic *Arabidopsis* plants, both elements are incapable of excisions/transpositions. This is in agreement with observations for similar *Ds*-type elements in transgenic tobacco (Coupland et al. 1988; Hehl and Baker 1989; Jones et al. 1990) and tomato (Lassner et al. 1989) and for the loss of mobility of an *Ac* element (*Ac*-18) detected in transgenic tobacco that had sustained a 4-bp deletion (Hehl and Baker 1989). Crossings between Ac_{cl} and Ds_A lines leading to the presence of both elements in one cell should, however, result in the transactivation of the Ds_A element by the Ac_{cl} element. This was observed to be the case. At least four of the five Ac_{cl} lines analyzed were able to transactivate Ds_A elements, however they differed in their efficiency. This trait was nearly exclusively attributed to the Ac_{cl} line, as the response of different Ds_A lines towards activation by one Ac_{cl} line did not greatly differ. Thus, it seems that the integration site/chromosomal location and/or the copy number of Ac_{cl} in a particular line is strongly influencing its ability to transactivate Ds_A elements whereas the chromosomal location of the Ds_A element seems to have little influence on Ds_A activation. It has to be mentioned that this conclusion is drawn from data derived from Ds_A elements located on a T-DNA fragment containing actively transcribed genes and that elements in inactive chromatin might behave differently.

With respect to the use of the Ac_{cl}/Ds_A system for gene tagging, the observation that Ds_A elements in different chromosomal locations can be transactivated to the same extent by one Ac_{cl} line is of great importance. This would allow the creation of *Arapidopsis* lines carrying multiple Ds_A insertions scattered throughout the chromosomes, all of them ideally becoming activated by the Ac_{cl} line at about the same rate. Though the presence of multiple Ds_A insertions would result in some slightly increased effort with respect to identification of the Ds_A insertion being responsible for a certain mutant phenotype, such lines would be advantageous for increasing both the number of independent Ds_A transpositions created in the progeny of one crossing and the chance of accessing all regions of the *Arabidopsis* genome by Ds_A insertions.

The DNA blot analysis of 18 germinal excision events has shown that in about 50% of the cases Ds_A excisions are accompanied by a scorable reinsertion. This value might actually be an underestimation of the true frequency as due to the selection for Kanamycin resistance the analysis performed was biased towards reintegration into genetically linked loci. These data compare well to the data obtained for *Ac* in transgenic tobacco (Jones et al. 1990). The reinsertion frequency of Ds_A observed here seems to be slightly lower than that found for a *Ds* element in transgenic tobacco (Masterson et al. 1989), which is most probably due to the different experimenal approach, as in the latter study all of the reinserted *Ds* elements were detected.

Another important criterium which needs to be fulfilled in order to enable tagging experiments with this two-component system to be carried out is the germinal transmission of Ds_A transpositions leading to plants homozygous for a certain Ds_A insertion and, therefore, the recognition of recessive mutant alleles. The data shown in the results section prove that a high proportion of fully Km^r plants appearing in either the F_2 or the F_3 progeny

of crossings between Ac_{cl} and Ds_A lines represent germinal excisions. The absolute frequency of germinal transpositions is about 1% as estimated from the number of fully Km^r plants with new Ds_A insertions detected in the F_3 progeny of variegated F_2 plants derived from two different $Ac_{cl} \times Ds_A$ crossings. Data obtained from transactivation of similar *Ds*-like elements in tobacco (Hehl and Baker 1989, 1990) indicate much higher transactivation frequencies. This comparison is complicated as in their study F_1 plants of crosses between parents carrying *Ac* and *Ds* elements were directly selected for Km resistance that most probably was conferred by very early and/or very frequent somatic excision events. The excision fragments created in the F_1 plants however were transmitted to the F_2 progeny at a very high frequency (Hehl and Baker 1989). Apparently much higher transactivation frequencies were obtained in tomato plants transgenic for *Ac* and *Ds* elements (Lassner et al. 1989). Our results do not indicate an obvious reason for the different behavior of Ac_{cl} and Ds_A in *Arabidopsis thaliana.*

In terms of the application of the Ac_{cl}/Ds_A system for gene tagging, more important than the absolute transactivation frequency is the result that fully Km^r plants were present in 90% of the F_3 progenies derived from variegated parents (Table 2). The observed frequencies will enable us to obtain at least one germinal transposition event among 100–200 progeny plants of most of the variegated parent plants. Due to the properties of *Arabidopsis thaliana* this number of progeny plants can easily be obtained from a single parent plant, and they can be grown for selection on a single standard petri dish. As the progeny of every variegated plant has to be treated individually in order to assure independent new Ds_A insertions to be obtained, it will not be the germinal transposition frequency of Ds_A but rather the time and the space needed to grow many individual progeny populations that will limit the number of new insertions that can be identified. Much higher transposition frequencies might even not be desired in this type of two-component system, as this would increase the probability that Ds_A excises again from its new location while Ac_{cl} still is present elsewhere in the same genome.

A further criterion for the value of a transposon tagging system is the distance that the mobile element can move away from its original position. Data obtained for transgenic tobacco show that *Ac* retains its property known from maize to transpose preferentially to genetically linkes sites (Jones et al. 1990). In the case of Ds_A, the observations that in F_2 plant number 3 of the crossing TA4/1/C × TA3/10/A (Fig. 4) new DNA fragments indicative of a transposed Ds_A element were present while no corresponding excision fragment was detectable and that separation of the excision fragment from the transposed Ds_A element in two other F_3 progenies occurred, suggest that Ds_A can move to genetically nonlinked sites in *Arabidopsis*. This indicates that transposition is not strictly limited to positions very near to the site of excision.

A final aspect of the two-component system described here concerning its application for gene tagging purposes in *Arabidopsis thaliana* is the fact that using Ac_{cl} line TA4/1/C and the two Ds_A lines TA3/10/A and TA3/15/A, for example (they have been analyzed in the most detail), it is possible to create in principle an unlimited number of independent (and thus ideally scattered) Ds_A insertions.

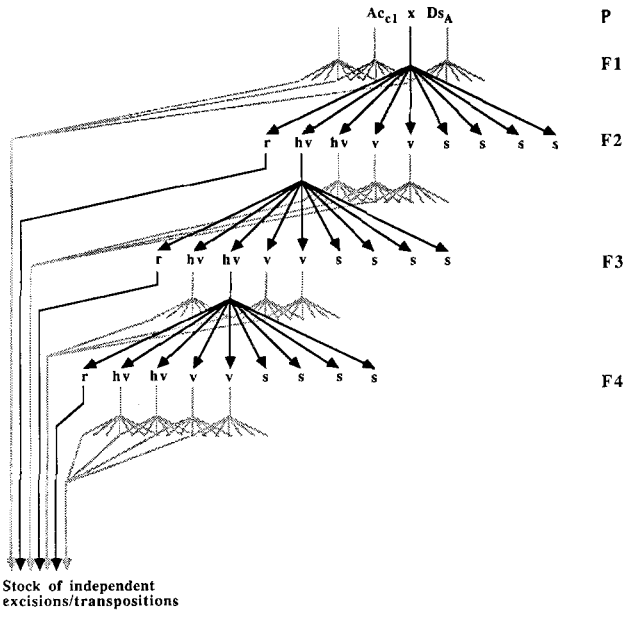

Fig. 8. Selection procedure for rapid isolation of high numbers of independent germinal Ds_A excision/transposition events. After crossing of an Ac_{cl} plant with a Ds_A plant the F_2 is the first generation in which germinal excisions can be found that are detected as fully Km^r plants (*r*). Excision/transposition events inherited by F_2 plants derived from different F_1 plants have to be independent as transactivation of Ds_A can not happen before onset of the life cycle of the F_1 plant when Ac_{cl} and Ds_A are first combined in one cell. As excision/transposition events inherited by F_2 plants derived from the same F_1 plant can be identical, all fully Km^r F_2 progeny plants of a particular F_1 plant will be termed as one family that will contain at least one excision/transposition event. Seeds of all plants belonging to one family will therefore be harvested together, but separately from the seeds of other families. In the same F_2 populations that contain fully resistant plants many partially Km^r plants (highly variegated, *hv*, or less variegated, *v*, according to the degree of resistance) will also be present in addition to completely sensitive plants (*s*). The partially Km^r F_2 plants, which do not inherit germinal excision events from their parent plant, will be the source for new (independent) excision/transposition events fixed in the next generation (F_3). Thus, each family of Km^r F_3 plants will again contain at least one excision/transposition event that is not present in any other family. As also partially resistant plants are again found in the F_3 populations (see Table 2), this scheme can be continued through further generations leading to the rapid accumulation of many families representing independent excision/transposition events

The basic principle for achieving this is the following: selection of F_2 progeny of the various crossings between highly activating Ac_{cl} lines and Ds_A lines on Kanamycin-containing plates results in at least three types of plants: fully resistant plants, variegated plants and sensitive ones. The fully resistant plants have been shown by molecular as well as genetic analysis to represent germinal excisions/transpositions of Ds_A, which therefore have most probably occurred premeiotically in the F_1 plant. They represent the stock of plants carrying Ds_A insertions suitable for mutant screening after selfing in order to obtain homozygotes for recognition of recessive mutant alleles. The variegated F_2 plants have by the same criteria been shown to be due to somatic transpositions of Ds_A. In the population of F_3 seedlings derived from the variegated F_2 plants the same three classes of plants as described above for the F_2 generation can be distinguished upon germination and growth in the presence of Kanamycin. Molecular analysis has demonstrated that about 30% of the fully resistant plants represent germinal Ds_A transpositions that are different from those obtained in the F_2 generation. Thus, the fully resistant F_3 plants represent new and independent Ds_A insertions, increasing the number of plants carrying putative insertion-mediated mutations that are suitable for mutant screening after selfing. On the other hand, the variegated F_3 plants comprise the source of new and independent germinal transpositions obtained in the F_4 generation. As in principle this scheme, which is shown in Fig. 8, can go on for an unlimited number of generations, it allows the creation of large numbers of *Arabidopsis* lines, all of which carry independent Ds_A insertions in their genome. Thus the Ac_{cl}/Ds_A system presented here fulfills the most important criterion for insertion mutagenesis i.e. the creation of a large number of independent insertions of the insertion mutagen in the desired host.

The development of other types of activating elements, including the tightly regulated expression of a highly active transposase protein (Houba-Hérin et al. 1990) will be important to overcome the bottle neck of selection for excisions/transpositions, which is very time- and space-intensive and thus limits the number of independent, stably inherited Ds_A insertions than can be obtained.

Further questions concerning the randomness of Ds_A insertions, the type of mutants created and the frequency at which they can be obtained will require the creation and analysis of a stock of several hundred lines carrying independent Ds_A insertions. From the data presented here we conclude that the Ac_{cl}/Ds_A system will allow us to obtain such a stock of lines and most probably to isolate Ds_A insertion mutants.

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